IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF NEW JERSEY

AMGEN INC. and AMGEN MANUFACTURING, LIMITED, Plaintiffs, v. ADELLO BIOLOGICS, LLC, AMNEAL PHARMACEUTICALS, LLC, and AMNEAL PHARMACEUTICALS, INC. Defendants.	Hon. Claire C. Cecchi No. 18-cv-3347-CCC-MF

AMGEN'S OPENING CLAIM CONSTRUCTION BRIEF

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Plaintiffs Amgen Inc. and Amgen Manufacturing, Limited (together, "Amgen") respectfully submit this brief in support of their proposed claim constructions for U.S. Patent Nos. 8,940,878 (Ex. 1); 9,643,997 (Ex. 2); 8,952,138 (Ex. 3); and 9,856,287 (Ex.4).[1]

I. INTRODUCTION

A. Amgen's Innovative Product, Neupogen®, and Adello's Filgrastim Biosimilar

Amgen Inc. discovers, develops, manufactures, and sells innovative therapeutic products based on advances in molecular biology, recombinant DNA technology, and chemistry. D.I. 84 ¶ 1. Amgen Manufacturing, Limited manufactures and sells biologic medicines for treating human diseases. *Id*. ¶ 2. Amgen's Neupogen® (filgrastim) is a recombinantly produced protein that stimulates the production of neutrophils, a type of white blood cell. *Id*. ¶¶ 37-39. Filgrastim is a pharmaceutical analog of a protein that is naturally produced in humans called granulocyte-colony stimulating factor ("G-CSF"). *Id.* ¶ 39. One use of Amgen's product is to counteract neutropenia, a neutrophil deficiency that makes a person highly susceptible to life-threatening infections. *Id.* Neutropenia is a common side effect of certain chemotherapeutic drugs. *Id*.

[1] All exhibits referred to in this brief are exhibits to the Sandel Declaration.

Amgen's Neupogen® was approved by the U.S. Food and Drug Administration under the traditional biologics regulatory pathway, 42 U.S.C. § 262(a), which requires that the applicant demonstrate that the biologic is "safe, pure, and potent." 42 U.S.C. § 262(a)(2)(C)(i)(I); D.I. 1 ¶ 38.

In contrast, Defendant Adello Biologics, LLC ("Adello")—which, in concert with other Defendants, develops, manufactures, and markets biopharmaceutical products—filed an abbreviated Biologics License Application under the Biologics Price Competition and Innovation Act of 2009's abbreviated pathway, 42 U.S.C. § 262(k), seeking approval to market a product based on biosimilarity using Amgen's Neupogen® as the reference product. (*See* D.I. 84 ¶¶ 3-5, 14-21.)[2]

B. The Patents-in-Suit

The Patents-in-Suit each generally relate to the production of proteins in non-mammalian expression systems using recombinant DNA technology. Scientists can harness the natural mechanisms by which cells, *e.g.*, bacterial cells, make their own proteins, and engineer those cells to make proteins for therapeutic use in humans and other living beings. *See, e.g.*, Declaration of Richard C. Page, Ph.D ¶¶ 28-30; *e.g.*, '287 Patent, 1:23-38. As an example, scientists can isolate a human gene that

[2] Adello has entered into a corporate transaction with Kashiv Pharma, LLC, and the resulting entity was renamed Kashiv BioSciences, LLC ("Kashiv"). Kashiv now owns the "aBLA", No. 761082, at issue here. D.I. 101, at 2 n.1. Amgen refers to the Defendants as Adello, because Kashiv is not yet a party to this action.

encodes for the production of a particular protein, then modify it, insert it into bacterial cells, and direct the cells to produce the human protein. Page Decl. ¶ 29. That protein can then be processed for therapeutic use in humans.

Proteins have three-dimensional structures that are typically critical for their functionality in the human body. *Id.* ¶ 25. Bacterial expression systems, however, produce misfolded and/or aggregated recombinant proteins. *Id.* ¶ 30; *e.g.*, '287 Patent, 1:25-38. The bacterial cells "may deposit the recombinant proteins into large relatively insoluble aggregates, such as inclusion bodies. These aggregates comprise protein that is typically not biologically active or less active than the completely folded native form of the protein." *E.g.*, '878 Patent, 11:66-12:5. Thus, before the expressed protein can be therapeutically useful it must undergo a series of processes. Page Decl. ¶¶ 25-30. This includes, for example, (1) separating the aggregated proteins and linearizing misfolded proteins, which can be done by using chemicals in a solubilization solution; (2) refolding the protein into its proper three-dimensional structure, which can be done by using chemicals in a refolding solution; and (3) purifying the protein by separating it from the chemicals introduced by the prior processing steps and from other contaminants, such as proteins expressed by the bacterial host cells. *Id.* ¶ 30; Declaration of Richard C. Willson, Ph.D ¶¶ 42, 45; *e.g.*, '878 Patent, 2:19-32. Purification is typically performed using a separation matrix, which utilizes the characteristics of the protein of interest, the chemicals,

and/or the contaminating proteins to isolate the protein of interest. Willson Decl. ¶¶ 42-45.

1. The '878 and '997 Patents

The '878 and the '997 Patents share the same specification, and "relate[] generally to processes for purifying proteins expressed in non-mammalian systems." *See* '878 Patent, 1:11-13; '997 Patent, 1:13-14. Protein purification is a critical step in the manufacture of biological products using recombinant DNA technology. Before the invention of the '878 Patent and the '997 Patent, it was believed in the art that certain of the specialized chemical compounds used to refold proteins needed to be diluted, reduced, or removed before applying the refold solution to a separation matrix for purification. *See, e.g.*, '878 Patent, 1:44-52; '997 Patent, 1:46-55; Willson Decl. ¶ 23. The conventional thinking was that these specialized chemical compounds in the refold solution could prevent or disrupt the interactions with a separation matrix necessary to achieve purification. '878 Patent, 15:25-42; '997 Patent, 15:50-67; Willson Decl. ¶ 23. In the prior art, processing steps, such as dilution, intervened between protein refolding and application to a first chromatographic separation matrix. *See, e.g.*, '878 Patent, 15:25-42; '997 Patent, 15:50-67; Willson Decl. ¶ 23. Such additional processing can be costly and time-consuming, particularly at a large manufacturing scale. '878 Patent, 11:58-63, 12:21-26, 15:25-42; '997 Patent, 12:14-20, 12:45-50, 15:50-67.

The ’878 and ’997 Patents reflect the inventors’ insight that protein purification can be achieved by applying a refold solution to a separation matrix, ***without*** certain intervening processing steps. ’878 Patent, 11:58-63, 15:25-42; ’997 Patent, 12:14-20; 15:50-67; Willson Decl. ¶¶ 24-25.

2. The ’138 and ’287 Patents

The ’138 and the ’287 Patents share the same specification and relate to improved methods for refolding proteins made in non-mammalian, *e.g.*, bacterial, cells. The inventors of the ’138 and the ’287 Patents discovered that efficient refolding of proteins expressed in non-mammalian expression systems is affected by the particular conditions relating to breaking (“reducing”) and forming (“oxidizing”) bonds within the protein (referred to as the “redox state” in the patents). Depending on the values of two related parameters, referred to as “thiol-pair ratio” and “thiol-pair buffer strength,” that are based on the amount of oxidant and reductant used to refold the protein, the distribution of the desired properly refolded and improperly folded species varies. *See, e.g.*, ’138 Patent, 4:15-58; ’287 Patent, 4:30-5:10; Page Decl. ¶¶ 47-48. The patents teach to advantageously control these parameters by working with amounts of oxidants and reductants to provide redox conditions in which the protein molecules in the desired, biologically active conformation, predominate. *See, e.g.*, ’138 Patent, 16:58-67; ’287 Patent, 17:32-41; Page Decl. ¶ 49.

C. The Level of Ordinary Skill in the Art

With respect to the '878 and '997 Patents, a person of ordinary skill in the art as of the priority date of the patents, June 25, 2009, would have a Ph.D. in biochemical engineering, biomedical engineering, biochemistry, or a related discipline, with at least two years of work experience in the field of protein chromatography. Additional training or study could substitute for additional work experience, and additional work experience or training could substitute for formal education.

With respect to the '138 and '287 Patents, a person of ordinary skill in the art as of the priority date of the patents, June 22, 2009, would have a Ph.D. in biochemistry, biochemical engineering, molecular biology, or a related biological/chemical/engineering discipline, or a master's degree in such a discipline and several years of industrial experience producing proteins in non-mammalian expression systems.

II. LEGAL STANDARD

Claim construction begins with the plain language of the claim. The words of a claim "are generally given their ordinary and customary meaning," which is "the meaning that the term would have to a person of ordinary skill in the art at the time of the invention." *Phillips v. AWH Corp.*, 415 F.3d 1303, 1312-13 (Fed. Cir. 2005). This starting point "is based on the well-settled understanding that inventors are

typically persons skilled in the field of the invention and that patents are addressed to and intended to be read by others of skill in the pertinent art." *Id.* at 1313.

The claims "must be read in view of the specification," which is "always highly relevant to the claim construction analysis." *Id.* at 1315. "Usually, it is dispositive; it is the single best guide to the meaning of a disputed term." *Id.*; *see Vitronics Corp. v. Conceptronic, Inc.*, 90 F.3d 1576, 1582 (Fed. Cir. 1996). However, "limitations from the specification are not to be read into the claims." *Comark Commc'ns, Inc. v. Harris Corp.*, 156 F.3d 1182, 1186-87 (Fed. Cir. 1998). While "[i]t is entirely proper to use the specification to interpret what the patentee meant by a word or phrase in the claim," a limitation from the specification should not be read into the claims "[w]here a specification does not *require* a limitation." *E.I. du Pont de Nemours & Co. v. Phillips Petroleum Co.*, 849 F.2d 1430, 1433 (Fed. Cir. 1988) (emphasis in original). Thus, "[a]lthough the specification may aid the court in interpreting the meaning of disputed claim language, particular embodiments and examples appearing in the specification will not generally be read into the claims." *Comark*, 156 F.3d at 1187.

A court "should also consider the patent's prosecution history," which is also intrinsic evidence. *Phillips*, 415 F.3d at 1315. "Like the specification, the prosecution history provides evidence of how the PTO and the inventor understood the patent." *Id.* The prosecution history informs "the meaning of the claim language

by demonstrating how the inventor understood the invention and whether the inventor limited the invention in the course of prosecution, making the claim scope narrower than it would otherwise be." *Id.*

Where the intrinsic record is ambiguous, and when necessary, courts may rely on extrinsic evidence, *Vitronics*, 90 F.3d at 1583, which "consists of all evidence external to the patent and prosecution history, including expert and inventor testimony, dictionaries, and learned treatises," *Phillips*, 415 F.3d at 1317. If the court consults extrinsic evidence to understand, for example, the background science or the meaning of a term in the relevant art during the relevant time period, it "will need to make subsidiary factual findings about that extrinsic evidence." *Teva Pharms. USA, Inc. v. Sandoz, Inc.*, 135 S. Ct. 831, 837, 841 (2015). Where a court resolves a dispute between experts and makes a factual finding that, in general, "a certain term of art had a particular meaning to a person of ordinary skill in the art at the time of the invention," it must then "conduct a legal analysis: whether a skilled artisan would ascribe that same meaning to that term *in the context of the specific patent claim under review*." *Id.* at 841 (emphasis in original). That is because "experts may be examined to explain terms of art, and the state of the art, at any given time," but they cannot be used to prove "the proper or legal construction of any instrument of writing." *Id.* Thus, the ultimate interpretation of the claim term is a legal conclusion. *Id.*

Defendants assert that at least one claim is indefinite. A claim term is definite where, when "read in light of the specification delineating the patent, and the prosecution history," it informs "with reasonable certainty, those skilled in the art about the scope of the invention." *Nautilus Inc. v. Biosig Instruments. Inc.*, 134 S.Ct. 2120, 2124 (2014). Certainty is not required. Definiteness "mandates clarity, while recognizing that absolute precision is unattainable." *Id.* at 2129.

III. AGREED-UPON CONSTRUCTIONS

The parties agree on these constructions:

'878 Patent

Claim Term	Agreed-Upon Construction
"a protein" (All asserted claims)	any chain of at least five naturally or non-naturally occurring amino acids linked by peptide bonds

'997 Patent

Claim Term	Agreed-Upon Construction
"a protein" (All asserted claims)	any chain of at least five naturally or non-naturally occurring amino acids linked by peptide bonds

'138 Patent

Claim Term	Agreed-Upon Construction
"protein" (All asserted claims)	any chain of at least five naturally or non-naturally occurring amino acids linked by peptide bonds
"a protein . . . present in a volume at a concentration of 2.0 g/L or greater (All asserted claims)	A protein as it existed in a volume before contacting the volume with a refold buffer. The protein concentration in the volume is 2.0 g/L or greater.

"non-aerobic conditions" (All asserted claims)	Any reaction or incubation condition that is performed without the intentional aeration of the mixture by mechanical or chemical means.

'287 Patent

Claim Term	Agreed-Upon Construction
"proteins" (All asserted claims)	More than one protein, where protein is defined as any chain of at least five naturally or non-naturally occurring amino acids linked by peptide bonds.
"solution" (Claims 16, 26, and all asserted claims depending therefrom)	refold mixture
"is calculated" (Claims 8-9, 14-15, 23-25, 30)	is determined using an equation as part of practicing the method, rather than using the equation in hindsight
"properly refolded" (Claims 1, 10, 16, 26 and all asserted claims depending therefrom)	having native secondary and tertiary structure reintroduced, such that the protein is biologically active

IV. DISPUTED CONSTRUCTIONS

A. '878 and '997 Patents

1. Disputed Term: "the protein" (All asserted claims of the '878 and '997 Patents)

Amgen's Proposed Construction	Defendants' Proposed Construction
a protein expressed in a non-native limited solubility form in a non-mammalian expression system	the protein to be purified

For only this litigation, and to reduce the number of disputes, Amgen will accept Defendants' proposed construction for the term "the protein," because it makes no difference here whether the "the protein" is limited to "the protein to be

purified." *See U.S. Surgical Corp. v. Ethicon, Inc.*, 103 F.3d 1554, 1568 (Fed. Cir. 1997) ("[Claim construction] is not an obligatory exercise in redundancy.").

2. Disputed Term: "the solubilization solution" (All asserted claims of the '878 and '997 Patents)

Patent	Amgen's Proposed Construction	Defendants' Proposed Construction
'878 Patent	the solution comprising the solubilized protein and one or more of a denaturant, a reductant, and a surfactant. "The solubilization solution" of 7(d) may differ from "a solubilization solution" of 7(c), at least because the 7(d) solubilization solution contains the solubilized protein that is the product of 7(c).	the "solubilization solution" in Step (d) of Claim 7 must refer to the same solubilization solution used to solubilize the protein in Step (c).
'997 Patent	the solution comprising the solubilized protein and one or more of a denaturant, a reductant, and a surfactant. "The solubilization solution" of 9(b) may differ from "a solubilization solution" of 9(a), at least because the 9(b) solubilization solution contains the solubilized protein that is the product of 9(a).	the 'solubilization solution' in Step (b) of Claim 9 must refer to the same solubilization solution used to solubilize the protein in Step (a)

The parties dispute whether "the solubilization solution" of claim element (d) must have exactly the same composition as the claimed "a solubilization solution"

of claim element (c).[3] Under Amgen's construction, the "solubilization solution" of element (c) differs from the "solubilization solution" of element (d), because (d) refers to the product of step (c) after the addition of protein. In other words, "the solubilization solution" of claim element (d) refers to the solution that results from "solubilizing the expressed protein in a solubilization solution comprising one or more of" the components listed in claim element (c). Amgen's construction is consistent with the language of the claim when read as a whole, is supported by the intrinsic evidence, and most closely aligns to what "the inventors actually invented and intended to envelop with the claim." *Phillips*, 415 F.3d at 1316. Here, the inventors claimed and described a protein-purification method that involves, among other things, (1) solubilizing the protein, and (2) taking the product of that solubilization and adding it to a refold buffer so that the protein can be refolded. Claim 7 thus recites (c) solubilizing the protein in a solubilization solution (which does not include the protein-to-be-solubilized) and (d) forming a refold solution with the product of the earlier solubilization, *i.e.*, using the solubilization solution including the now-solubilized protein. *See* '878 Patent, claim 7(c)-(d), '997 Patent, claim 9(a)-(b).

[3] Amgen uses the lettering scheme of the '878 Patent. Claim elements 7(c) and 7(d) of the '878 Patent are identical to claim elements 9(a) and 9(b) of the '997 Patent.

The language of the claim itself further demonstrates the difference between the solutions referred to in elements (c) and (d). Element (c) refers to "***a*** solubilization solution," whereas element (d) refers to "***the*** solubilization solution." The claim as a whole makes clear that the proteins are added to "a solubilization solution" such that they may be solubilized, and the product of that step, "the solubilization solution," is combined with a refold buffer to form the "refold solution." '878 Patent, claim 7(c)-(d); '997 Patent, claim 9(a)-(b). Moreover, element (c) requires "solubilizing the expressed protein ***in*** a solubilization solution" and not "solubilizing the expressed protein [***to form a***] solubilization solution." '878 Patent, claim 7(c)-(d); '997 Patent, claim 9(a)-(b) (emphasis added). Had the patent drafters intended the solution in claim element (c) to include the protein, they would have used the word "form" as they did in claim element (d). '878 Patent, claim 7(d); '997 Patent, claim 9(b). Defendants now seek to construe the claim to mean what it simply does not say.

The patent specification further confirms that the solubilization solution of element (d) is the product of the solubilizing in element (c), and thus that the solutions necessarily have different compositions. The specification thus teaches that: (1) a protein is first solubilized in a solubilization solution comprising one or more of a (i) a denaturant, (ii) a reductant, and (iii) a surfactant, '878 Patent, 13:42-44, '997 Patent, 13:65-67; and (2) the protein is then refolded by forming a

refold solution, which comprises "the solubilization solution ***(which comprises the protein),*** and a refold buffer." '878 Patent, 14:5-7; '997 Patent, 14:27-29 (emphasis added). The examples bear this out. In Example 3, protein from inclusion bodies was solubilized in a solution "containing guanidine and DTT." '878 Patent, 20:17-19; '997 Patent, 20:46-48. "After incubation for one hour, the protein solution was diluted in to a refold buffer containing appropriate levels of arginine, urea, glycerol, cysteine, and cystamine" for refolding. '878 Patent, 20:19-21; '997 Patent, 20:48-50. Thus, the solution that resulted from solubilizing the protein in guanidine and DTT was added to a refold buffer to form a refold solution for refolding. Similarly, in Example 2, the protein is combined into a solubilization solution, and that solution is then diluted into a refold buffer for refolding. '878 Patent, 18:65-19:3; '997 Patent, 19:22-27.

Additionally, the specification contemplates that certain components may be consumed during solubilization and thus the composition of the "solubilization solution" may be different before and after the protein is solubilized. For example, the specification describes the use of beta-mercaptoethanol in a solubilization solution for particular proteins. Beta-mercaptoethanol is a reductant that reacts with certain exposed residues on the protein, *see* '878 Patent, 13:55-61; '997 Patent, 14:10-16, or with other species that may be present like dissolved oxygen from the atmosphere, *see* '878 Patent, 9:52-55; '997 Patent, 10:4-7. The concentration of

such a reductant would be reduced as a result of such chemical reactions during solubilization.

3. Disputed Term: "refold buffer" (All asserted claims of the '878 and '997 Patents)

Amgen's Proposed Construction	Defendants' Proposed Construction
a pH-buffered solution that provides conditions for the protein to refold into its biologically active form, comprising one or more of a denaturant, an aggregation suppressor, a protein stabilizer and a redox component	a solution comprising one or more of the following: (i) a denaturant; (ii) an aggregation suppressor; (iii) a protein stabilizer; and (iv) a redox component. The refold buffer need not necessarily contain a buffering component or have the ability to buffer pH.

Although the parties agree that the refold buffer includes one or more of "a denaturant, an aggregation suppressor, a protein stabilizer and a redox component," they dispute (a) whether the refold buffer is pH-buffered. They may also dispute (b) whether the refold buffer provides conditions for the protein to refold into its biologically active form.

a. Amgen's proposed construction that the "refold buffer" is "a pH-buffered solution" is supported by the express language of the claim. Defendants' construction seeks to read out the word "***buffer***" and to replace it with "solution," expressly denying that the buffer must be able to buffer. Amgen's construction is further supported by the specification, which teaches that the "refold buffer" contains a "buffering component" such as "phosphate buffers, citrate buffers, tris

buffer, glycine buffer, CHAPS, CHES, and arginine-based buffers" and that "[t]he function of the buffer component of the refold solution is to maintain the pH of the refold solution and can comprise any buffer that buffers in the appropriate pH range." '878 Patent, 14:49-55; '997 Patent, 15:5-11.

Defendants' argument appears to rely on the fact that the other named components of the refold buffer—"(i) a denaturant; (ii) an aggregation suppressor; (iii) a protein stabilizer; and (iv) a redox component"—do not necessarily act as buffers or pH buffers. Defendants thus seek to read the list of components as exclusive, even though the claim specifically says the opposite, in that it requires only that the solution "comprise" those listed components. In patent law, the word "comprising" is an "inclusive or open-ended" term that "does not exclude unrecited elements." *Regeneron Pharmaceuticals, Inc. v. Merus N.V.*, 864 F.3d 1343, 1352 (Fed. Cir. 2017); *accord* MPEP § 2111.03. Thus, while the "refold buffer" must include one or more of the listed components, it is not limited to those components and, as the specification teaches, must also include a "buffer component" in order "to maintain the pH of the refold solution." '878 Patent, 14:49-51; '997 Patent, 15:5-7.

b. Regarding the second disagreement, while the parties agree that the '878 Patent and the '997 Patent "relate[] generally to processes for purifying proteins expressed in non-mammalian systems" and that the asserted claims are directed to

"proteins expressed in a non-native limited solubility form" that must be solubilized and "refolded into a biologically active form," *see* '878 Patent, 1:11-12, 12:5-8; '997 Patent, 1:13-14, 12:29-32, Defendants apparently disagree that the purpose of the "refold buffer" is to provide conditions suitable for refolding.

The Patents explain that "to produce a functional protein, these inclusion bodies often need to be carefully denatured so that the protein of interest can be extracted and refolded into a biologically active form." *See* '878 Patent, 12:5-8; '997 Patent, 12:29-32. Thus, after solubilizing the protein, the protein is refolded into its native three-dimensional structure. This is accomplished, for example, in claim 7 of the '878 Patent and claim 9 of the '997 Patent by "forming a refold solution comprising the solubilization solution and a refold buffer." *See* '878 Patent, 2:24-28; '997 Patent, 2:29-33. As the specification explains, the function of the (i) denaturant; (ii) aggregation suppressor; (iii) protein stabilizer; and/or (iv) redox component in the refold buffer is to modify "the thermodynamics of the solution, thereby shifting the equilibrium towards an optimal balance of native form . . . [,] preventing non-specific association . . . [and] promoting stable native protein structure." '878 Patent, 14:5-17; '997 Patent, 14:27-40. Thus, "what the inventors actually invented and intended to envelop with the claim" includes a refold buffer that provides conditions for the protein to refold into its biologically active native

form. *See Phillips*, 415 F.3d at 1316 (quoting *Renishaw PLC v. Marposs Societa' Per Azioni*, 158 F.3d 1243, 1250 (Fed. Cir. 1998)).

4. Disputed Term: "directly applying the refold solution to a separation matrix under conditions suitable for the protein to associate with the matrix" (All asserted claims of the '878 Patent)

Amgen's Proposed Construction	Defendants' Proposed Construction
applying the refold solution to the separation matrix without removing components of or diluting the refold solution under conditions suitable for protein to have specific, reversible interactions with a separation matrix in order to effect the separation of protein from its environment	applying the refold solution to a separation matrix without removing any components of or diluting the refold solution, under conditions suitable for the protein to be purified to bind to the matrix

a. The parties' first dispute is whether "directly applying" should be construed to mean applying "without removing or diluting components of the refold solution" (as Amgen proposes) or applying "without removing or diluting ***any*** components of the refold solution" (as Adello proposes, emphasis added). Adello has not identified why the addition of "any" is necessary or appropriate, or what effect it would have on this case. With no reason or basis for the addition, the Court should reject it.

b. The parties' second dispute is how to construe the claim verb to "associate." The Defendants propose to equate "associating" with "binding" to the purification matrix. As a matter of both chromatography and the specification, that

is wrong: "associating" includes specific, reversible interactions other than just binding, and the specification identifies several such interactions. '878 Patent, 7:11-23; '997 Patent, 7:25-37. However, in the interest of reducing the number of disputes before the Court, and solely for purposes of this litigation, Amgen will accept the Defendants' construction that to associate means to bind, because that construction will have no impact on this case. (Amgen reached the same, litigation-specific agreements in the *Mylan* and *Pfizer* Litigations: a protein that "binds" to the matrix is a protein that is "associated" with the matrix within the meaning of the '997 Patent. *See Amgen Inc. v. Mylan Inc.*, No. 2:17-cv-01235, 2018 WL 6061213 at *15 (W.D. Pa. Nov. 20, 2018).

5. Disputed term: "applying the refold solution to a separation matrix under conditions suitable for the protein to associate with the matrix" (All asserted claims of the '997 Patent)

Amgen's Proposed Construction	Defendants' Proposed Construction
applying the refold solution to a column that contains the separation matrix without intervening steps of dilution, centrifugation, dialysis, or precipitation under conditions suitable for protein to have specific, reversible interactions with a separation matrix in order to effect the separation of protein from its environment	applying the refold solution to a separation matrix, regardless of whether there are intermediate processing steps, under conditions suitable for the protein to be purified to bind to the matrix

Here, too, the parties dispute whether "associate" is equal to or more broad than "to bind," but Amgen will accept the Defendants' construction only for this case and only because it will make no substantive difference.

The parties' disagreement is thus whether there may be certain intervening processing steps (dilution, centrifugation, dialysis, or precipitation) before the refold solution is applied to the column containing the separation matrix. Amgen's construction would exclude these particular intervening steps; Defendants' construction would allow them.

Amgen's construction comes directly from the intrinsic evidence, including the specification and the prosecution history of the '997 Patent.

The specification of the '997 Patent explicitly describes the invention as "eliminat[ing] . . . the need to dilute the protein out of a refold solution prior to capturing it on a separation matrix." '997 Patent, 3:53-57. The specification teaches that, in the prior art, components that facilitate protein refolding could "inhibit purification," and that prior artists thought it "necessary to isolate or dilute the protein from these components for further processing, particularly before applying the protein to a separation matrix." *Id.,* 4:52-57. The inventors of the '997 Patent recognized that dilution can be time-consuming and resource-intensive, *see id.*, 12:45-46, and that it "significantly increases the volumes that need to be handled, as well as the associated tankage requirements, which can become limiting when working on large scales." *Id.*, 12:46-49. Their invention thus eliminated the need to dilute the components of the solution used for refolding the protein. *Id.*, 15:50-54.

The prosecution history of the '997 Patent also supports Amgen's proposed construction. In prosecution, Amgen surrendered three specific intervening steps that had been disclosed in a prior-art reference: dialysis, precipitation, and centrifugation. Claim 9 was initially rejected by the Patent Examiner as anticipated by and obvious over U.S. Patent No. 7,138,370 ("Oliner"). Ex. 5 (Excerpts from '997 Patent Prosecution History), at 8-9. Amgen distinguished Oliner stating:

> . . . . Claim 9 recites, *inter alia*, (b) forming a refold solution; and (c) applying the refold solution to a separation matrix under conditions suitable for the protein to associate with the matrix. In contrast, ***[Oliner] recites that the refolded protein is subject to dialysis, precipitation, and centrifugation***. *See*, [Oliner], col 76, lns 51-59. The supernatant of [Oliner] is then pH adjusted and loaded onto a column. Because ***[Oliner] does not recite forming a refold solution and applying the refold solution to a separation matrix***, [Oliner] fails to teach each and every element of claim [9].

Id. at 22 (emphases added); *see* Ex. 7 (Oliner), 76:51-61. Amgen unequivocally and repeatedly distinguished Oliner because of the dialysis, precipitation, and centrifugation that occurred between Oliner's forming its refold solution and applying the refold solution to a separation matrix. "[W]here the patentee has unequivocally disavowed a certain meaning to obtain [its] patent, the doctrine of prosecution disclaimer attaches and narrows the ordinary meaning of the claim congruent with the scope of the surrender." *Omega Eng'g, Inc. v. Raytek Corp.*, 334 F.3d 1314, 1324 (Fed. Cir. 2003). Amgen's proposed construction accordingly narrows the scope of the claim term "congruent with the scope of the surrender," by

expressly identifying and excluding the three steps recited in Oliner: dialysis, precipitation, and centrifugation.

* * * *

Notably, the specification and prosecution history do not exclude—and Amgen's proposed construction would not exclude—all intervening steps. The specification teaches that filtration, for example, may be an intervening step. *Id.*, 19:34-39, 20:56-62. Amgen's proposed construction would exclude only those intervening steps that the intrinsic evidence excludes: dilution because the specification excludes it, and dialysis, precipitation, and centrifugation because Amgen disclaimed them during prosecution. Other intervening steps are permitted.

B. '287 Patent

1. Disputed Term: "refold mixture" (Claims 1, 10, and all asserted claims depending therefrom)

Amgen's Proposed Construction	Defendants' Proposed Construction
A mixture formed from contacting (1) the proteins with (2) the refold buffer.	A mixture formed from contacting the entire protein-containing volume with the entire volume of the preparation. The refold mixture has a high protein concentration, where 'high protein concentration' is at or above about 1g/L protein.

The parties agree that the "refold mixture" is a mixture formed from contacting the protein with the preparation. Defendants' construction, however, adds two improper limitations: (1) that the "entire volumes" of the preparation and

the protein-containing volume be contacted and (2) that the refold mixture have a protein concentration at or above 1g/L protein. Neither limitation is warranted. Additionally, Amgen's construction makes explicit that the "preparation" of independent claims 1 and 10 is synonymous with what the specification calls the refold buffer.

a. Defendants' "entire volume" language is superfluous and confusing. The claim says that "the proteins" are contacted with "a preparation that supports the renaturation of at least one of the proteins to a biologically active form" to form a "refold mixture." '287 Patent, claims 1 and 10. The claims and the specification never say that the "entire" protein-containing volume must be contacted with the "entire volume of the preparation" to form a refold mixture. *See Digital Biometrics, Inc. v. Identix, Inc.*, 149 F.3d 1335, 1344 (Fed. Cir. 1998) ("The actual words of the claim are the controlling focus."). Rather, the claimed preparation simply comprises certain ingredients and supports the renaturation of at least one of the proteins. If a preparation meeting those requirements is contacted with proteins, the resulting liquid is a "refold mixture," regardless of whether the "entire volume" of the preparation and/or proteins is added.

A numerical example is instructive: If a scientist prepares a 1 L preparation according to the method of claim 1, and then contacts only 0.5 L of that 1 L preparation with a 0.3 L proteins-containing volume, the "preparation" would be the

0.5 L preparation that is actually contacted with the proteins. The "refold mixture" would then be the 0.8 L mixture formed by contacting the 0.3 L proteins-containing volume and the 0.5 L preparation. Nothing in the claims or the specification or the prosecution history mandates that the "refold mixture" must be the full preparation plus the full proteins-containing volume, as Defendants propose.

b. Defendants' proposed inclusion of a protein-concentration limitation is not supported by the claim language. Indeed, the independent claims contain ***no*** recitation regarding protein concentration, in the refold mixture or otherwise. '287 Patent, claims 1, 10, 16 and 26. Instead, some of the dependent claims contain protein-concentration limitations. *Id.*, claims 2, 3, 11, 13, 17, 18, 27, and 28. For example, claim 2 depends from claim 1 and adds "wherein the refold mixture has a protein concentration in a range of 1-40 g/L." Under the doctrine of claim differentiation, the dependent claims inform the construction of the independent claims, and they confirm that the independent claims should not be construed to require a protein concentration of one or more grams per liter.

c. Amgen's proposed construction makes explicit that the "preparation" named in the claims is synonymous with the "refold buffer" described in the specification. The specification's "refold buffer" and the claims' "preparation" each comprise the same ingredients (one or more of a denaturant, an aggregation suppressor and a protein stabilizer; an amount of oxidant; and an amount of

reductant); get contacted with protein to form a refold mixture; and support the renaturation of protein to a biologically active form. *See, e.g.*, '287 Patent, claim 1 and 10, 2:62-66, 1:50-53, 3:19-21, 3:39-40, 3:41-42, 10:51-53, 11:10-18, 11:56-60, 11:62-66, 15:44-48, 16:23-28. Persons of ordinary skill in the art reading the claims and the specification would understand that the "preparation" of the claims is synonymous with the "refold buffer" of the specification. Page Decl. ¶ 51.

2. Disputed Term: "thiol-pair ratio" (All asserted claims)

Amgen's Proposed Construction	Defendants' Proposed Construction
Defined by the following equation $\frac{[reductant]^2}{[oxidant]}$	Indefinite. *Alternatively*: Defined by the following equation: $\frac{[reductant]^2}{[oxidant]}$ where the concentrations are the concentrations in the redox component.

a. The term "thiol-pair ratio" is definite. Defendants assert that "because the patent does not specify a unit of measure for the claimed 'thiol-pair ratio,' it does not convey with reasonable certainty the scope of the invention claimed." D.I. 101 at 54. Not so. As is clear from the specification, the concentrations of oxidant and reductant that are in the thiol-pair ratio equation are measured in millimolar (mM), a common scientific notation of concentration expressing the number of millimoles of a substance in a defined volume of solution. A "mole" of a substance is its molecular weight in grams. (By way of example, the molecular weight of glucose is 180, so 1 mole of glucose is 180 g of glucose.) One "millimole" is a thousandth

of a mole. A "1 M" (one molar) solution of glucose has a concentration of 180 grams per liter, while a "1 mM" (one millimolar) solution of glucose has a concentration of 0.18 grams per liter. The concentrations at issue in the '287 Patent are expressed in terms of millimolarity. Thus, the thiol-pair ratio is determined using millimolar (mM) for the concentrations, and the specification so informs a person of ordinary skill with far-more-than-reasonable certainty.

The claims of '287 Patent use ***only*** the unit millimolar for concentration. Claims 4, 12, 19, and 29 recite that the thiol-pair buffer strength is "2 ***mM*** or greater" (emphasis added). The thiol-pair buffer strength equals twice the concentration of the oxidant plus the concentration of the reductant. As Dr. Page explains, because the thiol-pair buffer strength is expressed using the unit millimolar, a person of ordinary skill would understand the concentrations of the oxidant and the reductant to be measured in the same unit, and would use the numerical millimolar values in the thiol-pair-ratio equation. Page Decl. ¶¶ 54-55, 57.

The specification of the '287 Patent repeatedly and exclusively uses millimolarity for concentration of oxidants, the concentration of reductants, and the thiol-pair buffer strength. *E.g.*, '287 Patent figures 1a-1f, 2:67-3:1, 3:9-18, 9:18-22, 10:59-67, 11:24-34, 11:47-56, 12:11-20, 13:20-21, 15:5-6, 15:44-48, 16:28-30.

A person of ordinary skill calculating the thiol-pair ratio would use the numerical values of the millimolar concentrations that are consistently and

exclusively used by the claims and specification for the concentrations of oxidants and reductants. Page Decl. ¶¶ 56-57.

Adello itself uses millimolar to calculate the thiol-pair ratio. When Adello petitioned for post-grant review of the '287 Patent, both Adello and its expert used millimolar units for the concentrations of oxidant and reductant in the thiol-pair ratio equation. In determining the thiol-pair ratio used in a reference called Schlegl, Adello and its expert used 2 ***mM*** cysteine for the concentration of the reductant and 2 ***mM*** cystine for the concentration of the oxidant, to get a thiol-pair ratio of 2. Ex. 8 (Adello's Petition for Post Grant Review, dated Oct. 1, 2018), 55-56; Ex. 9 (Declaration of Anne S. Robinson, Ph.D) ¶ 122 n.7; Ex. 10 (Schlegl) ¶ [0075]. Adello's expert had no difficulty understanding the claim language that Adello now says is hopelessly indefinite. There is nothing indefinite about it.

b. With respect to Defendants' alternative proposed construction, the parties agree that the thiol-pair ratio is defined by the equation $\frac{[reductant]^2}{[oxidant]}$. The parties disagree about where the concentrations of reductant and oxidant are to be determined. The Defendants contend that the concentrations are always determined in the "redox component," for all claims. Amgen contends that different claims specify different locations for determining the concentrations. The parties have the same dispute with respect to the term "thiol-pair buffer strength."

Amgen's constructions are faithful to the claims, which specify in which liquid the thiol-pair ratio and the thiol-pair buffer strength should be determined. In independent claim 1, for example, the claim specifies that the thiol-pair values are determined in the "preparation." In claim 1, the "***preparation*** compris[es]…an amount of oxidant; and an amount of reductant, . . . ***wherein the thiol-pair ratio is in the range of 0.001-100***; and ***wherein the thiol-pair buffer strength maintains the solubility of the preparation*** . . . ." '287 Patent, claim 1 (emphasis added). In contrast, in independent claim 16, for example, the thiol-pair values are determined in the "solution": the "***solution*** compris[es]…an amount of oxidant; and an amount of reductant, . . . ***wherein the thiol-pair ratio is in the range of 0.001-100***, and ***wherein the thiol-pair buffer strength maintains the solubility of the solution*** . . . ." '287 Patent, claim 16 (emphasis added).

Defendants' assertion that the concentrations of the thiol-pair values are concentrations in the "redox component" is not supported by the claim language. Indeed, the claims do not even use the term "redox component."

3. Disputed Term: "thiol-pair buffer strength" (All asserted claims)

Amgen's Proposed Construction	Defendants' Proposed Construction
Defined by the following equation $2[oxidant] + [reductant]$	Defined by the following equation: $2[oxidant] + [reductant]$, where the concentrations are the concentrations in the redox component, and where the thiol-pair buffer strength

	is effectively bounded at a maximum of 100 mM.

The parties agree that the "thiol-pair buffer strength" is defined by the equation $2[oxidant] + [reductant]$.

a. As set forth immediately above, the parties disagree about whether the concentration is measured "in the redox component," which Defendants propose even though that term appears in none of the claims, or in the "preparation" or "solution" recited in independent claims 1 and 10 or 16 and 26, respectively, which Amgen proposes.

b. The parties also disagree about whether to include Defendants' proposed limitation that the "thiol pair buffer strength is effectively bounded at a maximum of 100 mM."

The claims do not support imposing a maximum thiol-pair buffer strength. Indeed, the claims recite ***no*** numerical limitation for the "thiol-pair buffer strength." The limitation is instead a functional one: the thiol-pair buffer strength must maintain the solubility of the preparation (claims 1 and 10) or the solution (claims 16 and 26). And the patentees knew how to include numerical limitations for the thiol-pair buffer strength had they chosen to do so. The specification, in discussing particular embodiments, several times repeats "a Thiol-pair buffer strength equal to or greater than 2 mM, for example greater than or equal to 2.25 mM, 2.5 mM, 2.75 mM, 3 mM, 5 mM, 7.5 mM, 10 mM, or 15 mM, wherein the thiol-pair buffer

strength is effectively bounded at a maximum of 100 mM." *E.g.*, '287 Patent, 3:10-14. And dependent claims 4, 12, 19, and 29 recite a numerical lower boundary of 2 mM. That none of the independent claims (1, 10, 16, and 26) includes any numerical limitation, let alone an upper boundary of 100 mM, confirms that no such boundary is intended. *See Digital Biometrics, Inc.*, 149 F.3d at 1344 ("The actual words of the claim are the controlling focus.").

The specification also rejects imposing a maximum thiol-pair buffer strength. The specification introduces the concept of a maximum boundary in the context of an embodiment, not as a limitation on the invention. The patent's Summary of the Invention section first describes the invention as a method comprising a thiol-pair buffer strength, without mention of an effective maximum boundary. '287 Patent, 2:62-3:4. The next paragraph discusses "various embodiments" and introduces exemplary ranges of thiol-pair buffer strengths and the effective maximum boundary of 100 mM. *Id.*, 3:5-18. Accordingly, the patent never describes the effective maximum boundary "as the present invention, as essential, or as important." *See GE Lighting Sols., LLC v. AgiLight, Inc.*, 750 F.3d 1304, 1309-10 (Fed. Cir. 2014); *see also Medegen MMS, Inc. v. ICU Med., Inc.*, 317 F. App'x 982, 986 (Fed. Cir. 2008) (nonprecedential) (looking to the summary of the invention section to determine whether to read a limitation into a claim term).

4. Disputed Term: "wherein the amounts of the oxidant and the reductant are related through a thiol-pair ratio and a thiol-pair buffer strength" (All asserted claims)

Amgen's Proposed Construction	Defendants' Proposed Construction
wherein the amounts of the oxidant and the reductant are defined by the following equations: $\frac{\left(\sqrt{bufferTPR^2 + 8 * bufferTPR * BS}\right) - bufferTPR}{4}$ and $\frac{(Concentration\ of\ Reduced\ Redox\ Component)^2}{TPR}$	wherein the amounts of the oxidant and the reductant are selected by performing the following equations: $\frac{\left(\sqrt{bufferTPR^2 + 8 * bufferTPR * BS}\right) - bufferTPR}{4}$ and $\frac{(Concentration\ of\ Reduced\ Redox\ Component)^2}{TPR}$

The parties' disagreement is not about Equations 3 and 4 themselves, but about how those equations are implemented. Amgen contends that Equations 3 and 4 are a way to define the amounts of the oxidant and the reductant. Under Amgen's interpretation, a process in which the amounts of oxidant and reductant are satisfied by Equations 3 and 4 is covered by the claim whether the person designing the process determined the amounts of oxidant and reductant by using the equations or by some other means, for example, independent experimental work. Defendants counter that the person who infringes the claim must actually use Equations 3 and 4 to determine the amounts of the oxidant and the reductant. Under Defendants' interpretation, whether a process is covered depends on whether the person that designed the process determined the amounts of oxidant and reductant using Equations 3 and 4.

Equations 3 and 4 of the '287 Patent specification provide the mathematical relationship between (1) the "Concentration of Reduced ***Redox Component***" and the "Concentration of Oxidized ***Redox Component***" and (2) the thiol-pair ratio and thiol-pair buffer strength. '287 Patent, 7:1-19 (emphases added). The specification defines a "redox component" as "any thiol-reactive chemical or solution comprising such a chemical that facilitates a reversible thiol exchange with another thiol or the cysteine residues of a protein." *Id.*, 7:20-23. The specification provides examples of such compounds: "glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine, beta-mercaptoethanol and combinations thereof." *Id.*, 7:23-27. Thus, not all amounts of oxidants and reductants are defined by Equations 3 and 4—only those amounts that are "redox components." As Dr. Page explains, Equations 3 and 4 account for the complex chemistry of "redox components," *i.e.*, those chemicals that facilitate reversible thiol exchanges (also known as reshuffling of disulfide bonds) during protein refolding. Page Decl. ¶ 50. Thus, "the amounts of the oxidant and the reductant are related through a thiol-pair ratio and a thiol-pair buffer strength" when they facilitate reshuffling of disulfide bonds, the only oxidants and reductants to which Equations 3 and 4 apply.

Defendants' contention that the infringer must actually use Equations 3 and 4 to determine the amounts of the oxidant and the reductant finds no support in the intrinsic evidence. Notably, dependent claims 8, 9, 14, 15, 23, 25, and 30 expressly

require the thiol-pair buffer strength and thiol-pair ratio to be "calculated," which (the parties agree) does require use of the Equations to determine the redox conditions under which to perform the refold. But the independent claims lack the requirement of calculation and instead speak only of the relationship between the variables of the Equations. "[D]ifferent words or phrases used in separate claims are presumed to indicate that the claims have different meanings and scope." *Karlin Tech., Inc. v. Surgical Dynamics, Inc.*, 177 F.3d 968, 972 (Fed. Cir. 1999). This "normally means that limitations stated in dependent claims are not to be read into the independent claim from which they depend." *Id.* A requirement of "calculating" should thus not be read into the independent claims.

Further, the prosecution history refutes Defendants' proposal. During prosecution, Amgen made arguments to overcome certain prior art cited by the examiner. Ex. 6 (Excerpts from '287 Patent Prosecution History), at 17-26. In its July 17, 2017 response, Amgen first addressed reasons why the independent claim (then-claim 25), which included the "related through" language at issue here, overcomes the prior art cited by the examiner. *Id.* at 17-18, 21-23. Amgen never said that the independent claim required the use of equations to choose redox conditions for the refold. *Id.* Amgen then specified that the dependent claims that required "calculat[ing]" (then-numbered claims 34 and 35) are also patentable for "additional reasons." *Id.* at 19-20, 23-25. Only then did Amgen make arguments

that the claims require, and the prior art lacks, the use of the equations. *Id.* at 20, 24-25. For example, Amgen argued that then-pending dependent claim 34 recited that the "thiol-pair ratio is calculated, and thus derived, according to the following equation: $\frac{[the\ reductant]^2}{[the\ oxidant]}$," and that the cited reference, Oliner, "does not even suggest that either equation is used to calculate the thiol-pair ratio value or the thiol-pair buffer strength." *Id.* at 20. If the "related through" term means that equations must be used *ab initio* to choose the redox conditions for refolding, as Defendants propose here, Amgen would have distinguished Oliner from the independent claim on the ground that the Oliner method lacks the use of equations (as Amgen did for the dependent claims).

5. Disputed Term: "wherein the thiol-pair buffer strength maintains the solubility of the preparation" (Claims 1, 10, and all asserted claims depending therefrom)

Amgen's Proposed Construction	Defendants' Proposed Construction
wherein the thiol-pair buffer strength maintains the solubility of the solutes in the refold buffer	Indefinite.

The term "wherein the thiol-pair buffer strength maintains the solubility of the preparation" is definite. Defendants state that a person of ordinary skill would "have noted that the plain language of the claims establishes that the 'preparation' does not contain proteins" and would have recognized that the preparation "cannot be 'solubilized' as that term is used in the '287 Patent specification," and assert that

"'solubility' is a concept inapplicable to the preparation under that construction." D.I. 101 at 64. While the parties agree that the claimed "preparation" does not contain proteins, the parties disagree that solubility is a "concept inapplicable to the preparation." The claims when read in light of the specification inform with at least reasonable certainty that "maintaining the solubility of the preparation" means maintaining the solubility of the solutes in the preparation (also known as the refold buffer), which include (1) at least one of a denaturant, an aggregation suppressor, and a protein stabilizer; (2) an oxidant; and (3) a reductant.

As Dr. Page explains, a person of ordinarily skill would know that solubility is a chemical property defining the amount of a given substance (the solute) that can dissolve in another substance (the solvent). Page Decl. ¶¶ 59-62. A dissolved solute is called "in solution." *Id.* ¶ 59. Skilled artisans would understand that there are solutes in the preparation whose solubility should be maintained. *Id.* ¶¶ 61-62. Indeed, the specification teaches that denaturants and reductants, which the preparation may comprise, should be in solution. '287 Patent, 13:12-15 ("The solubilized inclusion bodies are then diluted to achieve reduction of the denaturants and reductants ***in the solution*** to a level that allows the protein to refold." (emphasis added)). A skilled artisan would also know that other ingredients that the preparation may comprise are solutes, which are dissolved in the preparation. Page Decl. ¶ 61. The thiol-pair buffer strength can maintain the solubility of those solutes. As Dr.

Page explains, if the thiol-pair buffer strength is too high (meaning the concentrations of oxidant and reductant are too high), the solubility of one of the solutes in the preparation may be exceeded, causing one of the solutes to come out of solution. *Id.* ¶ 62. A person of ordinary skill would thus understand that the claim term requires the solutes of the preparation to remain in solution during the method. *Id.* ¶¶ 61-62.

C. '138 Patent

Disputed Terms		
Claim Term	**Amgen's Proposed Construction**	**Defendants' Proposed Construction**
"a redox component" (All asserted claims)	Any thiol-reactive chemical or combinations of such chemicals, or solution comprising such a chemical or chemicals that facilitates a reversible thiol exchange with another thiol or the cysteine residues of a protein.	A single volume consisting of a combination of reductant and oxidant that facilitates a reversible thiol exchange between thiols or with the cysteine residues of a protein. The redox component comprises a final thiol-pair ratio in the range of 0.001-100 and a redox buffer strength of 2mM or greater.
"final thiol-pair ratio having a range of 0.001 to 100" (All asserted claims)	The relationship of the reduced and oxidized redox species used in the redox component of the refold buffer as defined by the equation $\frac{[reductant]^2}{[oxidant]}$, having a range of 0.001 to 100	Indefinite. *Alternatively*: Defined by the following equation: $\frac{[reductant]^2}{[oxidant]}$ where the concentrations are the concentrations in the redox component.
"redox buffer strength" (All asserted claims)	$2[oxidant] + [reductant]$	Defined by the following equation: $2[oxidant] + [reductant]$

		where the concentrations are the concentrations in the redox component.
"2 mM or greater" (All asserted claims)	greater than or equal to 2 mM	2 mM or greater, wherein the redox buffer strength is effectively bounded at a maximum of 100 mM
"refold mixture" (All asserted claims)	A mixture formed from contacting (1) the protein with (2) a refold buffer.	A mixture formed from contacting (1) the entire volume in which the concentration of protein is 2.0g/L or greater with (2) the entire volume of refold buffer. The refold mixture has a high protein concentration, where 'high protein concentration' is at or above about 1g/L protein.

The '138 patent is currently the subject of an *inter partes* review ("IPR") proceeding, *Apotex Inc. v. Amgen Inc.*, IPR2016-01542, in which, in its final written decision, the Patent Trial and Appeal Board (the "Board"), after construing several claim terms, found claim 18 not unpatentable and found the other claims of the '138 Patent unpatentable. *See* Ex. 11 (Final Written Decision, *Apotex Inc. v. Amgen Inc.*, IPR2016-01542 (Feb. 15, 2018) (Paper 60)). The petitioners in that IPR, Apotex Inc. and Apotex Corp., requested reconsideration of the Board's finding that claim 18 is not unpatentable. *See* Ex. 12 (Apotex's Request for Rehearing, *Apotex Inc. v. Amgen Inc.*, IPR2016-01542 (Mar. 16, 2018) (Paper 61)). That request is pending with the Board. After the Board decides petitioners' request, Amgen will have the

right to appeal to the Federal Circuit. Amgen reserves its rights to appeal, including to challenge the Board's claim constructions.

Nevertheless, at present, the Board's constructions in the IPR proceeding are "an important part of the intrinsic record relevant to claim construction." *See Evolutionary Intelligence, LLC v. Sprint Nextel Corp.*, No. C-13-03587, 2014 WL 4802426, at *4 (N.D. Cal. Sept. 26, 2014); *see also Fairfield Indus., Inc. v. Wireless Seismic, Inc.*, No. 4:14-CV-2972, 2015 WL 1034275, at *5 (S.D. Tex. Mar. 10, 2015) ("Although PTAB applies a different construction standard than the district courts do, its claim construction analysis serves as further intrinsic evidence" as to the proper construction of a claim term.). The proposed constructions Amgen provides are the Board's constructions. While the Board did not expressly construe "a redox component," which was identified for construction by Defendants, the construction provided is consistent with the Board's constructions in that the Board construed the "final thiol-pair ratio" and "redox buffer strength" relationships to be in the refold buffer, not the redox component. *See* Ex. 11, Final Written Decision, *Apotex Inc. v. Amgen Inc.*, IPR2016-01542, at 10-11; *see also, e.g.*, *id.* at 31 (determining thiol-pair ratio in the "refold buffers in Hevehan," a prior-art reference asserted by the petitioners). The Board also did not construe the term "2 mM or greater." For this term, the parties have a similar dispute to that discussed above for the term "thiol-pair buffer strength" of the '287 Patent.

V. CONCLUSION

For the foregoing reasons, Amgen respectfully requests that the Court adopt Amgen's proposed constructions for the disputed claim terms of the Patents-in-Suit.

Dated: April 15, 2019
Newark, New Jersey

s/ Liza M. Walsh

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IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF NEW JERSEY

AMGEN INC. and AMGEN MANUFACTURING, LIMITED, Plaintiffs, v. ADELLO BIOLOGICS, LLC, AMNEAL PHARMACEUTICALS, LLC, and AMNEAL PHARMACEUTICALS, INC. Defendants.	Hon. Claire C. Cecchi No. 18-cv-3347-CCC-MF

DECLARATION OF PETER SANDEL IN SUPPORT OF AMGEN'S OPENING CLAIM CONSTRUCTION BRIEF

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I, Peter Sandel, declare and state as follows:

1. I am an attorney at law in the State of New York where I am a member in good standing of the bar and I have been granted admission to practice *pro hac vice* in the above-captioned matter. I am counsel at the law firm Paul, Weiss, Rifkind, Wharton & Garrison LLP, attorneys of record for plaintiffs Amgen Inc. and Amgen Manufacturing, Limited (together, "Amgen"). I have personal knowledge of the facts set forth in this Declaration which I submit in support of Amgen's Opening Claim Construction Brief.

2. Attached hereto as **Exhibit 1** is a true and correct copy of U.S. Patent No. 8,940,878 ("'878 Patent").

3. Attached hereto as **Exhibit 2** is a true and correct copy of U.S. Patent No. 9,643,997 ("'997 Patent").

4. Attached hereto as **Exhibit 3** is a true and correct copy of U.S. Patent No. 8,952,138 ("'138 Patent").

5. Attached hereto as **Exhibit 4** is a true and correct copy of U.S. Patent No. 9,856,287 ("'287 Patent").

6. Attached hereto as **Exhibit 5** is a true and correct copy of excerpts from the file history of U.S. Appl. No. 14/599,336, which later issued as U.S. Patent No. 9,643,997, to which sequential page numbers were added.

7. Attached hereto as **Exhibit 6** is a true and correct copy of excerpts from the file history of U.S. Appl. No. 15/422,327, which later issued as U.S. Patent No. 9,856,287, to which sequential page numbers were added.

8. Attached hereto as **Exhibit 7** is a true and correct copy of excerpts from U.S. Patent No. 7,138,370 ("Oliner") as produced by Adello, bearing Bates numbers AB066934-AB067099.

9. Attached hereto as **Exhibit 8** is a true and correct copy of excerpts from the Petition for Post-Grant Review of U.S. Patent No. 9,856,287 in *Adello Biologics LLC, v. Amgen Inc.*, PGR2019-00001 (Oct. 1, 2018) (Paper 3).

10. Attached hereto as **Exhibit 9** is a true and correct copy of excerpts from the Declaration of Anne S. Robinson, Ph.D. in *Adello Biologics LLC, v. Amgen Inc.*, PGR2019-00001 (Oct. 1, 2018) (Ex. 1002).

11. Attached hereto as **Exhibit 10** is a true and correct copy of U.S. Appl. No. 11/695,950 ("Schlegl") entitled "Method for Refolding a Protein" filed on

Apr. 3, 2007, as it was submitted in *Adello Biologics LLC, v. Amgen Inc.*, PGR2019-00001 (Oct. 1, 2018) (Ex. 1007).

12. Attached hereto as **Exhibit 11** is a true and correct copy of the Final Written Decision by the Patent Trial and Appeal Board in *Apotex Inc. v. Amgen Inc.*, IPR2016-01542 (Feb. 15, 2018) (Paper 60).

13. Attached hereto as **Exhibit 12** is a true and correct copy of excerpts from Apotex's Request for Rehearing, *Apotex Inc. v. Amgen Inc.*, IPR2016-01542 (Mar. 16, 2018) (Paper 61).

I declare under penalty of perjury under the laws of the United States that the foregoing is true and correct and that the foregoing was executed on April 15, 2019 in New York, New York.

Peter Sandel

Exhibit 1

US008940878B2

(12) **United States Patent**
Shultz et al.

(10) **Patent No.: US 8,940,878 B2**
(45) **Date of Patent: *Jan. 27, 2015**

(54) **CAPTURE PURIFICATION PROCESSES FOR PROTEINS EXPRESSED IN A NON-MAMMALIAN SYSTEM**

(75) Inventors: **Joseph Edward Shultz**, Santa Rosa Valley, CA (US); **Roger Hart**, Loveland, CO (US)

(73) Assignee: **Amgen Inc.**, Thousand Oaks, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 471 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **12/822,990**

(22) Filed: **Jun. 24, 2010**

(65) **Prior Publication Data**

US 2010/0331526 A1 Dec. 30, 2010

Related U.S. Application Data

(60) Provisional application No. 61/220,477, filed on Jun. 25, 2009.

(51) **Int. Cl.**

C07K 1/22	(2006.01)
C07K 1/14	(2006.01)
C07K 1/18	(2006.01)
C07K 1/32	(2006.01)

(52) **U.S. Cl.**

CPC . ***C07K 1/22*** (2013.01); ***C07K 1/145*** (2013.01); ***C07K 1/18*** (2013.01); ***C07K 1/32*** (2013.01)

USPC **530/413**

(58) **Field of Classification Search**

CPC C07K 1/22; C07K 1/18; C07K 1/32; C07K 1/145

See application file for complete search history.

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(57) **ABSTRACT**

Methods of purifying proteins expressed in non-mammalian expression systems in a non-native soluble form directly from cell lysate are disclosed. Methods of purifying proteins expressed in non-mammalian expression systems in a non-native limited solubility form directly from a refold solution are also disclosed. Resin regeneration methods are also provided.

25 Claims, 5 Drawing Sheets

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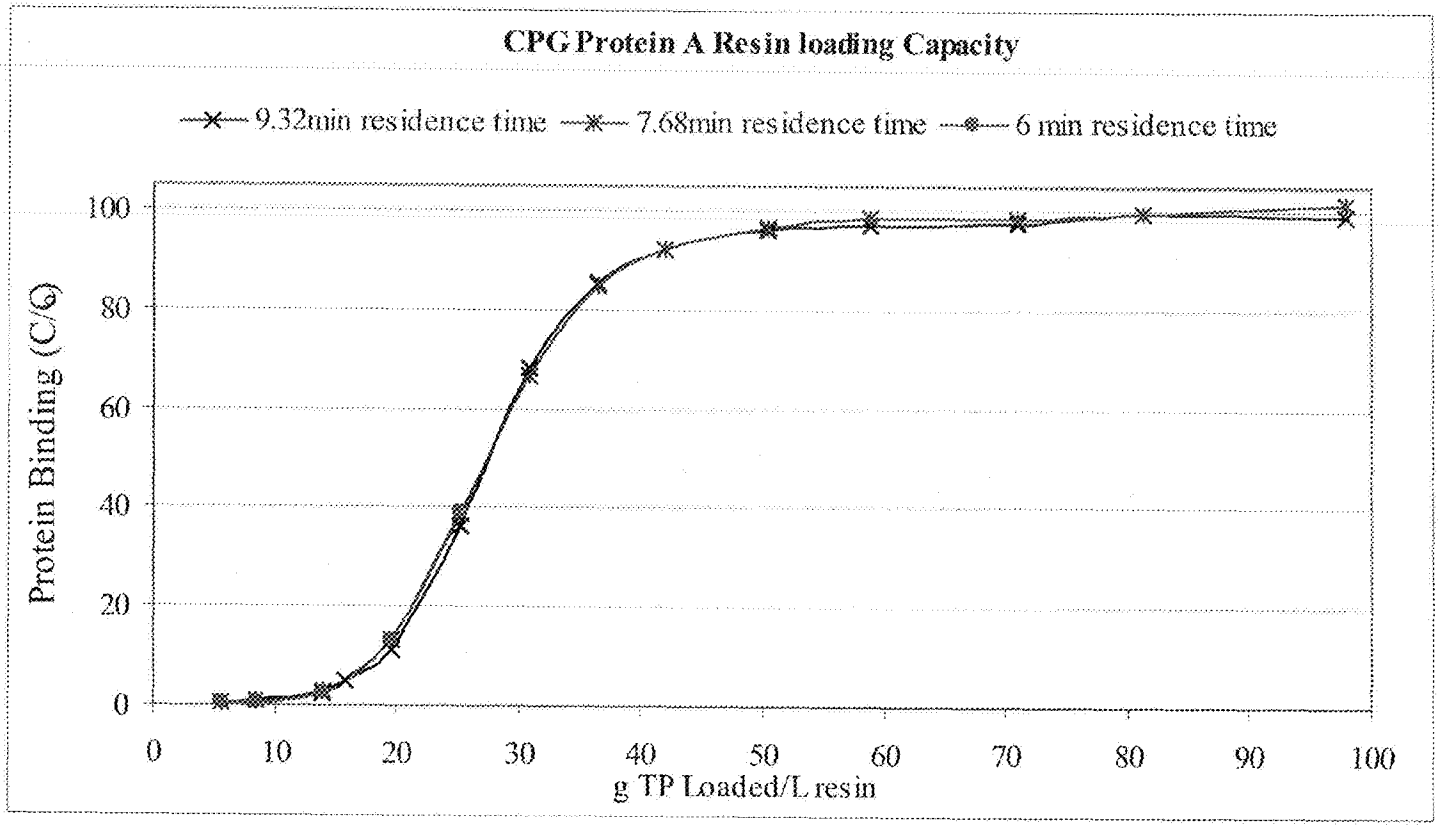
CPG Protein A Resin loading Capacity
9.32min residence time
7.68min residence time
6 min residence time
Protein Binding (C/G)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
100
g TP Loaded/L resin

Figure 1

		Average Purity					
		RP-HPLC Main Peak Purity (%)	SE-HPLC Main Peak Purity (%)	CE-SDS Main Peak Purity (%)	Host Protein Level (ppm)	DNA Level (pg/mg protein)	Average Yield (%)
Load	Average (n=13)	34.5	74.5	79.2	9100.0	>70000	-
	Std. Dev (n=13)	2.4	2.7	4.4	424.3	*	-
Purified Pool	Average (n=17)	41.3	68.8	84.7	41.0	215.2	81.7
	Std. Dev (n=17)	1.5	3.8	4.0	5.7	301.2	12.3

* Data limited to N=1

Figure 2

		Average Purity					
		RP-HPLC Main Peak Purity (%)	SE-HPLC Main Peak Purity (%)	CE-SDS Main Peak Purity (%)	Host Protein Level (ppm)	DNA Level (pg/mg protein)	Average Yield (%)
Load	Average (n=5)	36.0	76.1	75.5	1400.0	>70000	-
	Std. Dev (n=5)	0.9	1.9	1.5	*	*	-
Purified Pool	Average (150 cycles)	40.2	75.0	82.4	71.4	89.2	84.3
	Std. Dev (150 cycles)	2.5	8.7	4.6	23.0	175.0	18.8

* Data limited to N=1

Figure 3

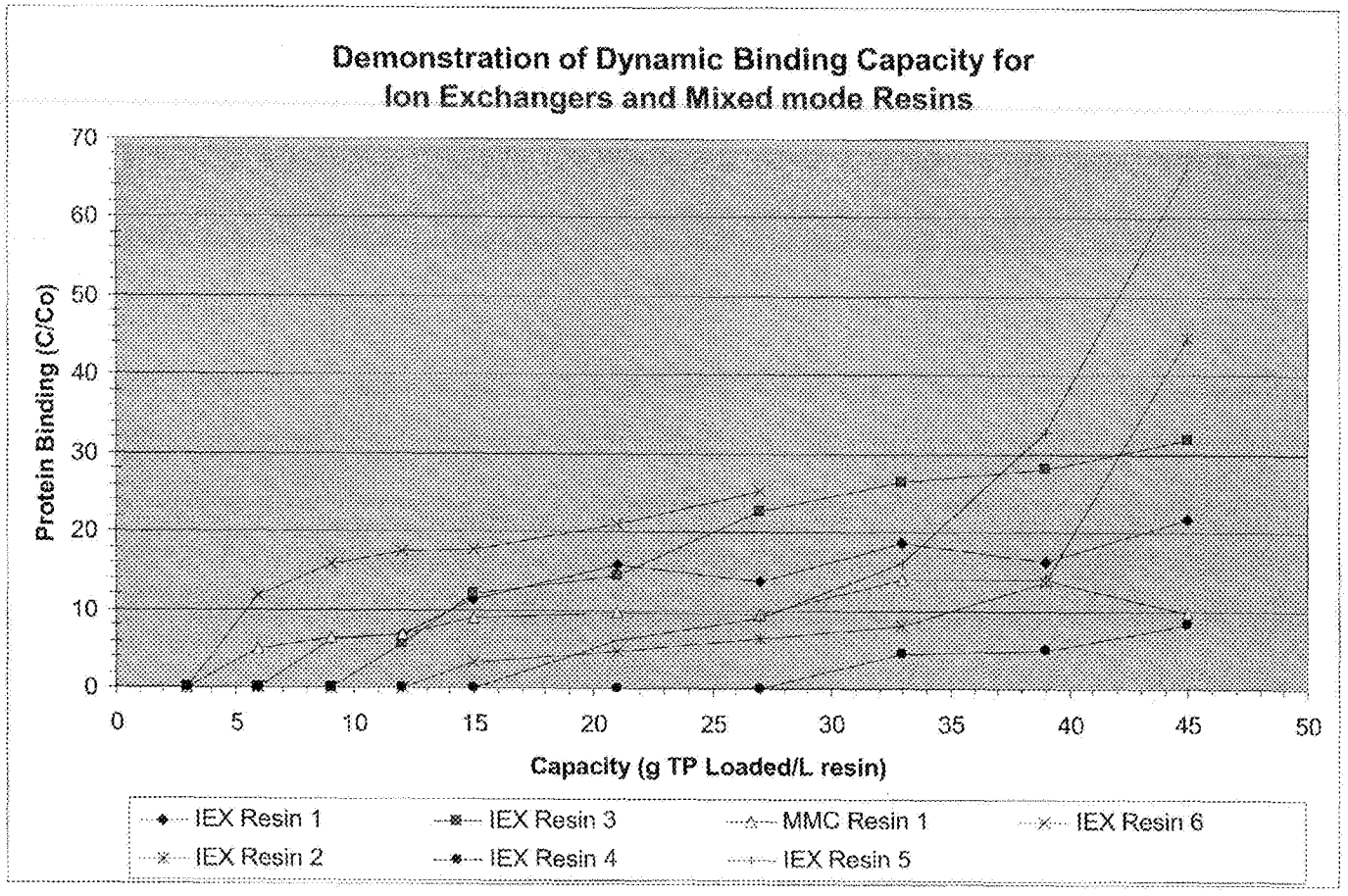
Demonstration of Dynamic Binding Capacity for Ion Exchangers and Mixed mode Resins
Protein Binding (C/Co)
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
50
Capacity (g TP Loaded/L resin)
IEX Resin 1
IEX Resin 3
MMC Resin 1
IEX Resin 6
IEX Resin 2
IEX Resin 4
IEX Resin 5

Figure 4

	RP-HPLC Main Peak Purity (%)	SE-HPLC Main Peak Purity (%)	Average Yield (%)
Load	29.8	64.6	-
CEX	46.0	80.3	62.0
AEX	30.9	75.7	85.0

Figure 5

CAPTURE PURIFICATION PROCESSES FOR PROTEINS EXPRESSED IN A NON-MAMMALIAN SYSTEM

This application claims the benefit of U.S. Provisional Application No. 61/220,477 filed Jun. 25, 2009, which is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates generally to processes for purifying proteins expressed in non-mammalian systems in both non-native soluble and non-native insoluble forms, and more particularly to the direct capture of such proteins from a refold mixture or a cell lysate pool by a separation matrix.

BACKGROUND OF THE INVENTION

Fc-containing proteins are typically expressed in mammalian cells, such as CHO cells. The use of affinity chromatography to purify Fc-containing proteins is documented (see, e.g., Shukla et al., (2007) *Journal of Chromatography B* 848 (1):28-39) and is successful, in part, due to the degree of Fc structure observed in proteins expressed in such systems. Fc-containing proteins expressed in non-mammalian cells, however, are often deposited in the expressing cells in limited solubility forms, such as inclusion bodies, that require refolding, and this has been a limiting factor in selecting non-mammalian systems for expressing Fc-containing proteins.

A drawback to the use of Protein A, Protein G and other chemistries is that in order for a protein comprising an Fc region to associate with the Protein A or Protein G molecule, the protein needs to have a minimum amount of structure. Often, the requisite amount of structure is absent from proteins expressed recombinantly in a soluble, but non-native, form and consequently Protein A chromatography is not performed in a purification process.

In the case of a protein expressed in an insoluble non-native form, Protein A chromatography is typically not performed in a purification process until after the protein has been refolded to a degree that it can associate with the Protein A molecule and has been subsequently diluted out of its refold solution. This is because it was believed that after a protein has been refolded it was necessary to dilute or remove the components of the refold mixture in a wash step, due to the tendency of the components that typically make up a refold solution to disrupt interactions between the target protein and the Protein A molecules (Wang et al., (1997). *Biochem. J.* 325 (Part 3):707-710). This dilution step can consume time and resources which, when working at a manufacturing scale of thousands of liters of culture, can be costly.

The present disclosure addresses these issues by providing simplified methods of purifying proteins comprising Fc regions that are expressed in non-mammalian expression systems in a non-native soluble form or in a non-native insoluble form.

SUMMARY OF THE INVENTION

A method of purifying a protein expressed in a non-native soluble form in a non-mammalian expression system is provided. In one embodiment the method comprises (a) lysing a non-mammalian cell in which the protein is expressed in a non-native soluble form to generate a cell lysate; (b) contacting the cell lysate with an separation matrix under conditions suitable for the protein to associate with the separation matrix; (c) washing the separation matrix; and (d) eluting the protein from the separation matrix.

The protein can be a complex protein, such as a protein is selected from the group consisting of a multimeric protein, an antibody and an Fc fusion protein. The non-mammalian expression system can comprise bacteria or yeast cells. The separation matrix can be an affinity resin, such as an affinity resin selected from the group consisting of Protein A, Protein G and a synthetic mimetic affinity resin, or it can be a non-affinity resin, such as a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin. The cell lysate can be filtered before it is contacted with the separation matrix. Although not required, the method can further comprise refolding the protein to its native form after it is eluted from the separation matrix.

A method of purifying a protein expressed in a non-native limited solubility form in a non-mammalian expression system is provided. In one embodiment that method comprises (a) expressing a protein in a non-native limited solubility form in a non-mammalian cell; (b) lysing a non-mammalian cell; (c) solubilizing the expressed protein in a solubilization solution comprising one or more of the following: (i) a denaturant; (ii) a reductant; and (iii) a surfactant; (d) forming a refold solution comprising the solubilization solution and a refold buffer, the refold buffer comprising one or more of the following: (i) a denaturant; (ii) an aggregation suppressor; (iii) a protein stabilizer; and (iv) a redox component; (e) applying the refold solution to a separation matrix under conditions suitable for the protein to associate with the matrix; (f) washing the separation matrix; and (g) eluting the protein from the separation matrix.

The non-native limited solubility form can be a component of an inclusion body. The protein can be a complex protein, such as a complex protein selected from the group consisting of a multimeric protein, an antibody, a peptibody, and an Fc fusion protein. The non-mammalian expression system can be bacteria or yeast cells. The denaturant can comprise one or more of urea, guanidinium salts, dimethyl urea, methylurea and ethylurea, the reductant can comprise one or more of cysteine, DTT, beta-mercaptoethanol and glutathione, the surfactant can comprise one or more of sarcosyl and sodium dodecylsulfate, the aggregation suppressor can be selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, tris, sodium sulfate, potassium sulfate and osmolytes, the protein stabilizer can comprise one or more of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, tris, sodium sulfate, potassium sulfate and osmolytes, and the redox component can comprise one or more of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol. The separation matrix can be an affinity resin such as an affinity resin selected from the group consisting of Protein A, Protein G, and synthetic mimetic affinity resin or the separation matrix can be a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin.

In other embodiments, the disclosed methods can further comprise the steps of (a) washing the separation matrix with a regeneration reagent; and (b) regenerating the separation matrix. The regeneration reagent can be one of a strong base, such as sodium hydroxide or a strong acid, such as phosphoric acid. The regenerating can comprise washing the separation matrix with a solution comprising one or both of a chaotrope present at a concentration of 4-6 M and a reductant. The

chaotrope can be one of urea, dimethyl urea, methylurea, ethylurea, and guanidinium, and the reductant can be one of cysteine, DTT, beta-mercaptoethanol and glutathione. In a particular embodiment the regenerating comprises washing the separation matrix with a solution comprising 50 mM Tris, 10 mM citrate, 6M urea, 50 mM DTT at pH 7.4.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a plot demonstrating the binding of refolded, non-mammalian non-native limited solubility fraction complex protein, to Protein A media; in the figure the X denotes resin loading at a 9.32 min residence time, star denotes resin loading at a 7.68 min residence time and solid circles denote resin loading at a 6 min residence time.

FIG. **2** is a table demonstrating purification of a complex protein comprising an Fc domain using Protein A resin.

FIG. **3** is a table demonstrating the reusability of Protein A resin when used to capture a non-mammalian non-native limited solubility complex protein over 150 cycles using the disclosed methods.

FIG. **4** is a plot demonstrating the binding profiles of a refolded, non-mammalian non-native limited solubility complex protein to six different ion exchange resins (IEX Resins 1, 2, 3, 4, 5, 6, corresponding to Toyopearl SP550C™, Toyopearl SP650M™, GigaCAP S™, POROS HS50™, Toyopearl SP650C™ and GE Healthcare SPxL™, respectively) and a mixed-mode resin (MMC Resin 1, GE Healthcare MMC™) following capture using the disclosed methods.

FIG. **5** is a table demonstrating purification levels achieved for a protein comprising an Fc domain using one anion exhange resin (Fractogel TMAE™) and one cation exchange resin (Fractogel SO_3^-™).

DETAILED DESCRIPTION OF THE INVENTION

The present disclosure provides methods of capturing on a separation matrix non-native proteins produced in microbial cells. In the case of the direct capture of a protein expressed in a non-native soluble form the advantages of the present invention over typical processes include enhanced protein concentration, volume reduction, and increased recovery over traditional methods, improved protein stability, and ultimately process cost savings.

In the case of the direct capture of a protein expressed in a non-native limited solubility form, the advantages of the present invention over typical processes include the elimination of the need to dilute the protein out of a refold solution prior to capturing it on a separation matrix.

Another advantage of the disclosed methods is that they may be performed at a range of scales, from laboratory scale (typically milliliter or liter scale), a pilot plant scale (typically hundreds of liters) or on an industrial scale (typically thousands of liters). The application of the disclosed methods on large scales may be particularly desirable, due to the potential savings in time and resources.

Non-mammalian, e.g., microbial, cells can naturally produce, or can be engineered to produce, proteins that are expressed in either a soluble or a limited solubility form. Most often, engineered non-mammalian cells will deposit the recombinant proteins into large limited solubility aggregates called inclusion bodies. However, certain cell growth conditions (e.g., temperature or pH) can be modified to drive the recombinant proteins to be expressed as intracellular, soluble monomers. As an alternative to producing a protein of interest in cells in which the protein is expressed in the form of limited solubility inclusion bodies, cell growth conditions can be modified such that proteins are expressed in a non-native yet soluble form. The cells can then be lysed and the protein can be isolated by capturing it directly from cell lysate using ion exchange chromatography, affinity chromatography or mixed mode chromatography, as described herein. The method can be particularly useful for purifying proteins comprising an Fc region.

In one aspect, therefore, the present disclosure relates to a method of isolating a protein of interest comprising an Fc region that is expressed in a non-mammalian cell in a non-native, yet soluble form, from a pool of lysate generated from the cell in which the protein was expressed. The method employs a separation matrix, such as Protein A. One beneficial aspect of the disclosed method is that it eliminates the need for a refolding step before the protein is applied to the separation matrix. That is, non-mammalian cells expressing the protein of interest in a non-native soluble form can be lysed, the lysate applied directly to the separation matrix and the protein subsequently eluted from the separation matrix. This process allows the separation of proteins from cell cultures in highly concentrated pools that can be subsequently refolded at high concentrations and can be of benefit when producing large quantities of protein, particularly since the method is scalable from bench scale, which involves cultures on the order of several liters, up to production scale, which involves cultures of thousands of liters.

Following isolation by the separation matrix, the protein of interest can optionally be subsequently refolded using any technique known or suspected to work well for the protein of interest.

In another aspect, the present invention relates to a method of isolating a protein of interest comprising an Fc region that is expressed in a non-native limited solubility form, for example in inclusion bodies, that needs to be refolded and isolated from the refold mixture. Commonly, a refold solution contains a denaturant (e.g., urea or other chaotrope, organic solvent or strong detergent), an aggregation suppressor (e.g., a mild detergent, arginine or low concentrations of PEG), a protein stabilizer (e.g., glycerol, sucrose or other osmolyte, salts) and/or a redox component (e.g., cysteine, cystine, cystamine, cysteamine, glutathione). While often beneficial for refolding proteins, these components can inhibit purification (see, e.g., Wang et al., (1997) *Biochemical Journal* 325 (Part 3):707-710) and it is necessary to isolate or dilute the protein from these components for further processing, particularly before applying the protein to a separation matrix.

In one embodiment of the disclosed method, purification is achieved by directly applying a protein of interest, which is present in a refold mixture, to a separation matrix. In this approach, following a refold step the entire refold mixture, including the protein of interest, is applied directly to a separation matrix, such as a Protein A or G resin. The protein of interest associates with the matrix in the presence of the components of refold buffer, impurities are washed away and the protein is eluted. Since the method omits the need for removing any components of the refold mixture before the refold mixture is applied to a separation matrix, the method can have the effect of saving steps, time and resources that are typically expended on removing the protein from refolding and dilution buffers in purification processes. In some cases, the method can also reduce or eliminate the need for subsequent purification steps.

The disclosed methods can also be employed to purify proteins expressed in a non-native soluble and non-native limited solubility forms in a non-mammalian expression system that have subsequently been derivatized. For example, following expression a protein comprising an Fc region can

be associated with a small molecule, such as a toxin. Such conjugates can be purified using the methods described herein.

I. DEFINITIONS

As used herein, the terms "a" and "an" mean one or more unless specifically indicated otherwise.

As used herein, the term "non-mammalian expression system" means a system for expressing proteins in cells derived from an organism other than a mammal, including but not limited to, prokaryotes, including bacteria such as *E. coli*, and yeast. Often a non-mammalian expression system is employed to express a recombinant protein of interest, while in other instances a protein of interest is an endogenous protein that is expressed by a non-mammalian cell. For purposes of the present disclosure, regardless of whether a protein of interest is endogenous or recombinant, if the protein is expressed in a non-mammalian cell then that cell is a "non-mammalian expression system." Similarly, a "non-mammalian cell" is a cell derived from an organism other than a mammal, examples of which include bacteria or yeast.

As used herein, the term "denaturant" means any compound having the ability to remove some or all of a protein's secondary and tertiary structure when placed in contact with the protein. The term denaturant refers to particular chemical compounds that affect denaturation, as well as solutions comprising a particular compound that affect denaturation. Examples of denaturants that can be employed in the disclosed method include, but are not limited to urea, guanidinium salts, dimethyl urea, methylurea, ethylurea and combinations thereof.

As used herein, the term "aggregation suppressor" means any compound having the ability to disrupt and decrease or eliminate interactions between two or more proteins. Examples of aggregation suppressors can include, but are not limited to, amino acids such as arginine, proline, and glycine; polyols and sugars such as glycerol, sorbitol, sucrose, and trehalose; surfactants such as, polysorbate-20, CHAPS, Triton X-100, and dodecyl maltoside; and combinations thereof.

As used herein, the term "protein stabilizer" means any compound having the ability to change a protein's reaction equilibrium state, such that the native state of the protein is improved or favored. Examples of protein stabilizers can include, but are not limited to, sugars and polyhedric alcohols such as glycerol or sorbitol; polymers such as polyethylene glycol (PEG) and α-cyclodextrin; amino acids salts such as arginine, proline, and glycine; osmolytes and certain Hoffmeister salts such as Tris, sodium sulfate and potassium sulfate; and combinations thereof.

As used herein, the terms "Fc" and "Fc region" are used interchangeably and mean a fragment of an antibody that comprises human or non-human (e.g., murine) C_{H2} and C_{H3} immunoglobulin domains, or which comprises two contiguous regions which are at least 90% identical to human or non-human C_{H2} and C_{H3} immunoglobulin domains. An Fc can but need not have the ability to interact with an Fc receptor. See, e.g., Hasemann & Capra, "Immunoglobulins: Structure and Function," in William E. Paul, ed., *Fundamental Immunology*, Second Edition, 209, 210-218 (1989), which is incorporated by reference herein in its entirety.

As used herein, the terms "protein" and "polypeptide" are used interchangeably and mean any chain of at least five naturally or non-naturally occurring amino acids linked by peptide bonds.

As used herein, the term "complex molecule" means any protein that is (a) larger than 20,000 MW, or comprises greater than 250 amino acid residues, and (b) comprises two or more disulfide bonds in its native form. A complex molecule can, but need not, form multimers. Examples of complex molecules include but are not limited to, antibodies, peptibodies and polypeptides comprising an Fc domain and other large proteins. Peptibodies are described in U.S. Pat. No. 6,660,843, U.S. Pat. No. 7,138,370 and U.S. Pat. No. 7,511,012.

As used herein, the term "peptibody" refers to a polypeptide comprising one or more bioactive peptides joined together, optionally via linkers, with an Fc domain. See U.S. Pat. No. 6,660,843, U.S. Pat. No. 7,138,370 and U.S. Pat. No. 7,511,012 for examples of peptibodies.

As used herein, the terms "Fc fusion" and "Fc fusion protein" are used interchangeably and refer to a peptide or polypeptide covalently attached to an Fc domain.

As used herein the term "Protein A" means any protein identical or substantially similar to Staphylococcal Protein A, including commercially available and/or recombinant forms of Protein A. For the purposes of this invention, Protein A specifically includes engineered Protein A derived media, such as Mab Select SuRe™ media (GE Healthcare), in which a single subunit (e.g., the B subunit) is replicated two or more times and joined in a contiguous sequence to form a recombinant Protein A molecule, and other non-naturally occurring Protein A molecules.

As used herein, the term "Protein G" means any protein identical or substantially similar to Streptococcal Protein G, including commercially available and/or recombinant forms of Protein G.

As used herein, the term "substantially similar," when used in the context of a protein, including Protein A, means proteins that are at least 80%, preferably at least 90% identical to each other in amino acid sequence and maintain or alter in a desirable manner the biological activity of the unaltered protein. Included in amino acids considered identical for the purpose of determining whether proteins are substantially similar are amino acids that are conservative substitutions, unlikely to affect biological activity, including the following: Ala for Ser, Val for Ile, Asp for Glu, Thr for Ser, Ala for Gly, Ala for Thr, Ser for Asn, Ala for Val, Ser for Gly, Tyr for Phe, Ala for Pro, Lys for Arg, Asp for Asn, Leu for Ile, Leu for Val, Ala for Glu, Asp for Gly, and these changes in the reverse. See, e.g., Neurath et al., *The Proteins*, Academic Press, New York (1979). The percent identity of two amino sequences can be determined by visual inspection and mathematical calculation, or more preferably, the comparison is done by comparing sequence information using a computer program such as the Genetics Computer Group (GCG; Madison, Wis.) Wisconsin package version 10.0 program, "GAP" (Devereux et al., 1984, *Nucl. Acids Res.* 12: 387) or other comparable computer programs. The preferred default parameters for the "GAP" program includes: (1) the weighted amino acid comparison matrix of Gribskov and Burgess ((1986), *Nucl. Acids Res.* 14: 6745), as described by Schwartz and Dayhoff, eds., *Atlas of Polypeptide Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358 (1979), or other comparable comparison matrices; (2) a penalty of 30 for each gap and an additional penalty of 1 for each symbol in each gap for amino acid sequences; (3) no penalty for end gaps; and (4) no maximum penalty for long gaps. Other programs used by those skilled in the art of sequence comparison can also be used.

As used herein, the terms "isolate" and "purify" are used interchangeably and mean to reduce by 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95%, or more, the

amount of heterogenous elements, for example biological macromolecules such as proteins or DNA, that may be present in a sample comprising a protein of interest. The presence of heterogenous proteins can be assayed by any appropriate method including High-performance Liquid Chromatography (HPLC), gel electrophoresis and staining and/or ELISA assay. The presence of DNA and other nucleic acids can be assayed by any appropriate method including gel electrophoresis and staining and/or assays employing polymerase chain reaction.

As used herein, the term "separation matrix" means any adsorbent material that utilizes specific, reversible interactions between synthetic and/or biomolecules, e.g., the property of Protein A to bind to an Fc region of an IgG antibody or other Fc-containing protein, in order to effect the separation of the protein from its environment. In other embodiments the specific, reversible interactions can be base on a property such as isoelectric point, hydrophobicity, or size. In one particular embodiment, a separation matrix comprises an adsorbent, such as Protein A, affixed to a solid support. See, e.g., Ostrove (1990) in "Guide to Protein Purification," *Methods in Enzymology* 182: 357-379, which is incorporated herein in its entirety.

As used herein, the terms "non-native" and "non-native form" are used interchangeably and when used in the context of a protein of interest, such as a protein comprising a Fc domain, mean that the protein lacks at least one formed structure attribute found in a form of the protein that is biologically active in an appropriate in vivo or in vitro assay designed to assess the protein's biological activity. Examples of structural features that can be lacking in a non-native form of a protein can include, but are not limited to, a disulfide bond, quaternary structure, disrupted secondary or tertiary structure or a state that makes the protein biologically inactive in an appropriate assay. A protein in a non-native form can but need not form aggregates.

As used herein, the term "non-native soluble form" when used in the context of a protein of interest, such as a protein comprising a Fc domain, means that the protein lacks at least one formed structure attribute found in a form of the protein that is biologically active in an appropriate in vivo or in vitro assay designed to assess the protein's biological activity, but in which the protein is expressed in a form or state that is soluble intracellularly (for example in the cell's cytoplasm) or extracellularly (for example, in a lysate pool).

As used herein, the term "non-native limited solubility form" when used in the context of a protein of interest, such as a protein comprising a Fc domain, means any form or state in which the protein lacks at least one formed structural feature found in a form of the protein that (a) is biologically active in an appropriate in vivo or in vitro assay designed to assess the protein's biological activity and/or (b) forms aggregates that require treatment, such as chemical treatment, to become soluble. The term specifically includes proteins existing in inclusion bodies, such as those sometimes found when a recombinant protein is expressed in a non-mammalian expression system.

As used herein, the term "soluble form" when used in the context of a protein of interest, such as a protein comprising a Fc domain, broadly refers to a form or state in which the protein is expressed in a form that is soluble in a intracellularly (for example in the cell's cytoplasm) or extracellularly (for example, in a cell lysate pool).

II. DIRECT CAPTURE OF A PROTEIN EXPRESSED IN A NON-NATIVE SOLUBLE FORM IN A NON-MAMMALIAN EXPRESSION SYSTEM

One advantage of the disclosed method over typical purification methods is the elimination of the need for a refolding step before the soluble protein is applied to the separation matrix. That is, a protein solublized in cell lysate can be directly applied to the separation matrix. This is advantageous because the method does not require any initial purification efforts, although an initial filtration step may be desirable in some cases.

In the case of a protein comprising a Fc domain, the Fc region must have a certain level of structure to be bound by protein A, (Wang et al., (1997) *Biochem. J.* 325 (Part 3):707-710). This fact has limited the application of separation matrices for purifying proteins that are expressed in a non-native soluble form, particularly proteins comprising an Fc region, because it is commonly believed that a soluble non-native Fc-containing protein would not have the requisite structural elements required to associate with a separation matrix. Furthermore, the Fc region of an antibody spontaneously forms a homodimer under non-reducing conditions and prior to the instant disclosure it was unexpected to observe that even in the reductive environment of the cell, the Fc-conjugated proteins and peptides not only form enough structure for protein to bind to the affinity resin, but that the individual peptide chains readily formed non-covalent dimers, even though the proteins had not yet been completely refolded to native form.

In view of prevailing beliefs, the success of the disclosed method was surprising and unanticipated because it was not expected that a non-mammalian, microbial cell fermentation could be induced to produce a protein that was soluble, yet still had enough structure to associate with the affinity separation matrix.

The disclosed method can be employed to purify a protein of interest that is expressed in a non-native soluble form in a non-mammalian cell expression system. The protein of interest can be produced by living host cells that either naturally produce the protein or that have been genetically engineered to produce the protein. Methods of genetically engineering cells to produce proteins are known in the art. See, e.g., Ausabel et al., eds. (1990), *Current Protocols in Molecular Biology* (Wiley, New York). Such methods include introducing nucleic acids that encode and allow expression of the protein into living host cells. In the context of the present disclosure, a host cell will be a non-mammalian cell, such as bacterial cells, fungal cells, yeast cells, and insect cells. Bacterial host cells include, but are not limited to, *Escherichia coli* cells. Examples of suitable *E. coli* strains include: HB101, DH5α, GM2929, JM109, KW251, NM538, NM539, and any *E. coli* strain that fails to cleave foreign DNA. Fungal host cells that can be used include, but are not limited to, *Saccharomyces cerevisiae, Pichia pastoris* and *Aspergillus* cells. New cell lines can be established using methods known to those skilled in the art (e.g., by transformation, viral infection, and/or selection). It is noted that the method can be performed on proteins that are endogenously expressed by the non-mammalian cell as well.

During the production of a non-mammalian culture, growth conditions can be identified and employed so as to favor the production of a protein of interest in an intracellular soluble form. Such conditions can be identified by systematic empirical optimization of the culture condition parameters, such as temperature or pH. This optimization can be achieved using analysis of multifactorial matrices. For example, a matrix or series of multifactorial matrices can be evaluated to optimize temperature and pH conditions favor production of a desired species (i.e., a non-native soluble form). An optimization screen can be set up to systematically evaluate temperature and pH in a full or partial factorial matrix, with each component varied over a range of at least three temperature or pH levels with all other parameters kept constant. The protein

can be expressed and the yield and quality of protein expressed in the desired form can be evaluated using standard multivariate statistical tools.

Initially, non-mammalian cells that express a particular protein of interest are grown to a desired target density under conditions designed to induce expression of the protein in a soluble form. In one embodiment, the cells express a wild type protein of interest. In another embodiment, the cells can be engineered using standard molecular biology techniques to recombinantly express a protein of interest, and induced to produce the protein of interest. The protein of interest can be any protein, for example a protein that comprises an Fc moiety. Such a protein can be, for example, an antibody, a peptibody or an Fc fusion protein, any of which can be joined to an Fc moiety via a linker.

Once the desired target density is reached, the non-mammalian cells are separated from the growth media. One convenient way of achieving separation is by centrifugation, however filtration and other clarification methods can also be used.

The cells are then collected and are resuspended to an appropriate volume in a resuspension solution. Examples of resuspension solutions that can be used in the disclosed methods include phosphate buffered saline, Tris buffered saline, or water. The selection of an appropriate buffer will be determined, in part, by the properties of the molecule of interest as well as any volume or concentration constraints.

Following resuspension, the non-mammalian cells are lysed to release the protein, which will be present in the cell lysate in a non-native soluble form to generate a cell lysate. The lysis can be performed using any convenient means, such as feeding the cell suspension through a high pressure homogenizer or by employing a chemical lysis process. Whichever lytic process is selected, the function of the lysis step is to break open the cells and to break down DNA. The lysis can be performed in multiple cycles to achieve a more complete lysis or to accommodate large volumes of cell suspension. For example, the cell suspension can be fed through a mechanical homogenizer several times. This process releases the intracellular contents, including the protein of interest, and forms a pool of cell lysate.

Following the lysis procedure, the cell lysate can optionally be filtered. Filtration can remove particulate matter and/or impurities, such as nucleic acids and lipids, and may be desirable in some cases, such as when one suspects that direct application of the cell lysate to the chromatography equipment or media may lead to fouling or clogging, or when the separation matrix is sensitive to fouling or difficult to clean in-place. The benefit of filtering the cell lysate prior to contacting it with the separation matrix can be determined on a case-by-case basis.

After the lysis procedure, the cell lysate can optionally be incubated for an appropriate amount of time in the presence of air or oxygen, or exposed to a redox component or redox thiol-pair. The incubation can facilitate and/or ensure the formation of the minimal secondary structure required to facilitate an association with a separation matrix. The particular length of the incubation can vary with the protein but is typically less than 72 hours (e.g., 0, 0.5, 1, 2, 3, 5, 7, 10, 12, 18, 24, 36, 48 or 72 hours). When an incubation is performed, the length of incubation time can be determined by empirical analysis for each protein, which in some cases will be shorter (or omitted) and other cases longer.

Following the incubation period the cell lysate, which comprises the released protein of interest, is contacted with a separation matrix under conditions suitable for the protein to associate with a binding element of the separation matrix. Representative conditions conducive to the association of a protein with an affinity matrix are provided in the Examples. The separation matrix can be any media by which the protein of interest can be separated from the components of the resuspension and/or lysis buffer, including impurities such as host cell proteins, DNA, lipids and chemical impurities introduced by the components of the resuspension and/or lysis buffer.

Proteins A and G are often employed to purify antibodies, peptibodies and other fusion proteins comprising a Fc region by affinity chromatography. See, e.g., Vola et al. (1994), *Cell Biophys.* 24-25: 27-36; Aybay and Imir (2000), *J. Immunol. Methods* 233(1-2): 77-81; Ford et al. (2001), *J. Chromatogr. B* 754: 427-435. Proteins A and G are useful in this regard because they bind to the Fc region of these types of proteins. Recombinant fusion proteins comprising an Fc region of an IgG antibody can be purified using similar methods. Proteins A and G can be employed in the disclosed methods as an adsorbent component of a separation matrix.

Thus, examples of separation matrices that can be employed in the present invention include Protein A resin, which is known to be, and is commonly employed as, an effective agent for purifying molecules comprising an Fc moiety, as well as Protein G and synthetic mimetic affinity resins, such as MEP HyperCel® chromatography resin.

After the protein of interest has been associated with the separation matrix by contacting the cell lysate containing the protein with the separation matrix, thereby allowing the protein to associate with the adsorbent component of the separation matrix, the separation matrix is washed to remove unbound lysate and impurities.

The wash buffer can be of any composition, as long as the composition and pH of the wash buffer is compatible with both the protein and the matrix, and maintains the interaction between the protein and the matrix. Examples of suitable wash buffers that can be employed include solutions containing glycine, Tris, citrate, or phosphate; typically at levels of 5-100 mM (e.g., 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75 or 100 mM). These solutions can also contain an appropriate salt ion, such as chloride, sulfate or acetate at levels of 5-500 mM (e.g., 5, 10, 12, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450 or 500 mM). The resin can be washed once or any number of times. The exact composition of a wash buffer will vary with the protein being purified.

After the separation matrix with which the protein has associated has been washed, the protein of interest is eluted from the matrix using an appropriate solution. The protein of interest can be eluted using a solution that interferes with the binding of the adsorbent component of the separation matrix to the protein, for example by disrupting the interactions between the separation matrix and the protein of interest. This solution can include an agent that can either increase or decrease pH, and/or a salt. For example, the pH can be lowered to about 4.5 or less, for example to between about 3.3 and about 4.0, e.g., 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4 or 4.5. A solution comprising citrate or acetate, for example, can be employed to lower the pH. Other methods of elution are also known, such as via the use of chaotropes (see, e.g., Ejima et al. (2005) *Analytical Biochemistry* 345(2):250-257) or amino acid salts (see, e.g., Arakawa et al. (2004) *Protein Expression & Purification* 36(2):244-248). Protocols for such affinity chromatography are well known in the art. See, e.g., Miller and Stone (1978), *J. Immunol. Methods* 24(1-2): 111-125. Conditions for binding and eluting can be readily optimized by those skilled in the art. The exact composition of an elution buffer will vary with the protein being purified. The protein can then optionally be further purified from the elution pool and refolded as necessary. In other

situations the protein need not be further purified and instead can be refolded directly from the elution pool. Refolding directly from the elution pool may or may not require denaturation or reduction of the protein prior to incubation in a refolding solution and will depend in part on the properties of the protein.

In some cases it will be desirable to provide the separation matrix in a column format. In such cases a chromatography column can be prepared and then equilibrated before the cell suspension is loaded. Techniques for generating a chromatography column are well known and can be employed. An optional preparation and equilibration step can comprise washing the column with a buffer having an appropriate pH and salt condition that is conducive to protein-matrix interactions. This step can provide the benefit of removing impurities present in the separation matrix and can enhance the binding of the protein to be isolated to the adsorbent component of a separation matrix.

As noted, the separation matrix can be disposed in a column. The column can be run with or without pressure and from top to bottom or bottom to top. The direction of the flow of fluid in the column can be reversed during the purification process. Purifications can also be carried out using a batch process in which the solid support is separated from the liquid used to load, wash, and elute the sample by any suitable means, including gravity, centrifugation, or filtration. Moreover, purifications can also be carried out by contacting the sample with a filter that adsorbs or retains some molecules in the sample more strongly than others, such as anion exchange membrane chromatography.

If desired, the protein concentration of a sample at any given step of the disclosed method can be determined, and any suitable method can be employed. Such methods are well known in the art and include: 1) colorimetric methods such as the Lowry assay, the Bradford assay, the Smith assay, and the colloidal gold assay; 2) methods utilizing the UV absorption properties of proteins; and 3) visual estimation based on stained protein bands on gels relying on comparison with protein standards of known quantity on the same gel. See, e.g., Stoschek (1990), "Quantitation of Protein," in "Guide to Protein Purification," *Methods in Enzymology* 182: 50-68. Periodic determinations of protein concentration can be useful for monitoring the progress of the method as it is performed.

It is noted that any or all steps of the disclosed methods can be carried out manually or by any convenient automated means, such as by employing automated or computer-controlled systems.

III. DIRECT CAPTURE OF NON-NATIVE LIMITED SOLUBILITY PROTEIN FORMS FROM A REFOLD SOLUTION FOLLOWING EXPRESSION IN NON-MAMMALIAN CELLS

In another aspect of the present disclosure, a method of purifying a protein expressed in a non-native limited solubility form in a non-mammalian expression system is disclosed. An advantage of the disclosed method is that the method eliminates the need for removing or diluting the refold solution before applying the protein to a separation matrix, thereby saving the time and resources associated with what is a typical step in a purification process for isolating proteins expressed in a non-native limited solubility form.

Non-mammalian cells, e.g., microbial cells, can produce recombinant proteins that are expressed intracellularly in either a soluble or a limited solubility form. When the growth conditions are not directed to force expression of the protein in a soluble form, the cells may deposit the recombinant proteins into large relatively insoluble aggregates, such as inclusion bodies. These aggregates comprise protein that is typically not biologically active or less active than the completely folded native form of the protein. In order to produce a functional protein, these inclusion bodies often need to be carefully denatured so that the protein of interest can be extracted and refolded into a biologically active form.

In typical approaches, the inclusion bodies need to be captured, washed, exposed to a denaturing and/or reducing solubilization solution and the denaturing solution is then diluted with a solution to generate a condition that allows the protein to refold into an active form and form a structure that is found in the native protein. Subsequently, it is necessary to remove the components of the diluted denaturing solution from the immediate location of the protein. In order to do this, the refold solution comprising the solubilization solution and the refolded protein is typically diluted with a buffered solution before it is applied to a separation matrix, such as a Protein A ion exchange or other mixed-mode adsorbents. This process can be time-consuming and resource-intensive. It also significantly increases the volumes that need to be handled, as well as the associated tankage requirements, which can become limiting when working on large scales. The disclosed method eliminates the need for such a dilution step

The disclosed method is particularly useful for purifying a protein of interest that is expressed in a non-native limited solubility form in a non-mammalian cell expression system. The protein of interest can be produced by living host cells that either naturally produce the protein or that have been genetically engineered to produce the protein. Methods of genetically engineering cells to produce proteins are well known in the art. See, e.g., Ausabel et al., eds. (1990), *Current Protocols in Molecular Biology* (Wiley, New York). Such methods include introducing nucleic acids that encode and allow expression of the protein into living host cells. In the context of the present disclosure, these host cells will be non-mammalian cells, such as bacterial cells, fungal cells. Bacterial host cells include, but are not limited to *Escherichia coli* cells. Examples of suitable *E. coli* strains include: HB101, DH5α, GM2929, JM109, KW251, NM538, NM539, and any *E. coli* strain that fails to cleave foreign DNA. Fungal host cells that can be used include, but are not limited to, *Saccharomyces cerevisiae, Pichia pastoris* and *Aspergillus* cells. New cell lines can be established using methods well know by those skilled in the art (e.g., by transformation, viral infection, and/or selection). It is noted that the method can be performed on endogenous proteins that are naturally expressed by the non-mammalian cell as well.

Initially, non-mammalian cells that express a particular protein of interest are grown to a desired target density. In one embodiment, the cells can be expressing a particular wild type microbial protein of interest. In another embodiment, the cells can be engineered using standard molecular biology techniques to recombinantly express a protein of interest, and in this context they can be induced to overproduce the protein of interest. The protein of interest can be any protein, for example a protein that comprises an Fc moiety. Such a protein can be, for example, an antibody, a peptibody or an Fc fusion protein, any of which can be joined to an Fc moiety via a linker.

Once the desired target density is reached, the non-mammalian cells can be separated from the growth media. One convenient way of achieving separation is by centrifugation, however filtration and other clarification methods can also be used.

The cells are then collected and are resuspended to an appropriate volume in a resuspension solution. Examples of resuspension solutions that can be used in the present invention include phosphate-buffered saline, Tris-buffered saline, or water. The selection of an appropriate buffer will be determined, in part, by the properties of the molecule of interest as well as any volume or concentration constraints.

In order to release the limited solubility non-native protein from the cells, the non-mammalian cells are lysed to form a cell lysate comprising the released the limited solubility non-native protein. The lysis can be performed in any convenient way, such as feeding the cell suspension through a high pressure homogenizer or by employing a chemical lysis process. Whichever lysis process is selected, the function of the lysis step is to break open the cells and to break down DNA. The lysis can be performed in multiple cycles to achieve a more complete lysis or to accommodate large volumes of cell suspension. For example, the cell suspension can be fed through a mechanical homogenizer several times. This process releases the intracellular contents, including the naturally-occurring or recombinant protein of interest, and forms a pool of cell lysate.

Next, the limited solubility non-native protein is separated from the rest of the lysis pool. This can be done, for example, by centrifugation. Representative conditions for a centrifuge-mediated separation or washing typically include removal of excess water from the cell lysate, resuspension of the resulting slurry in a resuspension solution. This washing process may be performed once or multiple times. Examples of typical centrifuge types include, but are not limited to, disk-stack, continuous discharge, and tube bowl. Examples of resuspension solutions that can be used in the present invention include phosphate-buffered saline, Tris-buffered saline, or water and can include other agents, such as ETDA or other salts. The selection of an appropriate buffer will be determined, in part, by the properties of the molecule of interest as well as any volume or concentration constraints. The exact composition of an resuspension buffer will vary with the protein being purified.

The expressed protein is then solubilized in a solubilization solution comprising one or more of (i) a denaturant, (ii) a reductant and (iii) a surfactant. The denaturant can be included as a means of unfolding the limited solubility protein, thereby removing any existing structure, exposing buried residues and making the protein more soluble.

Any denaturant can be employed in the solubilization solution. Examples of some common denaturants that can be employed in the refold buffer include urea, guanidinium, dimethyl urea, methylurea, or ethylurea. The specific concentration of the denaturant can be determined by routine optimization.

The reductant can be included as a means to reduce exposed residues that have a propensity to form covalent intra or intermolecular-protein bonds and minimize non-specific bond formation. Examples of suitable reductants include, but are not limited to, cysteine, DTT, beta-mercaptoethanol and glutathione. The specific concentration of the reductant can be determined by routine optimization.

A surfactant can be included as a means of unfolding the limited solubility non-native protein, thereby exposing buried residues and making the protein more soluble. Examples of suitable surfactants include, but are not limited to, sarcosyl and sodium dodecylsulfate. The specific concentration of the surfactant can be determined by routine optimization.

Although the composition of a solubilization solution will vary with the protein being purified, in one particular embodiment the solubilization solution comprises 4-6 M guanidine, 50 mM DTT.

Continuing, a refold solution comprising the solubilization solution (which comprises the protein), and a refold buffer is formed. The refold buffer comprises one or more of (i) a denaturant; (ii) an aggregation suppressor; (iii) a protein stabilizer; and (iv) a redox component. The denaturant can be included as a means of modifying the thermodynamics of the solution, thereby shifting the equilibrium towards an optimal balance of native form. The aggregation suppressor can be included as a means of preventing non-specific association of one protein with another, or with one region of a protein with another region of the same protein. The protein stabilizer can be included as a means of promoting stable native protein structure and may also suppress aggregation.

In various embodiments, the denaturant in the refold buffer can be selected from the group consisting of urea, guanidinium salts, dimethyl urea, methylurea and ethylurea.

In various embodiments, the protein stabilizer in the refold buffer can be selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes.

In various embodiments, the aggregation suppressor can be selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes.

In various embodiments, the thiol-pairs can comprise at least one component selected from the group consisting of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol.

The specific concentrations of the components of a refold buffer can be determined by routine optimization. For example, a matrix or series of multifactorial matrices can be evaluated to optimize the refolding buffer for conditions that optimize yield and distributions of desired species. An optimization screen can be set up to systematically evaluate denaturant, aggregation suppressor, protein stabilizer and redox component concentrations and proportions in a full or partial factorial matrix, with each component varied over a range of concentrations with all other parameters kept constant. The completed reactions can be evaluated by RP-HPLC and SE-HPLC analysis for yield and product quality using standard multivariate statistical tools.

The function of the buffer component of the refold solution is to maintain the pH of the refold solution and can comprise any buffer that buffers in the appropriate pH range. Examples of the buffering component of a refold buffer that can be employed in the method include, but are not limited to, phosphate buffers, citrate buffers, tris buffer, glycine buffer, CHAPS, CHES, and arginine-based buffers, typically at levels of 5-100 mM (e.g., 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 80, 85, 90, 95 or 100, mM).

Although the composition of an refold buffer will vary with the protein being purified, in one embodiment a refold buffer comprises arginine, urea, glycerol, cysteine and cystamine.

The refold solution can then be incubated for a desired period of time. The incubation period can be of any length but is typically between 0 and 72 hours (e.g., 0, 0.5, 1, 2, 3, 5, 7, 10, 12, 18, 24, 36, 48 or 72 hours).

After an appropriate incubation time, the refold solution is then applied to a separation matrix under conditions suitable for the protein to associate with the matrix. The separation

matrix can be any media by which the protein of interest can be separated from the components of the resuspension and/or lysis buffer, including impurities such as host cell proteins, DNA and chemical impurities introduced by the components of the solubilization and/or lysis buffer.

Proteins A and G are often employed to purify antibodies, peptibodies and other fusion proteins comprising a Fc region by affinity chromatography. See, e.g., Vola et al. (1994), *Cell Biophys.* 24-25: 27-36; Aybay and Imir (2000), *J. Immunol. Methods* 233(1-2): 77-81; Ford et al. (2001), *J. Chromatogr. B* 754: 427-435. Proteins A and G are useful in this regard because they bind to the Fc region of these types of proteins. Recombinant fusion proteins comprising an Fc region of an IgG antibody can be purified using similar methods. Proteins A and G can be employed in the disclosed methods as an adsorbent component of a separation matrix.

Thus, examples of affinity separation matrices that can be employed in the present invention include Protein A resin, which is know to be, and is commonly employed as, an effective agent for purifying molecules comprising an Fc moiety, as well as Protein G and synthetic mimetic affinity resins. Other materials that can be employed include HIC and ion exchange resins (see Example 4), depending on the properties of the protein to be purified.

It is noted that when performing the method, the refold solution comprising the refolded protein of interest is applied directly to the separation matrix, without the need for diluting or removing the components of the solution required for refolding the protein. This is an advantage of the disclosed method. Initially, it was expected that the highly ionic and/or chaotropic compounds and various other components of the refold solution would inhibit the association of the protein with the separation matrix. However, in contrast to reports in the literature (e.g., Wang et al. (1997) *Biochemical Journal.* 325 (Part 3):707-710), it was surprising to observe that the protein was in fact able to associate with the separation matrix in the presence of the components of the refold solution. The unexpected finding that the protein could associate with the separation matrix in the presence of the components of the refold solution facilitates the elimination of a dilution step or buffer exchange operation, providing a savings of time and resources.

After the protein of interest has associated with the separation matrix the separation matrix is washed to remove unbound protein, lysate, impurities and unwanted components of the refold solution.

The wash buffer can be of any composition, as long as the composition and pH of the wash buffer is compatible with both the protein and the matrix. Examples of suitable wash buffers that can include, but are limited to, solutions containing glycine, tris, citrate, or phosphate. These solutions may also contain an appropriate salt. Suitable salts include, but are not limited to, sodium, potassium, ammonium, magnesium, calcium, chloride, fluoride, acetate, phosphate, and/or citrate. The pH range is chosen to optimize the chromatography conditions, preserve protein binding, and to retain the desired characteristics of the protein of interest. The resin can be washed once or any number of times. The exact composition of a wash buffer will vary with the protein being purified.

After the separation matrix with which the protein has associated has been washed, the protein of interest is eluted using an appropriate solution (e.g., a low pH buffered solution or a salt solution) to form an elution pool comprising the protein of interest.

The protein of interest can be eluted using a solution that interferes with the binding of the adsorbent component of the separation matrix to the protein, for example by disrupting the interactions between Protein A and the Fc region of a protein of interest. This solution may include an agent that can either increase or decrease pH, and/or a salt. In various embodiments, the elution solution can comprise acetic acid, glycine, or citric acid. Elution can be achieved by lowering the pH. For example, the pH can be lowered to about 4.5 or less, for example to between about 3.3 to about 4.2 (e.g., 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1 or 4.2, using a solution comprising citrate or acetate, among other possibilities.

In some situations, the protein can then be further purified from the elution pool and can be further refolded, if necessary. In other situations the protein need not be further purified and instead can be further refolded directly in the elution pool, if necessary.

Protocols for such affinity chromatography are known in the art. See, e.g., Miller and Stone (1978), *J. Immunol. Methods* 24(1-2): 111-125. In the cases that utilize ion exchange, mixed-mode, or hydrophobic interaction chromatography, the concentration of salt can be increased or decreased to disrupt ionic interaction between bound protein and a separation matrix. Solutions appropriate to effect such elutions can include, but are not limited to, sodium, potassium, ammonium, magnesium, calcium, chloride, fluoride, acetate, phosphate, and/or citrate. Other methods of elution are also known. Conditions for binding and eluting can be readily optimized by those skilled in the art.

The exact composition of an elution buffer will vary with the protein being purified and the separation matrix being employed.

In some cases it will be desirable to situate the separation matrix in a column format. In such cases a column can be prepared and then equilibrated before the cell suspension is loaded. Techniques for generating a chromatography column are well known and can be employed. The optional preparation and equilibration step can comprise washing the column with a buffer having an appropriate pH and composition that will prepare the media to bind a protein of interest. This step has the benefit of removing impurities present in the separation matrix and can enhance the binding of the protein to be isolated to the adsorbent component of a separation matrix.

It is noted that any or all steps of the invention can be carried out by any mechanical means. As noted, the separation matrix can be disposed in a column. The column can be run with or without pressure and from top to bottom or bottom to top. The direction of the flow of fluid in the column can be reversed during the purification process. Purifications can also be carried out using a batch process in which the solid support is separated from the liquid used to load, wash, and elute the sample by any suitable means, including gravity, centrifugation, or filtration. Moreover, purifications can also be carried out by contacting the sample with a filter that adsorbs or retains some molecules in the sample more strongly than others.

If desired, the protein concentration of a sample at any given step of the disclosed method can be determined by any suitable method. Such methods are well known in the art and include: 1) colorimetric methods such as the Lowry assay, the Bradford assay, the Smith assay, and the colloidal gold assay; 2) methods utilizing the UV absorption properties of proteins; and 3) visual estimation based on stained protein bands on gels relying on comparison with protein standards of known quantity on the same gel. See, e.g., Stoschek (1990), "Quantitation of Protein," in "Guide to Protein Purification," *Methods in Enzymology* 182: 50-68. Periodic determinations of protein concentration can be useful for monitoring the progress of the method as it is performed.

It is noted that any or all steps of the disclosed methods can be carried out manually or by any convenient automated means, such as by employing automated or computer-controlled systems.

IV. COLUMN CLEANING

In another aspect the present disclosure relates to the observation that in many cases the separation matrix employed in the methods provided herein can be cleaned after multiple separations and reused. This unexpected property of the method provides a significant cost and resource savings, particularly on the manufacturing scale, since the separation matrix need not be discarded after a separation is complete.

Common wisdom in the industry suggests that after a separation matrix, such as Protein A, is repeatedly exposed to highly heterogenous feedstocks comprising high lipid and host protein content it becomes irreversibly contaminated and unusable when treated with the mild regeneration solutions commonly utilized for protein-based affinity resins. The disclosed methods, however, avoid this situation and extend the usable lifetime of a separation matrix. In the context of a large scale manufacturing process this can translate into a measurable savings of time and money. Moreover, the cleaning step can be performed, as disclosed in the Examples, in-place and with no need to extract the separation matrix from a column or other matrix retaining device for cleaning, thus saving time and resources.

In one embodiment of a cleaning operation of a separation matrix, following a separation employing the disclosed method the separation matrix is washed with a regeneration reagent, such as sodium hydroxide, or an acidic reagent, such as phosphoric acid.

In one particular embodiment of a cleaning operation, Protein A is the separation matrix and a column containing Protein A resin is washed with 5 column volumes of 150 mM phosphoric acid and held for >15 minutes over the column. Following the wash with the acid, the column can be flushed with water, regenerated with 5 column volumes of 50 mM Tris, 10 mM citrate, 6M urea, 50 mM DTT; pH 7.4, subsequently washed with water, and then flushed with 3 column volumes of 150 mM phosphoric acid. This cleaning protocol has been utilized to achieve over 200 cycles of protein A resin. FIG. **3** highlights the results achievable using the disclosed cleaning methods.

EXAMPLES

The following examples demonstrate embodiments and aspects of the present invention and are not intended to be limiting.

Example 1

Direct Capture of Proteins Expressed in a Soluble Form Using Protein A Affinity Chromatography

The following experiment demonstrates that a protein comprising a plurality of polypeptides joined to an Fc moiety can be separated from an *E. coli* cell lysate slurry using a Protein A affinity media.

A protein comprising a plurality of polypeptides joined to an Fc moiety was expressed in an *E. coli* fermentation induced at 30° C. and driven to express soluble-form protein product. The fermentation broth was centrifuged, the liquid fraction removed, and the cell paste was collected. The cells were resuspended in a 10 mM potassium phosphate, 5 mM EDTA; pH 6.8 buffer solution, to approximately 100% of the original volume. The cells were then lysed by means of three passes through a high pressure homogenizer. After the cells were lysed, the cell lysate was filtered through a 0.1 μm filter to reduce particulate levels. The material was then stored in a closed bottle for ~24 hours at approximately 5° C.

In a separate operation, a packed column comprising GE Healthcare Mab Select™ Protein A affinity resin was prepared and equilibrated with 5 column volumes (CV) of 10 mM Tris; pH 8.0.

An aliquot of a protein comprising an Fc moiety was sampled directly from a lysate. The protein mixture was loaded to approximately 0.02 millimoles total protein/L resin at a 6-10 minute residence time. See FIG. **1**, which correlates protein bound and protein loaded as a function of residence time.

After loading, the column was washed with 10 mM Tris; pH 8.0, for 5 CV at up to 220 cm/hr. The protein of interest was recovered from the resin by elution with 50 mM sodium acetate, pH 3.1 at up to 220 cm/hr. The elution pool yielded greater than 90% recovery of the soluble material in the initial cell broth. The collected protein in the elution pool was stored at 2-8° C. until the next purification step was carried out.

Following the separation, the resin media was cleaned in-place by flowing 5 CV of 6 M Guanidine, pH 8.0 at 220 cm/hr.

The results of this separation demonstrated that a soluble protein expressed in a non-mammalian system can be captured and purified, with high yield, directly from cell lysate broth without having to refold the protein prior to application to a separation matrix.

Example 2

Capture of a Fc-Containing Protein Expressed in a Limited Solubility Form from a Refold Mixture Using Protein A Affinity Chromatography

The following experiments demonstrate that an Fc-containing protein can be separated from a refold mixture comprising glycerol, guanidine, urea, and arginine using Protein A affinity media.

In one experiment, a recombinant protein comprising a biologically active peptide linked to the C-terminus of the Fc moiety of an IgG1 molecule via a linker and having a molecular weight of about 57 kDa and comprising 8 disulfide bonds, in a non-mammalian expression system, namely *E. coli*, harvested, refolded under appropriate conditions, and captured using Protein A affinity media.

The growth media in which the cells were growing was centrifuged and the liquid fraction removed, leaving the cells as a paste. The cells were resuspended in water to approximately 60% of the original volume. The cells were lysed by means of three passes through a high pressure homogenizer.

After the cells were lysed, the lysate was centrifuged in a disc-stack centrifuge to collect the protein in the solid fraction, which was expressed in a limited solubility non-native form, namely as inclusion bodies.

The protein slurry was washed multiple times by resuspending the slurry in water to between 50 and 80% of the original fermentation broth volume, mixing, and centrifugation to collect the protein in the solid fraction.

The concentrated protein was then combined in a solubilization solution containing the protein, guanidine, urea, and DTT.

After incubation for one hour, the protein solution was diluted in to a refold buffer containing appropriate levels of arginine, urea, glycerol, cysteine, and cystamine.

In a separate operation, a packed column comprising ProSep VA Ultra™ Protein A affinity resin with dimensions of 1.1 cm internal diameter and ~25 cm height, was prepared and equilibrated with 5 column volumes (CV) of 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution.

An aliquot of a protein comprising an Fc moiety from the refold solution was filtered through a series of depth and/or membrane filter to remove particulates. The conditioned and filtered protein mixture was loaded to approximately 0.35 millimoles total protein/L resin at a 6-10 minute residence time. See FIG. **1**, which correlates protein bound and protein loaded as a function of residence time.

After loading, the column was washed with 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution, for 4.5 CV at up to 400 cm/hr. The Fc-contiaing protein was recovered from the resin by elution with 100 mM sodium acetate, pH 3.7 at up to 300 cm/hr. The average level of purity achieved is shown in FIG. **3**.

Following the separation, the resin media was cleaned in-place by flowing 5 CV of 150 mM phosphoric acid. The column was regenerated with 5 CV of 50 mM Tris, 10 mM citrate, 6M urea and 50 mM DTT; pH 7.4, washed with water, and then flushed with 3 CV of 150 mM phosphoric acid.

The results of this separation demonstrate that an insoluble protein expressed in a non-mammalian system can be purified directly from a refold buffer without having to dilute the refold buffer prior to application to a separation matrix for more than 150 cycles, as indicated by the table presented in FIG. **3**.

In another separation, the Protein A column was cycled with the above procedure 8-10 times and then the final cycle was run as follows: The media was equilibrated with 5 column volumes (CV) of 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution. An aliquot of protein sampled directly from a refold buffer was filtered through a series of depth and/or membrane filter to remove particulates. The conditioned and filtered protein mixture was then loaded on the column to 0.35 millimoles total protein/L resin at a 6-10 minute residence time. See FIG. **1**, which correlates protein bound and protein loaded as a function of residence time.

After loading, the column was washed with 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution, for 4.5 CV at up to 400 cm/hr. The protein of interest was recovered from the resin by eluting with 100 mM sodium acetate, pH 3.7 at up to 300 cm/hr. The resin media was cleaned in-place by flowing 5 CV of 150 mM phosphoric acid over it. Finally, the column was flushed with water, regenerated with 5 CV of 50 mM Tris, 10 mM citrate, 6M urea, and 50 mM DTT; pH 7.4, washed with water, and then flushed with 3 CV of 150 mM phosphoric acid. Subsequent analysis of the resin showed no protein carry-over between cycles, demonstrating the ability to re-use the resin after both cleaning methods.

Example 3

Separation of an Fc-Containing Protein from a Refold Mixture Using Cation Exchange Chromatography

The following experiments demonstrate that an Fc-containing protein can be separated from a refold mixture comprising glycerol, guanidine, urea, and arginine using cation exchange media.

In one experiment, a recombinant protein comprising a biologically active peptide linked to the C-terminus of the Fc moiety of an IgG1 molecule via a linker and having a molecular weight of about 57 kDa and comprising 8 disulfide bonds, was expressed in a non-mammalian expression system, namely *E. coli*, harvested, refolded under appropriate conditions, and captured using cation exchange media.

The growth media in which the cells were growing was centrifuged and the liquid fraction removed, leaving the cells as a paste. The cells were resuspended in water. The cells were lysed by means of multiple passes through a high pressure homogenizer. After the cells were lysed, the lysate was centrifuged to collect the protein, which was expressed in a limited solubility non-native form, namely as inclusion bodies. The protein slurry was washed multiple times by resuspending the slurry in water, mixing, and centrifugation to collect the protein. The concentrated protein was then transferred to a solubilization buffer containing guanidine and DTT. After incubation for one hour, the protein solution was diluted in to a refold buffer containing appropriate levels of arginine, urea, glycerol, cysteine, and cystamine.

In a separate operation, a packed column comprising EMD Fractogel SO_3^- cation exchange resin with dimensions of 1.1 cm internal diameter and 20 cm height, was prepared and equilibrated with 5 column volumes of 30 mM MES; pH 4.5 buffered solution.

An aliquot of a protein comprising an Fc moiety was sampled directly from a refold solution, was diluted 3-fold with water, titrated with 50% hydrochloric acid to ~pH 4.5 and was filtered through a series of depth and/or membrane filter to remove particulates. The conditioned and filtered protein mixture was loaded to approximately 0.96 millimoles total protein/L resin at 60 cm/hr.

After loading, the column was washed with 30 mM MES; pH 4.5, for 3 CV at 60 cm/hr, then washed with an additional 3 CV of 30 mM MES; pH 6.0. The protein of interest was recovered from the resin by gradient elution over 25 CV between 30 mM MES; pH 6.0 and 30 mM MES, 500 mM NaCl; pH 6.0 at 60 cm/hr. The collected protein in the elution pool was stored at 2-8° C. until the next purification step was carried out.

Purity levels achieved, as determined by SEC and RP-HPLC are shown in FIG. **5**.

Following the separation, the resin media was cleaned in-place by flowing 3 CV of 1 M sodium hydroxide, at 120 cm/hr and held for 60 minutes prior an additional 3 CV wash with 1 m sodium hydroxide.

The results of this separation demonstrate that an insoluble protein expressed in a non-mammalian system can be captured and purified from a refold buffer with a variety of separation matrices, including an ion-exchange separation matrix.

Example 4

Re-Usability of Protein A Affinity Resin Used to Isolate a Fc-Containing Protein Directly from a Refold Buffer by Affinity Chromatography

In another aspect of the method, a range of column cleaning methods can be employed in conjunction with the methods described herein, allowing the chromatography resins to be reused to an extent that make the method economically feasible. As described in Examples 2 and 3 for the case of Protein A affinity resins, cleaning protocols have been developed and demonstrated to remove product and non-product contaminants from the resin to allow reuse. The cleaning

agents include caustic (e.g. sodium or potassium hydroxide), detergents (e.g. SDS or Triton X-100), denaturants (e.g. urea or guanidine-derivatives), and reductants (e.g. DTT, or thioglycolates). These agents can be used in combination or alone.

In order to demonstrate the reusability of column resins following application of the direct capture methods described, an aliquot of pH adjusted and filtered Fc-containing protein was loaded on new, unused resin and resin that had been previously cycled 94 times to evaluate the cleaning of the Protein A resin and the effect on purification binding and separation of an Fc-containing protein with regard to resin history.

The media was equilibrated with 5 column volumes (CV) of 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution. An aliquot of protein sampled directly from a refold buffer was filtered through a series of depth and/or membrane filter to remove particulates. The conditioned and filtered protein mixture was then loaded on the column to approximately 0.35 millimoles total protein/mL resin at a 6-10 minute residence time. See FIG. **1**, which correlates protein bound and protein loaded as a function of residence time.

After loading, the column was washed with 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution, for 4.5 CV at up to 400 cm/hr. The protein of interest was recovered from the resin by eluting with 100 mM sodium acetate, pH 3.7 at up to 300 cm/hr. Each column was regenerated using 5 CV phosphoric acid and 5 CV of an acidic buffered solution containing 50 mM Tris, 10 mM citrate, 6M urea, and 50 mM DTT; pH 7.4.

This procedure was repeated for greater than 100 cycles. Selected samples from this reuse study were submitted for SEC-HPLC analysis. The goal was to track the % MP purity, % HMW and % dimer species from the pools as well as to understand the change of purity level from the load. No major differences were observed between the used columns and new columns.

This Example demonstrates that not only can a complex protein be captured from a complex chemical solution, but that the resin can be cycled repeatedly and cleaned and reused reproducibly over a number of industrially-relevant cycles.

What is claimed is:

1. A method of purifying a protein expressed in a non-native soluble form in a non-mammalian expression system comprising:

(a) lysing a non-mammalian cell in which the protein is expressed in a non-native soluble form to generate a cell lysate;

(b) contacting the cell lysate with a separation matrix under conditions suitable for the protein to associate with the separation matrix;

(c) washing the separation matrix; and

(d) eluting the protein from the separation matrix, wherein the separation matrix is an affinity resin selected from the group consisting of Protein A, Protein G and a synthetic mimetic affinity resin.

2. The method of claim **1**, wherein the protein is a complex protein.

3. The method of claim **2**, wherein the complex protein is selected from the group consisting of a multimeric protein, an antibody and an Fc fusion protein.

4. The method of claim **1**, wherein the non-mammalian expression system comprises bacteria or yeast cells.

5. The method of claim **1**, wherein the cell lysate is filtered before it is contacted with the separation matrix.

6. The method of claim **1**, further comprising refolding the protein to its native form after it is eluted.

7. A method of purifying a protein expressed in a non-native limited solubility form in a non-mammalian expression system comprising:

(a) expressing a protein in a non-native limited solubility form in a non-mammalian cell;

(b) lysing a non-mammalian cell;

(c) solubilizing the expressed protein in a solubilization solution comprising one or more of the following:

(i) a denaturant;

(ii) a reductant; and

(iii) a surfactant;

(d) forming a refold solution comprising the solubilization solution and a refold buffer, the refold buffer comprising one or more of the following:

(i) a denaturant;

(ii) an aggregation suppressor;

(iii) a protein stabilizer; and

(iv) a redox component;

(e) directly applying the refold solution to a separation matrix under conditions suitable for the protein to associate with the matrix;

(f) washing the separation matrix; and

(g) eluting the protein from the separation matrix, wherein the separation matrix is a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin.

8. The method of claim **7**, wherein the non-native limited solubility form is a component of an inclusion body.

9. The method of claim **7**, wherein the protein is a complex protein.

10. The method of claim **7**, wherein the complex protein is selected from the group consisting of a multimeric protein, an antibody, a peptibody, and an Fc fusion protein.

11. The method of claim **7**, wherein the non-mammalian expression system is bacteria or yeast cells.

12. The method of claim **7**, wherein the denaturant of the solubilization solution or the refold buffer comprises one or more of urea, guanidinium salts, dimethyl urea, methylurea and ethylurea.

13. The method of claim **7**, wherein the reductant comprises one or more of cysteine, dithiothreitol (DTT), beta-mercaptoethanol and glutathione.

14. The method of claim **7**, wherein the surfactant comprises one or more of sarcosyl and sodium dodecylsulfate.

15. The method of claim **7**, wherein the aggregation suppressor is selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes.

16. The method of claim **7**, wherein the protein stabilizer comprises one or more of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, tris, sodium sulfate, potassium sulfate and osmolytes.

17. The method of claim **7**, wherein the redox component comprises one or more of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol.

18. The method of claim **1** or **7**, further comprising the step of washing the separation matrix with a regeneration reagent.

19. The method of claim **18**, wherein the regeneration reagent is one of a strong base or a strong acid.

20. The method of claim **19**, wherein the strong acid is phosphoric acid.

21. The method of claim **19**, wherein the strong base is sodium hydroxide.

22. The method of claim **18**, wherein the regenerating comprises washing the separation matrix with a solution comprising one or both of a chaotrope present at a concentration of 4-6 M and a reductant.

23. The method of claim **22**, wherein the chaotrope is one of urea, dimethyl urea, methylurea, ethylurea, and guanidinium.

24. The method of claim **22**, wherein the reductant is one of cysteine, dithiothreitol (DTT), beta-mercaptoethanol and glutathione.

25. The method of claim **18**, wherein the regenerating comprises washing the separation matrix with a solution comprising 50 mM Tris, 10 mM citrate, 6 M urea, 50 mM dithiothreitol (DTT) at pH 7.4.

* * * * *

Exhibit 2

US009643997B2

(12) **United States Patent**
Shultz et al.

(10) **Patent No.: US 9,643,997 B2**
(45) **Date of Patent: *May 9, 2017**

(54) **CAPTURE PURIFICATION PROCESSES FOR PROTEINS EXPRESSED IN A NON-MAMMALIAN SYSTEM**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **14/599,336**

(22) Filed: **Jan. 16, 2015**

(65) **Prior Publication Data**

US 2015/0361130 A1 Dec. 17, 2015

Related U.S. Application Data

(62) Division of application No. 12/822,990, filed on Jun. 24, 2010, now Pat. No. 8,940,878.

(60) Provisional application No. 61/220,477, filed on Jun. 25, 2009.

(51) **Int. Cl.**

C07K 1/14	(2006.01)
C07K 1/22	(2006.01)
C07K 1/18	(2006.01)
C07K 1/32	(2006.01)
C07K 16/00	(2006.01)

(52) **U.S. Cl.**

CPC ***C07K 1/22*** (2013.01); ***C07K 1/145*** (2013.01); ***C07K 1/18*** (2013.01); ***C07K 1/32*** (2013.01); ***C07K 16/00*** (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

Methods of purifying proteins expressed in non-mammalian expression systems in a non-native soluble form directly from cell lysate are disclosed. Methods of purifying proteins expressed in non-mammalian expression systems in a non-native limited solubility form directly from a refold solution are also disclosed. Resin regeneration methods are also provided.

30 Claims, 5 Drawing Sheets

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Document 186: Trial Brief Apotex's Supplemental Claim Construction Brief in Support of Their Construction for the Term 'protein' As Used in Claim 1 of U.S. Pat. No. 8,952,138 by Apotex Corp., Apotex Inc.pp. 1-18 (Attachments: #1 Exhibit 1 U.S. Pat. No. 8,952,138; pp. 1-19; # 2 Exhibit 6—Response to Office Action dated Jun. 22, 2012)(Brier, Simeon) (Entered: Jun. 22, 2016); pp. 1-6.

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Document 260: Reply to Response to Motion re 244 Motion for Judgment on Partial Findings Pursuant to Fed. R. Civ. P. 52(c) filed by Apotex Corp., Apotex Inc. pp. 1-11; Attachments: # 1 Exhibit 1—Trial Transcript Day 2 (Jul. 12, 2016) (Willson), pp. 1-7. # 2 Exhibit 2—Trial Transcript Day 3 (Jul. 13, 2016) (Dowd), pp. 1-4. # 3 Exhibit 3—Trial Transcript Day 4 (Jul. 14, 2016) (Robinson), pp. 1-4. # 4 Exhibit 4—Trial Transcript Day 5 (Jul. 18, 2016), pp. 1-3.

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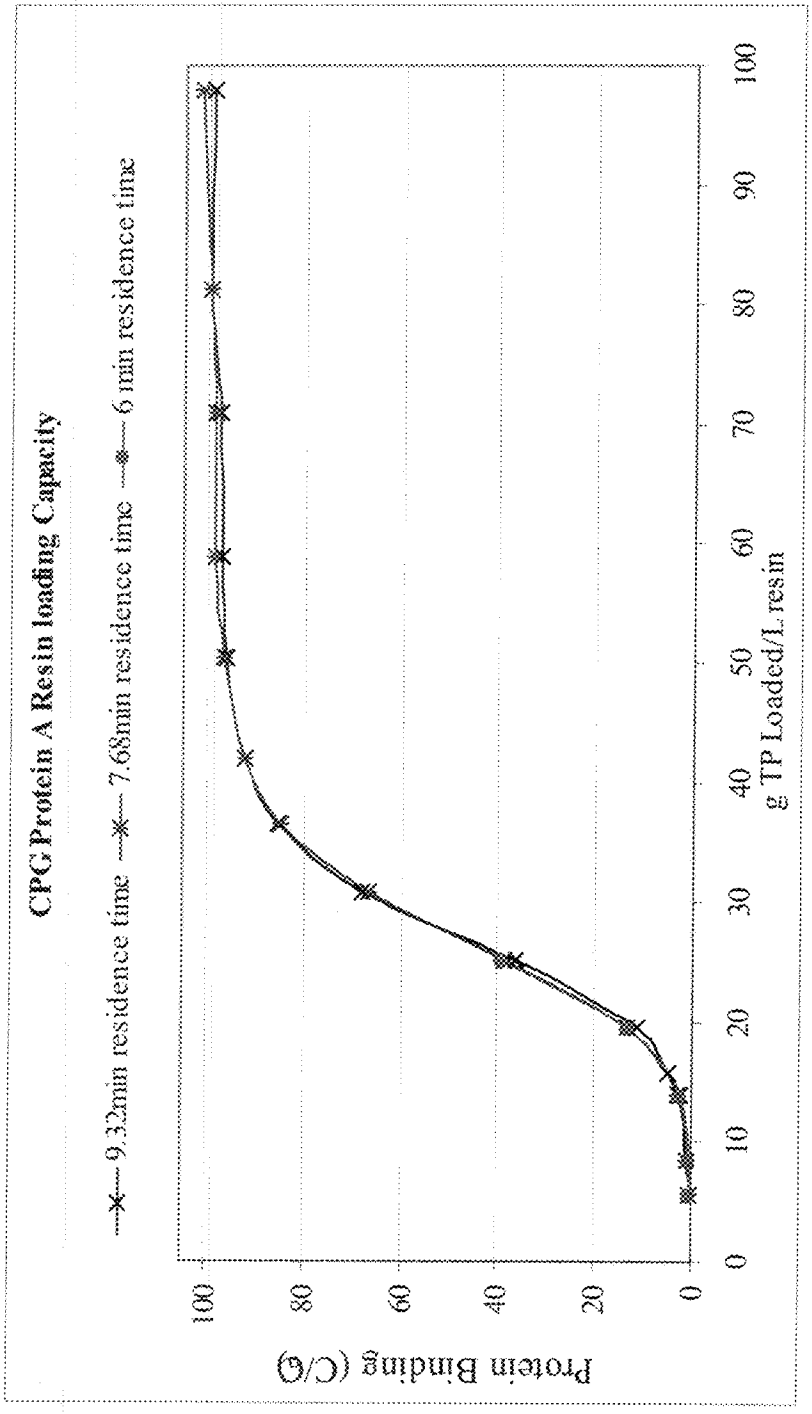
CPG Protein A Resin loading Capacity
9.32min residence time
7.68min residence time
6 min residence time
Protein Binding (C/C0)
0
20
40
60
80
100
0
10
20
30
40
50
60
70
80
90
100
g TP Loaded/L resin

Figure 1

		Average Purity					
		RP-HPLC Main Peak Purity (%)	SE-HPLC Main Peak Purity (%)	CE-SDS Main Peak Purity (%)	Host Protein Level (ppm)	DNA Level (pg/mg protein)	Average Yield (%)
Load	Average (n=13)	34.5	74.5	79.2	9100.0	>70000	-
	Std. Dev (n=13)	2.4	2.7	4.4	424.3	*	-
Purified Pool	Average (n=17)	41.3	68.8	84.7	41.0	215.2	81.7
	Std. Dev (n=17)	1.5	3.8	4.0	5.7	301.2	12.3

* Data limited to N=1

Figure 2

		Average Purity					
		RP-HPLC Main Peak Purity (%)	SE-HPLC Main Peak Purity (%)	CE-SDS Main Peak Purity (%)	Host Protein Level (ppm)	DNA Level (pg/mg protein)	Average Yield (%)
Load	Average (n=5)	36.0	76.1	75.5	1400.0	>70000	-
	Std. Dev (n=5)	0.9	1.9	1.5	*	*	-
Purified Pool	Average (150 cycles)	40.2	75.0	82.4	71.4	89.2	84.3
	Std. Dev (150 cycles)	2.5	8.7	4.6	23.0	175.0	18.8

* Data limited to N=1

Figure 3

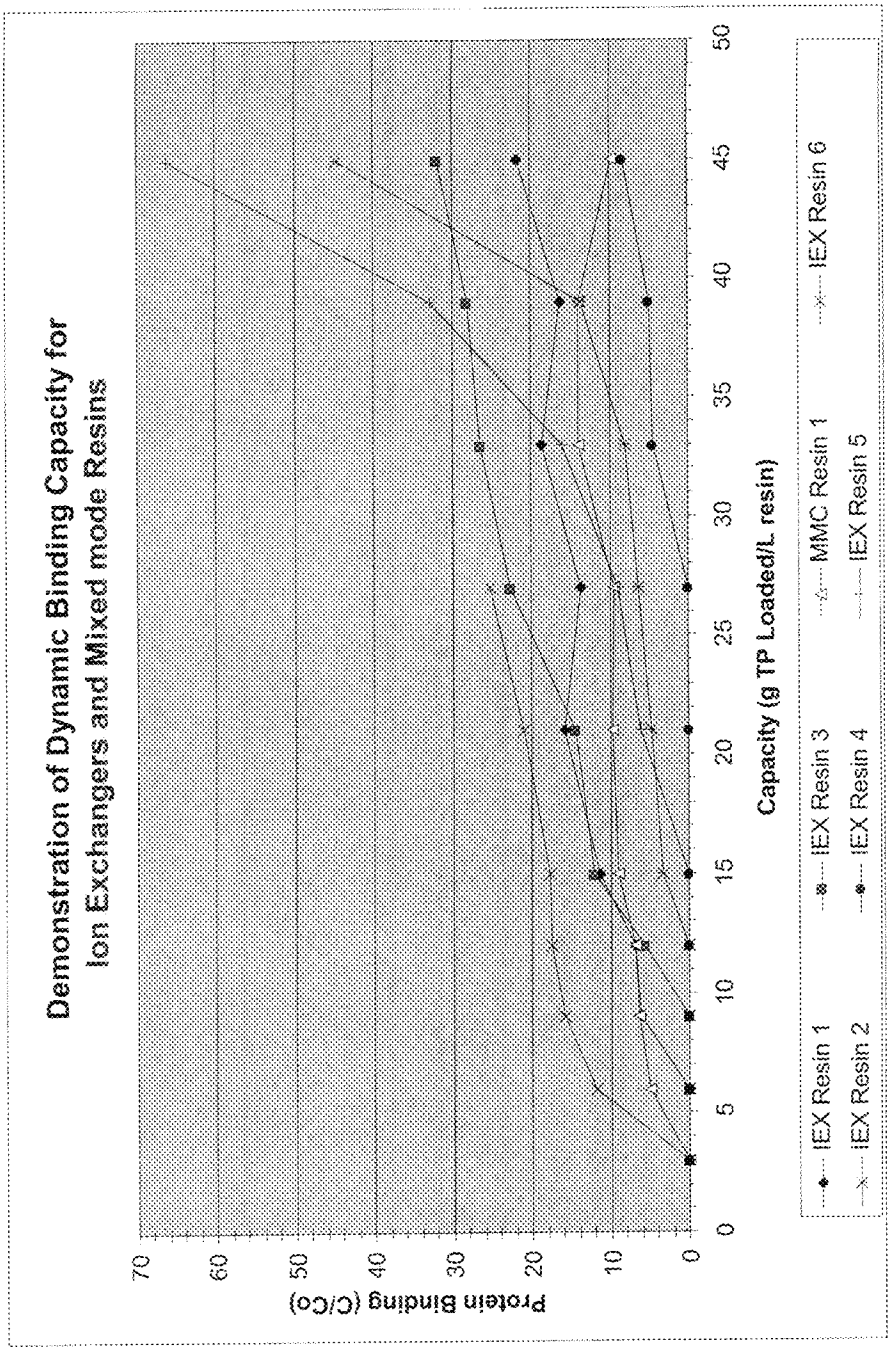
Demonstration of Dynamic Binding Capacity for Ion Exchangers and Mixed mode Resins
Protein Binding (C/Co)
0
10
20
30
40
50
60
70
Capacity (g TP Loaded/L resin)
0
5
10
15
20
25
30
35
40
45
50
IEX Resin 1
IEX Resin 2
IEX Resin 3
IEX Resin 4
MMC Resin 1
IEX Resin 5
IEX Resin 6

Figure 4

	RP-HPLC Main Peak Purity (%)	SE-HPLC Main Peak Purity (%)	Average Yield (%)
Load	29.8	64.6	-
CEX	46.0	80.3	62.0
AEX	30.9	75.7	85.0

Figure 5

CAPTURE PURIFICATION PROCESSES FOR PROTEINS EXPRESSED IN A NON-MAMMALIAN SYSTEM

This application is a divisional of U.S. application Ser. No. 12/822,990, filed on Jun. 24, 2010, now U.S. Pat. No. 8,940,878; which claims the benefit of U.S. Provisional Application No. 61/220,477 filed Jun. 25, 2009, which is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates generally to processes for purifying proteins expressed in non-mammalian systems in both non-native soluble and non-native insoluble forms, and more particularly to the direct capture of such proteins from a refold mixture or a cell lysate pool by a separation matrix.

BACKGROUND OF THE INVENTION

Fc-containing proteins are typically expressed in mammalian cells, such as CHO cells. The use of affinity chromatography to purify Fc-containing proteins is documented (see, e.g., Shukla et al., (2007) *Journal of Chromatography B* 848(1):28-39) and is successful, in part, due to the degree of Fc structure observed in proteins expressed in such systems. Fc-containing proteins expressed in non-mammalian cells, however, are often deposited in the expressing cells in limited solubility forms, such as inclusion bodies, that require refolding, and this has been a limiting factor in selecting non-mammalian systems for expressing Fc-containing proteins.

A drawback to the use of Protein A, Protein G and other chemistries is that in order for a protein comprising an Fc region to associate with the Protein A or Protein G molecule, the protein needs to have a minimum amount of structure. Often, the requisite amount of structure is absent from proteins expressed recombinantly in a soluble, but non-native, form and consequently Protein A chromatography is not performed in a purification process.

In the case of a protein expressed in an insoluble non-native form, Protein A chromatography is typically not performed in a purification process until after the protein has been refolded to a degree that it can associate with the Protein A molecule and has been subsequently diluted out of its refold solution. This is because it was believed that after a protein has been refolded it was necessary to dilute or remove the components of the refold mixture in a wash step, due to the tendency of the components that typically make up a refold solution to disrupt interactions between the target protein and the Protein A molecules (Wang et al., (1997). *Biochem. J.* 325(Part 3):707-710). This dilution step can consume time and resources which, when working at a manufacturing scale of thousands of liters of culture, can be costly.

The present disclosure addresses these issues by providing simplified methods of purifying proteins comprising Fc regions that are expressed in non-mammalian expression systems in a non-native soluble form or in a non-native insoluble form.

SUMMARY OF THE INVENTION

A method of purifying a protein expressed in a non-native soluble form in a non-mammalian expression system is provided. In one embodiment the method comprises (a) lysing a non-mammalian cell in which the protein is expressed in a non-native soluble form to generate a cell lysate; (b) contacting the cell lysate with an separation matrix under conditions suitable for the protein to associate with the separation matrix; (c) washing the separation matrix; and (d) eluting the protein from the separation matrix.

The protein can be a complex protein, such as a protein is selected from the group consisting of a multimeric protein, an antibody and an Fc fusion protein. The non-mammalian expression system can comprise bacteria or yeast cells. The separation matrix can be an affinity resin, such as an affinity resin selected from the group consisting of Protein A, Protein G and a synthetic mimetic affinity resin, or it can be a non-affinity resin, such as a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin. The cell lysate can be filtered before it is contacted with the separation matrix. Although not required, the method can further comprise refolding the protein to its native form after it is eluted from the separation matrix.

A method of purifying a protein expressed in a non-native limited solubility form in a non-mammalian expression system is provided. In one embodiment that method comprises (a) expressing a protein in a non-native limited solubility form in a non-mammalian cell; (b) lysing a non-mammalian cell; (c) solubilizing the expressed protein in a solubilization solution comprising one or more of the following: (i) a denaturant; (ii) a reductant; and (iii) a surfactant; (d) forming a refold solution comprising the solubilization solution and a refold buffer, the refold buffer comprising one or more of the following: (i) a denaturant; (ii) an aggregation suppressor; (iii) a protein stabilizer; and (iv) a redox component; (e) applying the refold solution to a separation matrix under conditions suitable for the protein to associate with the matrix; (f) washing the separation matrix; and (g) eluting the protein from the separation matrix.

The non-native limited solubility form can be a component of an inclusion body. The protein can be a complex protein, such as a complex protein selected from the group consisting of a multimeric protein, an antibody, a peptibody, and an Fc fusion protein. The non-mammalian expression system can be bacteria or yeast cells. The denaturant can comprise one or more of urea, guanidinium salts, dimethyl urea, methylurea and ethylurea, the reductant can comprise one or more of cysteine, DTT, beta-mercaptoethanol and glutathione, the surfactant can comprise one or more of sarcosyl and sodium dodecylsulfate, the aggregation suppressor can be selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, tris, sodium sulfate, potassium sulfate and osmolytes, the protein stabilizer can comprise one or more of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, tris, sodium sulfate, potassium sulfate and osmolytes, and the redox component can comprise one or more of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol. The separation matrix can be an affinity resin such as an affinity resin selected from the group consisting of Protein A, Protein G, and synthetic mimetic affinity resin or the separation matrix can be a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin.

In other embodiments, the disclosed methods can further comprise the steps of (a) washing the separation matrix with

a regeneration reagent; and (b) regenerating the separation matrix. The regeneration reagent can be one of a strong base, such as sodium hydroxide or a strong acid, such as phosphoric acid. The regenerating can comprise washing the separation matrix with a solution comprising one or both of a chaotrope present at a concentration of 4-6 M and a reductant. The chaotrope can be one of urea, dimethyl urea, methylurea, ethylurea, and guanidinium, and the reductant can be one of cysteine, DTT, beta-mercaptoethanol and glutathione. In a particular embodiment the regenerating comprises washing the separation matrix with a solution comprising 50 mM Tris, 10 mM citrate, 6M urea, 50 mM DTT at pH 7.4.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a plot demonstrating the binding of refolded, non-mammalian non-native limited solubility fraction complex protein, to Protein A media; in the figure the X denotes resin loading at a 9.32 min residence time, star denotes resin loading at a 7.68 min residence time and solid circles denote resin loading at a 6 min residence time.

FIG. **2** is a table demonstrating purification of a complex protein comprising an Fc domain using Protein A resin.

FIG. **3** is a table demonstrating the reusability of Protein A resin when used to capture a non-mammalian non-native limited solubility complex protein over 150 cycles using the disclosed methods.

FIG. **4** is a plot demonstrating the binding profiles of a refolded, non-mammalian non-native limited solubility complex protein to six different ion exchange resins (IEX Resins 1, 2, 3, 4, 5, 6, corresponding to Toyopearl SP550C™, Toyopearl SP650M™, GigaCAP S™, POROS HS50™, Toyopearl SP650C™ and GE Healthcare SPxL™, respectively) and a mixed-mode resin (MMC Resin 1, GE Healthcare MMC™) following capture using the disclosed methods.

FIG. **5** is a table demonstrating purification levels achieved for a protein comprising an Fc domain using one anion exhange resin (Fractogel TMAE™) and one cation exchange resin (Fractogel SO_3^-™).

DETAILED DESCRIPTION OF THE INVENTION

The present disclosure provides methods of capturing on a separation matrix non-native proteins produced in microbial cells. In the case of the direct capture of a protein expressed in a non-native soluble form the advantages of the present invention over typical processes include enhanced protein concentration, volume reduction, and increased recovery over traditional methods, improved protein stability, and ultimately process cost savings.

In the case of the direct capture of a protein expressed in a non-native limited solubility form, the advantages of the present invention over typical processes include the elimination of the need to dilute the protein out of a refold solution prior to capturing it on a separation matrix.

Another advantage of the disclosed methods is that they may be performed at a range of scales, from laboratory scale (typically milliliter or liter scale), a pilot plant scale (typically hundreds of liters) or on an industrial scale (typically thousands of liters). The application of the disclosed methods on large scales may be particularly desirable, due to the potential savings in time and resources.

Non-mammalian, e.g., microbial, cells can naturally produce, or can be engineered to produce, proteins that are expressed in either a soluble or a limited solubility form. Most often, engineered non-mammalian cells will deposit the recombinant proteins into large limited solubility aggregates called inclusion bodies. However, certain cell growth conditions (e.g., temperature or pH) can be modified to drive the recombinant proteins to be expressed as intracellular, soluble monomers. As an alternative to producing a protein of interest in cells in which the protein is expressed in the form of limited solubility inclusion bodies, cell growth conditions can be modified such that proteins are expressed in a non-native yet soluble form. The cells can then be lysed and the protein can be isolated by capturing it directly from cell lysate using ion exchange chromatography, affinity chromatography or mixed mode chromatography, as described herein. The method can be particularly useful for purifying proteins comprising an Fc region.

In one aspect, therefore, the present disclosure relates to a method of isolating a protein of interest comprising an Fc region that is expressed in a non-mammalian cell in a non-native, yet soluble form, from a pool of lysate generated from the cell in which the protein was expressed. The method employs a separation matrix, such as Protein A. One beneficial aspect of the disclosed method is that it eliminates the need for a refolding step before the protein is applied to the separation matrix. That is, non-mammalian cells expressing the protein of interest in a non-native soluble form can be lysed, the lysate applied directly to the separation matrix and the protein subsequently eluted from the separation matrix. This process allows the separation of proteins from cell cultures in highly concentrated pools that can be subsequently refolded at high concentrations and can be of benefit when producing large quantities of protein, particularly since the method is scalable from bench scale, which involves cultures on the order of several liters, up to production scale, which involves cultures of thousands of liters.

Following isolation by the separation matrix, the protein of interest can optionally be subsequently refolded using any technique known or suspected to work well for the protein of interest.

In another aspect, the present invention relates to a method of isolating a protein of interest comprising an Fc region that is expressed in a non-native limited solubility form, for example in inclusion bodies, that needs to be refolded and isolated from the refold mixture. Commonly, a refold solution contains a denaturant (e.g., urea or other chaotrope, organic solvent or strong detergent), an aggregation suppressor (e.g., a mild detergent, arginine or low concentrations of PEG), a protein stabilizer (e.g., glycerol, sucrose or other osmolyte, salts) and/or a redox component (e.g., cysteine, cystine, cystamine, cysteamine, glutathione). While often beneficial for refolding proteins, these components can inhibit purification (see, e.g., Wang et al., (1997) *Biochemical Journal* 325 (Part 3):707-710) and it is necessary to isolate or dilute the protein from these components for further processing, particularly before applying the protein to a separation matrix.

In one embodiment of the disclosed method, purification is achieved by directly applying a protein of interest, which is present in a refold mixture, to a separation matrix. In this approach, following a refold step the entire refold mixture, including the protein of interest, is applied directly to a separation matrix, such as a Protein A or G resin. The protein of interest associates with the matrix in the presence of the components of refold buffer, impurities are washed away and the protein is eluted. Since the method omits the need for removing any components of the refold mixture before the

refold mixture is applied to a separation matrix, the method can have the effect of saving steps, time and resources that are typically expended on removing the protein from refolding and dilution buffers in purification processes. In some cases, the method can also reduce or eliminate the need for subsequent purification steps.

The disclosed methods can also be employed to purify proteins expressed in a non-native soluble and non-native limited solubility forms in a non-mammalian expression system that have subsequently been derivatized. For example, following expression a protein comprising an Fc region can be associated with a small molecule, such as a toxin. Such conjugates can be purified using the methods described herein.

I. DEFINITIONS

As used herein, the terms "a" and "an" mean one or more unless specifically indicated otherwise.

As used herein, the term "non-mammalian expression system" means a system for expressing proteins in cells derived from an organism other than a mammal, including but not limited to, prokaryotes, including bacteria such as *E. coli*, and yeast. Often a non-mammalian expression system is employed to express a recombinant protein of interest, while in other instances a protein of interest is an endogenous protein that is expressed by a non-mammalian cell. For purposes of the present disclosure, regardless of whether a protein of interest is endogenous or recombinant, if the protein is expressed in a non-mammalian cell then that cell is a "non-mammalian expression system." Similarly, a "non-mammalian cell" is a cell derived from an organism other than a mammal, examples of which include bacteria or yeast.

As used herein, the term "denaturant" means any compound having the ability to remove some or all of a protein's secondary and tertiary structure when placed in contact with the protein. The term denaturant refers to particular chemical compounds that affect denaturation, as well as solutions comprising a particular compound that affect denaturation. Examples of denaturants that can be employed in the disclosed method include, but are not limited to urea, guanidinium salts, dimethyl urea, methylurea, ethylurea and combinations thereof.

As used herein, the term "aggregation suppressor" means any compound having the ability to disrupt and decrease or eliminate interactions between two or more proteins. Examples of aggregation suppressors can include, but are not limited to, amino acids such as arginine, proline, and glycine; polyols and sugars such as glycerol, sorbitol, sucrose, and trehalose; surfactants such as, polysorbate-20, CHAPS, Triton X-100, and dodecyl maltoside; and combinations thereof.

As used herein, the term "protein stabilizer" means any compound having the ability to change a protein's reaction equilibrium state, such that the native state of the protein is improved or favored. Examples of protein stabilizers can include, but are not limited to, sugars and polyhedric alcohols such as glycerol or sorbitol; polymers such as polyethylene glycol (PEG) and α-cyclodextrin; amino acids salts such as arginine, proline, and glycine; osmolytes and certain Hoffmeister salts such as Tris, sodium sulfate and potassium sulfate; and combinations thereof.

As used herein, the terms "Fc" and "Fc region" are used interchangeably and mean a fragment of an antibody that comprises human or non-human (e.g., murine) C_{H2} and C_{H3} immunoglobulin domains, or which comprises two contiguous regions which are at least 90% identical to human or non-human C_{H2} and C_{H3} immunoglobulin domains. An Fc can but need not have the ability to interact with an Fc receptor. See, e.g., Hasemann & Capra, "Immunoglobulins: Structure and Function," in William E. Paul, ed., *Fundamental Immunology*, Second Edition, 209, 210-218 (1989), which is incorporated by reference herein in its entirety.

As used herein, the terms "protein" and "polypeptide" are used interchangeably and mean any chain of at least five naturally or non-naturally occurring amino acids linked by peptide bonds.

As used herein, the term "complex molecule" means any protein that is (a) larger than 20,000 MW, or comprises greater than 250 amino acid residues, and (b) comprises two or more disulfide bonds in its native form. A complex molecule can, but need not, form multimers. Examples of complex molecules include but are not limited to, antibodies, peptibodies and polypeptides comprising an Fc domain and other large proteins. Peptibodies are described in U.S. Pat. No. 6,660,843, U.S. Pat. No. 7,138,370 and U.S. Pat. No. 7,511,012.

As used herein, the term "peptibody" refers to a polypeptide comprising one or more bioactive peptides joined together, optionally via linkers, with an Fc domain. See U.S. Pat. No. 6,660,843, U.S. Pat. No. 7,138,370 and U.S. Pat. No. 7,511,012 for examples of peptibodies.

As used herein, the terms "Fc fusion" and "Fc fusion protein" are used interchangeably and refer to a peptide or polypeptide covalently attached to an Fc domain.

As used herein the term "Protein A" means any protein identical or substantially similar to Staphylococcal Protein A, including commercially available and/or recombinant forms of Protein A. For the purposes of this invention, Protein A specifically includes engineered Protein A derived media, such as Mab Select SuRe™ media (GE Healthcare), in which a single subunit (e.g., the B subunit) is replicated two or more times and joined in a contiguous sequence to form a recombinant Protein A molecule, and other non-naturally occurring Protein A molecules.

As used herein, the term "Protein G" means any protein identical or substantially similar to Streptococcal Protein G, including commercially available and/or recombinant forms of Protein G.

As used herein, the term "substantially similar," when used in the context of a protein, including Protein A, means proteins that are at least 80%, preferably at least 90% identical to each other in amino acid sequence and maintain or alter in a desirable manner the biological activity of the unaltered protein. Included in amino acids considered identical for the purpose of determining whether proteins are substantially similar are amino acids that are conservative substitutions, unlikely to affect biological activity, including the following: Ala for Ser, Val for Ile, Asp for Glu, Thr for Ser, Ala for Gly, Ala for Thr, Ser for Asn, Ala for Val, Ser for Gly, Tyr for Phe, Ala for Pro, Lys for Arg, Asp for Asn, Leu for Ile, Leu for Val, Ala for Glu, Asp for Gly, and these changes in the reverse. See, e.g., Neurath et al., *The Proteins*, Academic Press, New York (1979). The percent identity of two amino sequences can be determined by visual inspection and mathematical calculation, or more preferably, the comparison is done by comparing sequence information using a computer program such as the Genetics Computer Group (GCG; Madison, Wis.) Wisconsin package version 10.0 program, "GAP" (Devereux et al., 1984, *Nucl. Acids Res.* 12: 387) or other comparable computer programs. The preferred default parameters for the "GAP" program includes: (1) the weighted amino acid comparison matrix of

Gribskov and Burgess ((1986), *Nucl. Acids Res.* 14: 6745), as described by Schwartz and Dayhoff, eds., *Atlas of Polypeptide Sequence and Structure,* National Biomedical Research Foundation, pp. 353-358 (1979), or other comparable comparison matrices; (2) a penalty of 30 for each gap and an additional penalty of 1 for each symbol in each gap for amino acid sequences; (3) no penalty for end gaps; and (4) no maximum penalty for long gaps. Other programs used by those skilled in the art of sequence comparison can also be used.

As used herein, the terms "isolate" and "purify" are used interchangeably and mean to reduce by 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95%, or more, the amount of heterogenous elements, for example biological macromolecules such as proteins or DNA, that may be present in a sample comprising a protein of interest. The presence of heterogenous proteins can be assayed by any appropriate method including High-performance Liquid Chromatography (HPLC), gel electrophoresis and staining and/or ELISA assay. The presence of DNA and other nucleic acids can be assayed by any appropriate method including gel electrophoresis and staining and/or assays employing polymerase chain reaction.

As used herein, the term "separation matrix" means any adsorbent material that utilizes specific, reversible interactions between synthetic and/or biomolecules, e.g., the property of Protein A to bind to an Fc region of an IgG antibody or other Fc-containing protein, in order to effect the separation of the protein from its environment. In other embodiments the specific, reversible interactions can be base on a property such as isoelectric point, hydrophobicity, or size. In one particular embodiment, a separation matrix comprises an adsorbent, such as Protein A, affixed to a solid support. See, e.g., Ostrove (1990) in "Guide to Protein Purification," *Methods in Enzymology* 182: 357-379, which is incorporated herein in its entirety.

As used herein, the terms "non-native" and "non-native form" are used interchangeably and when used in the context of a protein of interest, such as a protein comprising a Fc domain, mean that the protein lacks at least one formed structure attribute found in a form of the protein that is biologically active in an appropriate in vivo or in vitro assay designed to assess the protein's biological activity. Examples of structural features that can be lacking in a non-native form of a protein can include, but are not limited to, a disulfide bond, quaternary structure, disrupted secondary or tertiary structure or a state that makes the protein biologically inactive in an appropriate assay. A protein in a non-native form can but need not form aggregates.

As used herein, the term "non-native soluble form" when used in the context of a protein of interest, such as a protein comprising a Fc domain, means that the protein lacks at least one formed structure attribute found in a form of the protein that is biologically active in an appropriate in vivo or in vitro assay designed to assess the protein's biological activity, but in which the protein is expressed in a form or state that is soluble intracellularly (for example in the cell's cytoplasm) or extracellularly (for example, in a lysate pool).

As used herein, the term "non-native limited solubility form" when used in the context of a protein of interest, such as a protein comprising a Fc domain, means any form or state in which the protein lacks at least one formed structural feature found in a form of the protein that (a) is biologically active in an appropriate in vivo or in vitro assay designed to assess the protein's biological activity and/or (b) forms aggregates that require treatment, such as chemical treatment, to become soluble. The term specifically includes proteins existing in inclusion bodies, such as those sometimes found when a recombinant protein is expressed in a non-mammalian expression system.

As used herein, the term "soluble form" when used in the context of a protein of interest, such as a protein comprising a Fc domain, broadly refers to a form or state in which the protein is expressed in a form that is soluble in a intracellularly (for example in the cell's cytoplasm) or extracellularly (for example, in a cell lysate pool).

II. DIRECT CAPTURE OF A PROTEIN EXPRESSED IN A NON-NATIVE SOLUBLE FORM IN A NON-MAMMALIAN EXPRESSION SYSTEM

One advantage of the disclosed method over typical purification methods is the elimination of the need for a refolding step before the soluble protein is applied to the separation matrix. That is, a protein solublized in cell lysate can be directly applied to the separation matrix. This is advantageous because the method does not require any initial purification efforts, although an initial filtration step may be desirable in some cases.

In the case of a protein comprising a Fc domain, the Fc region must have a certain level of structure to be bound by protein A, (Wang et al., (1997) *Biochem. J.* 325(Part 3):707-710). This fact has limited the application of separation matrices for purifying proteins that are expressed in a non-native soluble form, particularly proteins comprising an Fc region, because it is commonly believed that a soluble non-native Fc-containing protein would not have the requisite structural elements required to associate with a separation matrix. Furthermore, the Fc region of an antibody spontaneously forms a homodimer under non-reducing conditions and prior to the instant disclosure it was unexpected to observe that even in the reductive environment of the cell, the Fc-conjugated proteins and peptides not only form enough structure for protein to bind to the affinity resin, but that the individual peptide chains readily formed non-covalent dimers, even though the proteins had not yet been completely refolded to native form.

In view of prevailing beliefs, the success of the disclosed method was surprising and unanticipated because it was not expected that a non-mammalian, microbial cell fermentation could be induced to produce a protein that was soluble, yet still had enough structure to associate with the affinity separation matrix.

The disclosed method can be employed to purify a protein of interest that is expressed in a non-native soluble form in a non-mammalian cell expression system. The protein of interest can be produced by living host cells that either naturally produce the protein or that have been genetically engineered to produce the protein. Methods of genetically engineering cells to produce proteins are known in the art. See, e.g., Ausabel et al., eds. (1990), *Current Protocols in Molecular Biology* (Wiley, New York). Such methods include introducing nucleic acids that encode and allow expression of the protein into living host cells. In the context of the present disclosure, a host cell will be a non-mammalian cell, such as bacterial cells, fungal cells, yeast cells, and insect cells. Bacterial host cells include, but are not limited to, *Escherichia coli* cells. Examples of suitable *E. coli* strains include: HB101, DH5α, GM2929, JM109, KW251, NM538, NM539, and any *E. coli* strain that fails to cleave foreign DNA. Fungal host cells that can be used include, but are not limited to, *Saccharomyces cerevisiae, Pichia pasto-*

ris and *Aspergillus* cells. New cell lines can be established using methods known to those skilled in the art (e.g., by transformation, viral infection, and/or selection). It is noted that the method can be performed on proteins that are endogenously expressed by the non-mammalian cell as well.

During the production of a non-mammalian culture, growth conditions can be identified and employed so as to favor the production of a protein of interest in an intracellular soluble form. Such conditions can be identified by systematic empirical optimization of the culture condition parameters, such as temperature or pH. This optimization can be achieved using analysis of multifactorial matrices. For example, a matrix or series of multifactorial matrices can be evaluated to optimize temperature and pH conditions favor production of a desired species (i.e., a non-native soluble form). An optimization screen can be set up to systematically evaluate temperature and pH in a full or partial factorial matrix, with each component varied over a range of at least three temperature or pH levels with all other parameters kept constant. The protein can be expressed and the yield and quality of protein expressed in the desired form can be evaluated using standard multivariate statistical tools.

Initially, non-mammalian cells that express a particular protein of interest are grown to a desired target density under conditions designed to induce expression of the protein in a soluble form. In one embodiment, the cells express a wild type protein of interest. In another embodiment, the cells can be engineered using standard molecular biology techniques to recombinantly express a protein of interest, and induced to produce the protein of interest. The protein of interest can be any protein, for example a protein that comprises an Fc moiety. Such a protein can be, for example, an antibody, a peptibody or an Fc fusion protein, any of which can be joined to an Fc moiety via a linker.

Once the desired target density is reached, the non-mammalian cells are separated from the growth media. One convenient way of achieving separation is by centrifugation, however filtration and other clarification methods can also be used.

The cells are then collected and are resuspended to an appropriate volume in a resuspension solution. Examples of resuspension solutions that can be used in the disclosed methods include phosphate buffered saline, Tris buffered saline, or water. The selection of an appropriate buffer will be determined, in part, by the properties of the molecule of interest as well as any volume or concentration constraints.

Following resuspension, the non-mammalian cells are lysed to release the protein, which will be present in the cell lysate in a non-native soluble form to generate a cell lysate. The lysis can be performed using any convenient means, such as feeding the cell suspension through a high pressure homogenizer or by employing a chemical lysis process. Whichever lytic process is selected, the function of the lysis step is to break open the cells and to break down DNA. The lysis can be performed in multiple cycles to achieve a more complete lysis or to accommodate large volumes of cell suspension. For example, the cell suspension can be fed through a mechanical homogenizer several times. This process releases the intracellular contents, including the protein of interest, and forms a pool of cell lysate.

Following the lysis procedure, the cell lysate can optionally be filtered. Filtration can remove particulate matter and/or impurities, such as nucleic acids and lipids, and may be desirable in some cases, such as when one suspects that direct application of the cell lysate to the chromatography equipment or media may lead to fouling or clogging, or when the separation matrix is sensitive to fouling or difficult to clean in-place. The benefit of filtering the cell lysate prior to contacting it with the separation matrix can be determined on a case-by-case basis.

After the lysis procedure, the cell lysate can optionally be incubated for an appropriate amount of time in the presence of air or oxygen, or exposed to a redox component or redox thiol-pair. The incubation can facilitate and/or ensure the formation of the minimal secondary structure required to facilitate an association with a separation matrix. The particular length of the incubation can vary with the protein but is typically less than 72 hours (e.g., 0, 0.5, 1, 2, 3, 5, 7, 10, 12, 18, 24, 36, 48 or 72 hours). When an incubation is performed, the length of incubation time can be determined by empirical analysis for each protein, which in some cases will be shorter (or omitted) and other cases longer.

Following the incubation period the cell lysate, which comprises the released protein of interest, is contacted with a separation matrix under conditions suitable for the protein to associate with a binding element of the separation matrix. Representative conditions conducive to the association of a protein with an affinity matrix are provided in the Examples. The separation matrix can be any media by which the protein of interest can be separated from the components of the resuspension and/or lysis buffer, including impurities such as host cell proteins, DNA, lipids and chemical impurities introduced by the components of the resuspension and/or lysis buffer.

Proteins A and G are often employed to purify antibodies, peptibodies and other fusion proteins comprising a Fc region by affinity chromatography. See, e.g., Vola et al. (1994), *Cell Biophys.* 24-25: 27-36; Aybay and Imir (2000), *J. Immunol. Methods* 233(1-2): 77-81; Ford et al. (2001), *J. Chromatogr. B* 754: 427-435. Proteins A and G are useful in this regard because they bind to the Fc region of these types of proteins. Recombinant fusion proteins comprising an Fc region of an IgG antibody can be purified using similar methods. Proteins A and G can be employed in the disclosed methods as an adsorbent component of a separation matrix.

Thus, examples of separation matrices that can be employed in the present invention include Protein A resin, which is known to be, and is commonly employed as, an effective agent for purifying molecules comprising an Fc moiety, as well as Protein G and synthetic mimetic affinity resins, such as MEP HyperCel® chromatography resin.

After the protein of interest has been associated with the separation matrix by contacting the cell lysate containing the protein with the separation matrix, thereby allowing the protein to associate with the adsorbent component of the separation matrix, the separation matrix is washed to remove unbound lysate and impurities.

The wash buffer can be of any composition, as long as the composition and pH of the wash buffer is compatible with both the protein and the matrix, and maintains the interaction between the protein and the matrix. Examples of suitable wash buffers that can be employed include solutions containing glycine, Tris, citrate, or phosphate; typically at levels of 5-100 mM (e.g., 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75 or 100 mM). These solutions can also contain an appropriate salt ion, such as chloride, sulfate or acetate at levels of 5-500 mM (e.g., 5, 10, 12, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450 or 500 mM). The resin can be washed once or any number of times. The exact composition of a wash buffer will vary with the protein being purified.

After the separation matrix with which the protein has associated has been washed, the protein of interest is eluted from the matrix using an appropriate solution. The protein of interest can be eluted using a solution that interferes with the

binding of the adsorbent component of the separation matrix to the protein, for example by disrupting the interactions between the separation matrix and the protein of interest. This solution can include an agent that can either increase or decrease pH, and/or a salt. For example, the pH can be lowered to about 4.5 or less, for example to between about 3.3 and about 4.0, e.g., 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4 or 4.5. A solution comprising citrate or acetate, for example, can be employed to lower the pH. Other methods of elution are also known, such as via the use of chaotropes (see, e.g., Ejima et al. (2005) *Analytical Biochemistry* 345(2):250-257) or amino acid salts (see, e.g., Arakawa et al. (2004) *Protein Expression & Purification* 36(2):244-248). Protocols for such affinity chromatography are well known in the art. See, e.g., Miller and Stone (1978), *J. Immunol. Methods* 24(1-2): 111-125. Conditions for binding and eluting can be readily optimized by those skilled in the art. The exact composition of an elution buffer will vary with the protein being purified. The protein can then optionally be further purified from the elution pool and refolded as necessary. In other situations the protein need not be further purified and instead can be refolded directly from the elution pool. Refolding directly from the elution pool may or may not require denaturation or reduction of the protein prior to incubation in a refolding solution and will depend in part on the properties of the protein.

In some cases it will be desirable to provide the separation matrix in a column format. In such cases a chromatography column can be prepared and then equilibrated before the cell suspension is loaded. Techniques for generating a chromatography column are well known and can be employed. An optional preparation and equilibration step can comprise washing the column with a buffer having an appropriate pH and salt condition that is conducive to protein-matrix interactions. This step can provide the benefit of removing impurities present in the separation matrix and can enhance the binding of the protein to be isolated to the adsorbent component of a separation matrix.

As noted, the separation matrix can be disposed in a column. The column can be run with or without pressure and from top to bottom or bottom to top. The direction of the flow of fluid in the column can be reversed during the purification process. Purifications can also be carried out using a batch process in which the solid support is separated from the liquid used to load, wash, and elute the sample by any suitable means, including gravity, centrifugation, or filtration. Moreover, purifications can also be carried out by contacting the sample with a filter that adsorbs or retains some molecules in the sample more strongly than others, such as anion exchange membrane chromatography.

If desired, the protein concentration of a sample at any given step of the disclosed method can be determined, and any suitable method can be employed. Such methods are well known in the art and include: 1) colorimetric methods such as the Lowry assay, the Bradford assay, the Smith assay, and the colloidal gold assay; 2) methods utilizing the UV absorption properties of proteins; and 3) visual estimation based on stained protein bands on gels relying on comparison with protein standards of known quantity on the same gel. See, e.g., Stoschek (1990), "Quantitation of Protein," in "Guide to Protein Purification," *Methods in Enzymology* 182: 50-68. Periodic determinations of protein concentration can be useful for monitoring the progress of the method as it is performed.

It is noted that any or all steps of the disclosed methods can be carried out manually or by any convenient automated means, such as by employing automated or computer-controlled systems.

III. DIRECT CAPTURE OF NON-NATIVE LIMITED SOLUBILITY PROTEIN FORMS FROM A REFOLD SOLUTION FOLLOWING EXPRESSION IN NON-MAMMALIAN CELLS

In another aspect of the present disclosure, a method of purifying a protein expressed in a non-native limited solubility form in a non-mammalian expression system is disclosed. An advantage of the disclosed method is that the method eliminates the need for removing or diluting the refold solution before applying the protein to a separation matrix, thereby saving the time and resources associated with what is a typical step in a purification process for isolating proteins expressed in a non-native limited solubility form.

Non-mammalian cells, e.g., microbial cells, can produce recombinant proteins that are expressed intracellularly in either a soluble or a limited solubility form. When the growth conditions are not directed to force expression of the protein in a soluble form, the cells may deposit the recombinant proteins into large relatively insoluble aggregates, such as inclusion bodies. These aggregates comprise protein that is typically not biologically active or less active than the completely folded native form of the protein. In order to produce a functional protein, these inclusion bodies often need to be carefully denatured so that the protein of interest can be extracted and refolded into a biologically active form.

In typical approaches, the inclusion bodies need to be captured, washed, exposed to a denaturing and/or reducing solubilization solution and the denaturing solution is then diluted with a solution to generate a condition that allows the protein to refold into an active form and form a structure that is found in the native protein. Subsequently, it is necessary to remove the components of the diluted denaturing solution from the immediate location of the protein. In order to do this, the refold solution comprising the solubilization solution and the refolded protein is typically diluted with a buffered solution before it is applied to a separation matrix, such as a Protein A ion exchange or other mixed-mode adsorbents. This process can be time-consuming and resource-intensive. It also significantly increases the volumes that need to be handled, as well as the associated tankage requirements, which can become limiting when working on large scales. The disclosed method eliminates the need for such a dilution step

The disclosed method is particularly useful for purifying a protein of interest that is expressed in a non-native limited solubility form in a non-mammalian cell expression system. The protein of interest can be produced by living host cells that either naturally produce the protein or that have been genetically engineered to produce the protein. Methods of genetically engineering cells to produce proteins are well known in the art. See, e.g., Ausabel et al., eds. (1990), *Current Protocols in Molecular Biology* (Wiley, New York). Such methods include introducing nucleic acids that encode and allow expression of the protein into living host cells. In the context of the present disclosure, these host cells will be non-mammalian cells, such as bacterial cells, fungal cells. Bacterial host cells include, but are not limited to *Escherichia coli* cells. Examples of suitable *E. coli* strains include: HB101, DH5α, GM2929, JM109, KW251, NM538, NM539, and any *E. coli* strain that fails to cleave foreign

DNA. Fungal host cells that can be used include, but are not limited to, *Saccharomyces cerevisiae, Pichia pastoris* and *Aspergillus* cells. New cell lines can be established using methods well know by those skilled in the art (e.g., by transformation, viral infection, and/or selection). It is noted that the method can be performed on endogenous proteins that are naturally expressed by the non-mammalian cell as well.

Initially, non-mammalian cells that express a particular protein of interest are grown to a desired target density. In one embodiment, the cells can be expressing a particular wild type microbial protein of interest. In another embodiment, the cells can be engineered using standard molecular biology techniques to recombinantly express a protein of interest, and in this context they can be induced to overproduce the protein of interest. The protein of interest can be any protein, for example a protein that comprises an Fc moiety. Such a protein can be, for example, an antibody, a peptibody or an Fc fusion protein, any of which can be joined to an Fc moiety via a linker.

Once the desired target density is reached, the non-mammalian cells can be separated from the growth media. One convenient way of achieving separation is by centrifugation, however filtration and other clarification methods can also be used.

The cells are then collected and are resuspended to an appropriate volume in a resuspension solution. Examples of resuspension solutions that can be used in the present invention include phosphate-buffered saline, Tris-buffered saline, or water. The selection of an appropriate buffer will be determined, in part, by the properties of the molecule of interest as well as any volume or concentration constraints.

In order to release the limited solubility non-native protein from the cells, the non-mammalian cells are lysed to form a cell lysate comprising the released the limited solubility non-native protein. The lysis can be performed in any convenient way, such as feeding the cell suspension through a high pressure homogenizer or by employing a chemical lysis process. Whichever lysis process is selected, the function of the lysis step is to break open the cells and to break down DNA. The lysis can be performed in multiple cycles to achieve a more complete lysis or to accommodate large volumes of cell suspension. For example, the cell suspension can be fed through a mechanical homogenizer several times. This process releases the intracellular contents, including the naturally-occurring or recombinant protein of interest, and forms a pool of cell lysate.

Next, the limited solubility non-native protein is separated from the rest of the lysis pool. This can be done, for example, by centrifugation. Representative conditions for a centrifuge-mediated separation or washing typically include removal of excess water from the cell lysate, resuspension of the resulting slurry in a resuspension solution. This washing process may be performed once or multiple times. Examples of typical centrifuge types include, but are not limited to, disk-stack, continuous discharge, and tube bowl. Examples of resuspension solutions that can be used in the present invention include phosphate-buffered saline, Tris-buffered saline, or water and can include other agents, such as ETDA or other salts. The selection of an appropriate buffer will be determined, in part, by the properties of the molecule of interest as well as any volume or concentration constraints. The exact composition of an resuspension buffer will vary with the protein being purified.

The expressed protein is then solubilized in a solubilization solution comprising one or more of (i) a denaturant, (ii) a reductant and (iii) a surfactant. The denaturant can be included as a means of unfolding the limited solubility protein, thereby removing any existing structure, exposing buried residues and making the protein more soluble.

Any denaturant can be employed in the solubilization solution. Examples of some common denaturants that can be employed in the refold buffer include urea, guanidinium, dimethyl urea, methylurea, or ethylurea. The specific concentration of the denaturant can be determined by routine optimization.

The reductant can be included as a means to reduce exposed residues that have a propensity to form covalent intra or intermolecular-protein bonds and minimize non-specific bond formation. Examples of suitable reductants include, but are not limited to, cysteine, DTT, beta-mercaptoethanol and glutathione. The specific concentration of the reductant can be determined by routine optimization.

A surfactant can be included as a means of unfolding the limited solubility non-native protein, thereby exposing buried residues and making the protein more soluble. Examples of suitable surfactants include, but are not limited to, sarcosyl and sodium dodecylsulfate. The specific concentration of the surfactant can be determined by routine optimization.

Although the composition of a solubilization solution will vary with the protein being purified, in one particular embodiment the solubilization solution comprises 4-6 M guanidine, 50 mM DTT.

Continuing, a refold solution comprising the solubilization solution (which comprises the protein), and a refold buffer is formed. The refold buffer comprises one or more of (i) a denaturant; (ii) an aggregation suppressor; (iii) a protein stabilizer; and (iv) a redox component. The denaturant can be included as a means of modifying the thermodynamics of the solution, thereby shifting the equilibrium towards an optimal balance of native form. The aggregation suppressor can be included as a means of preventing non-specific association of one protein with another, or with one region of a protein with another region of the same protein. The protein stabilizer can be included as a means of promoting stable native protein structure and may also suppress aggregation.

In various embodiments, the denaturant in the refold buffer can be selected from the group consisting of urea, guanidinium salts, dimethyl urea, methylurea and ethylurea.

In various embodiments, the protein stabilizer in the refold buffer can be selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes.

In various embodiments, the aggregation suppressor can be selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes.

In various embodiments, the thiol-pairs can comprise at least one component selected from the group consisting of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol.

The specific concentrations of the components of a refold buffer can be determined by routine optimization. For example, a matrix or series of multifactorial matrices can be evaluated to optimize the refolding buffer for conditions that optimize yield and distributions of desired species. An optimization screen can be set up to systematically evaluate denaturant, aggregation suppressor, protein stabilizer and redox component concentrations and proportions in a full or partial factorial matrix, with each component varied over a

range of concentrations with all other parameters kept constant. The completed reactions can be evaluated by RP-HPLC and SE-HPLC analysis for yield and product quality using standard multivariate statistical tools.

The function of the buffer component of the refold solution is to maintain the pH of the refold solution and can comprise any buffer that buffers in the appropriate pH range. Examples of the buffering component of a refold buffer that can be employed in the method include, but are not limited to, phosphate buffers, citrate buffers, tris buffer, glycine buffer, CHAPS, CHES, and arginine-based buffers, typically at levels of 5-100 mM (e.g., 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 80, 85, 90, 95 or 100, mM).

Although the composition of an refold buffer will vary with the protein being purified, in one embodiment a refold buffer comprises arginine, urea, glycerol, cysteine and cystamine.

The refold solution can then be incubated for a desired period of time. The incubation period can be of any length but is typically between 0 and 72 hours (e.g., 0, 0.5, 1, 2, 3, 5, 7, 10, 12, 18, 24, 36, 48 or 72 hours).

After an appropriate incubation time, the refold solution is then applied to a separation matrix under conditions suitable for the protein to associate with the matrix. The separation matrix can be any media by which the protein of interest can be separated from the components of the resuspension and/or lysis buffer, including impurities such as host cell proteins, DNA and chemical impurities introduced by the components of the solubilization and/or lysis buffer.

Proteins A and G are often employed to purify antibodies, peptibodies and other fusion proteins comprising a Fc region by affinity chromatography. See, e.g., Vola et al. (1994), *Cell Biophys.* 24-25: 27-36; Aybay and Imir (2000), *J. Immunol. Methods* 233(1-2): 77-81; Ford et al. (2001), *J. Chromatogr. B* 754: 427-435. Proteins A and G are useful in this regard because they bind to the Fc region of these types of proteins. Recombinant fusion proteins comprising an Fc region of an IgG antibody can be purified using similar methods. Proteins A and G can be employed in the disclosed methods as an adsorbent component of a separation matrix.

Thus, examples of affinity separation matrices that can be employed in the present invention include Protein A resin, which is know to be, and is commonly employed as, an effective agent for purifying molecules comprising an Fc moiety, as well as Protein G and synthetic mimetic affinity resins. Other materials that can be employed include HIC and ion exchange resins (see Example 4), depending on the properties of the protein to be purified.

It is noted that when performing the method, the refold solution comprising the refolded protein of interest is applied directly to the separation matrix, without the need for diluting or removing the components of the solution required for refolding the protein. This is an advantage of the disclosed method. Initially, it was expected that the highly ionic and/or chaotropic compounds and various other components of the refold solution would inhibit the association of the protein with the separation matrix. However, in contrast to reports in the literature (e.g., Wang et al. (1997) *Biochemical Journal.* 325(Part 3):707-710), it was surprising to observe that the protein was in fact able to associate with the separation matrix in the presence of the components of the refold solution. The unexpected finding that the protein could associate with the separation matrix in the presence of the components of the refold solution facilitates the elimination of a dilution step or buffer exchange operation, providing a savings of time and resources.

After the protein of interest has associated with the separation matrix the separation matrix is washed to remove unbound protein, lysate, impurities and unwanted components of the refold solution.

The wash buffer can be of any composition, as long as the composition and pH of the wash buffer is compatible with both the protein and the matrix. Examples of suitable wash buffers that can include, but are limited to, solutions containing glycine, tris, citrate, or phosphate. These solutions may also contain an appropriate salt. Suitable salts include, but are not limited to, sodium, potassium, ammonium, magnesium, calcium, chloride, fluoride, acetate, phosphate, and/or citrate. The pH range is chosen to optimize the chromatography conditions, preserve protein binding, and to retain the desired characteristics of the protein of interest. The resin can be washed once or any number of times. The exact composition of a wash buffer will vary with the protein being purified.

After the separation matrix with which the protein has associated has been washed, the protein of interest is eluted using an appropriate solution (e.g., a low pH buffered solution or a salt solution) to form an elution pool comprising the protein of interest.

The protein of interest can be eluted using a solution that interferes with the binding of the adsorbent component of the separation matrix to the protein, for example by disrupting the interactions between Protein A and the Fc region of a protein of interest. This solution may include an agent that can either increase or decrease pH, and/or a salt. In various embodiments, the elution solution can comprise acetic acid, glycine, or citric acid. Elution can be achieved by lowering the pH. For example, the pH can be lowered to about 4.5 or less, for example to between about 3.3 to about 4.2 (e.g., 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1 or 4.2, using a solution comprising citrate or acetate, among other possibilities.

In some situations, the protein can then be further purified from the elution pool and can be further refolded, if necessary. In other situations the protein need not be further purified and instead can be further refolded directly in the elution pool, if necessary.

Protocols for such affinity chromatography are known in the art. See, e.g., Miller and Stone (1978), *J. Immunol. Methods* 24(1-2): 111-125. In the cases that utilize ion exchange, mixed-mode, or hydrophobic interaction chromatography, the concentration of salt can be increased or decreased to disrupt ionic interaction between bound protein and a separation matrix. Solutions appropriate to effect such elutions can include, but are not limited to, sodium, potassium, ammonium, magnesium, calcium, chloride, fluoride, acetate, phosphate, and/or citrate. Other methods of elution are also known. Conditions for binding and eluting can be readily optimized by those skilled in the art.

The exact composition of an elution buffer will vary with the protein being purified and the separation matrix being employed.

In some cases it will be desirable to situate the separation matrix in a column format. In such cases a column can be prepared and then equilibrated before the cell suspension is loaded. Techniques for generating a chromatography column are well known and can be employed. The optional preparation and equilibration step can comprise washing the column with a buffer having an appropriate pH and composition that will prepare the media to bind a protein of interest. This step has the benefit of removing impurities present in the separation matrix and can enhance the binding of the protein to be isolated to the adsorbent component of a separation matrix.

It is noted that any or all steps of the invention can be carried out by any mechanical means. As noted, the separation matrix can be disposed in a column. The column can be run with or without pressure and from top to bottom or bottom to top. The direction of the flow of fluid in the column can be reversed during the purification process. Purifications can also be carried out using a batch process in which the solid support is separated from the liquid used to load, wash, and elute the sample by any suitable means, including gravity, centrifugation, or filtration. Moreover, purifications can also be carried out by contacting the sample with a filter that adsorbs or retains some molecules in the sample more strongly than others.

If desired, the protein concentration of a sample at any given step of the disclosed method can be determined by any suitable method. Such methods are well known in the art and include: 1) colorimetric methods such as the Lowry assay, the Bradford assay, the Smith assay, and the colloidal gold assay; 2) methods utilizing the UV absorption properties of proteins; and 3) visual estimation based on stained protein bands on gels relying on comparison with protein standards of known quantity on the same gel. See, e.g., Stoschek (1990), "Quantitation of Protein," in "Guide to Protein Purification," *Methods in Enzymology* 182: 50-68. Periodic determinations of protein concentration can be useful for monitoring the progress of the method as it is performed.

It is noted that any or all steps of the disclosed methods can be carried out manually or by any convenient automated means, such as by employing automated or computer-controlled systems.

IV. COLUMN CLEANING

In another aspect the present disclosure relates to the observation that in many cases the separation matrix employed in the methods provided herein can be cleaned after multiple separations and reused. This unexpected property of the method provides a significant cost and resource savings, particularly on the manufacturing scale, since the separation matrix need not be discarded after a separation is complete.

Common wisdom in the industry suggests that after a separation matrix, such as Protein A, is repeatedly exposed to highly heterogenous feedstocks comprising high lipid and host protein content it becomes irreversibly contaminated and unusable when treated with the mild regeneration solutions commonly utilized for protein-based affinity resins. The disclosed methods, however, avoid this situation and extend the usable lifetime of a separation matrix. In the context of a large scale manufacturing process this can translate into a measurable savings of time and money. Moreover, the cleaning step can be performed, as disclosed in the Examples, in-place and with no need to extract the separation matrix from a column or other matrix retaining device for cleaning, thus saving time and resources.

In one embodiment of a cleaning operation of a separation matrix, following a separation employing the disclosed method the separation matrix is washed with a regeneration reagent, such as sodium hydroxide, or an acidic reagent, such as phosphoric acid.

In one particular embodiment of a cleaning operation, Protein A is the separation matrix and a column containing Protein A resin is washed with 5 column volumes of 150 mM phosphoric acid and held for >15 minutes over the column. Following the wash with the acid, the column can be flushed with water, regenerated with 5 column volumes of 50 mM Tris, 10 mM citrate, 6M urea, 50 mM DTT; pH 7.4, subsequently washed with water, and then flushed with 3 column volumes of 150 mM phosphoric acid. This cleaning protocol has been utilized to achieve over 200 cycles of protein A resin. FIG. **3** highlights the results achievable using the disclosed cleaning methods.

EXAMPLES

The following examples demonstrate embodiments and aspects of the present invention and are not intended to be limiting.

Example 1

Direct Capture of Proteins Expressed in a Soluble Form Using Protein A Affinity Chromatography

The following experiment demonstrates that a protein comprising a plurality of polypeptides joined to an Fc moiety can be separated from an *E. coli* cell lysate slurry using a Protein A affinity media.

A protein comprising a plurality of polypeptides joined to an Fc moiety was expressed in an *E. coli* fermentation induced at 30° C. and driven to express soluble-form protein product. The fermentation broth was centrifuged, the liquid fraction removed, and the cell paste was collected. The cells were resuspended in a 10 mM potassium phosphate, 5 mM EDTA; pH 6.8 buffer solution, to approximately 100% of the original volume. The cells were then lysed by means of three passes through a high pressure homogenizer. After the cells were lysed, the cell lysate was filtered through a 0.1 μm filter to reduce particulate levels. The material was then stored in a closed bottle for ~24 hours at approximately 5° C.

In a separate operation, a packed column comprising GE Healthcare Mab Select™ Protein A affinity resin was prepared and equilibrated with 5 column volumes (CV) of 10 mM Tris; pH 8.0.

An aliquot of a protein comprising an Fc moiety was sampled directly from a lysate. The protein mixture was loaded to approximately 0.02 millimoles total protein/L resin at a 6-10 minute residence time. See FIG. **1**, which correlates protein bound and protein loaded as a function of residence time.

After loading, the column was washed with 10 mM Tris; pH 8.0, for 5 CV at up to 220 cm/hr. The protein of interest was recovered from the resin by elution with 50 mM sodium acetate, pH 3.1 at up to 220 cm/hr. The elution pool yielded greater than 90% recovery of the soluble material in the initial cell broth. The collected protein in the elution pool was stored at 2-8° C. until the next purification step was carried out.

Following the separation, the resin media was cleaned in-place by flowing 5 CV of 6 M Guanidine, pH 8.0 at 220 cm/hr.

The results of this separation demonstrated that a soluble protein expressed in a non-mammalian system can be captured and purified, with high yield, directly from cell lysate broth without having to refold the protein prior to application to a separation matrix.

Example 2

Capture of a Fc-Containing Protein Expressed in a Limited Solubility Form from a Refold Mixture Using Protein A Affinity Chromatography

The following experiments demonstrate that an Fc-containing protein can be separated from a refold mixture comprising glycerol, guanidine, urea, and arginine using Protein A affinity media.

In one experiment, a recombinant protein comprising a biologically active peptide linked to the C-terminus of the Fc moiety of an IgG1 molecule via a linker and having a molecular weight of about 57 kDa and comprising 8 disulfide bonds, in a non-mammalian expression system, namely *E. coli*, harvested, refolded under appropriate conditions, and captured using Protein A affinity media.

The growth media in which the cells were growing was centrifuged and the liquid fraction removed, leaving the cells as a paste. The cells were resuspended in water to approximately 60% of the original volume. The cells were lysed by means of three passes through a high pressure homogenizer.

After the cells were lysed, the lysate was centrifuged in a disc-stack centrifuge to collect the protein in the solid fraction, which was expressed in a limited solubility non-native form, namely as inclusion bodies.

The protein slurry was washed multiple times by resuspending the slurry in water to between 50 and 80% of the original fermentation broth volume, mixing, and centrifugation to collect the protein in the solid fraction.

The concentrated protein was then combined in a solubilization solution containing the protein, guanidine, urea, and DTT.

After incubation for one hour, the protein solution was diluted in to a refold buffer containing appropriate levels of arginine, urea, glycerol, cysteine, and cystamine.

In a separate operation, a packed column comprising ProSep VA Ultra™ Protein A affinity resin with dimensions of 1.1 cm internal diameter and ~25 cm height, was prepared and equilibrated with 5 column volumes (CV) of 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution.

An aliquot of a protein comprising an Fc moiety from the refold solution was filtered through a series of depth and/or membrane filter to remove particulates. The conditioned and filtered protein mixture was loaded to approximately 0.35 millimoles total protein/L resin at a 6-10 minute residence time. See FIG. **1**, which correlates protein bound and protein loaded as a function of residence time.

After loading, the column was washed with 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution, for 4.5 CV at up to 400 cm/hr. The Fc-containing protein was recovered from the resin by elution with 100 mM sodium acetate, pH 3.7 at up to 300 cm/hr. The average level of purity achieved is shown in FIG. **3**.

Following the separation, the resin media was cleaned in-place by flowing 5 CV of 150 mM phosphoric acid. The column was regenerated with 5CV of 50 mM Tris, 10 mM citrate, 6M urea and 50 mM DTT; pH 7.4, washed with water, and then flushed with 3CV of 150 mM phosphoric acid.

The results of this separation demonstrate that an insoluble protein expressed in a non-mammalian system can be purified directly from a refold buffer without having to dilute the refold buffer prior to application to a separation matrix for more than 150 cycles, as indicated by the table presented in FIG. **3**.

In another separation, the Protein A column was cycled with the above procedure 8-10 times and then the final cycle was run as follows: The media was equilibrated with 5 column volumes (CV) of 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution. An aliquot of protein sampled directly from a refold buffer was filtered through a series of depth and/or membrane filter to remove particulates. The conditioned and filtered protein mixture was then loaded on the column to 0.35 millimoles total protein/L resin at a 6-10 minute residence time. See FIG. **1**, which correlates protein bound and protein loaded as a function of residence time.

After loading, the column was washed with 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution, for 4.5 CV at up to 400 cm/hr. The protein of interest was recovered from the resin by eluting with 100 mM sodium acetate, pH 3.7 at up to 300 cm/hr. The resin media was cleaned in-place by flowing 5 CV of 150 mM phosphoric acid over it. Finally, the column was flushed with water, regenerated with 5CV of 50 mM Tris, 10 mM citrate, 6M urea, and 50 mM DTT; pH 7.4, washed with water, and then flushed with 3CV of 150 mM phosphoric acid. Subsequent analysis of the resin showed no protein carry-over between cycles, demonstrating the ability to reuse the resin after both cleaning methods.

Example 3

Separation of an Fc-Containing Protein from a Refold Mixture Using Cation Exchange Chromatography

The following experiments demonstrate that an Fc-containing protein can be separated from a refold mixture comprising glycerol, guanidine, urea, and arginine using cation exchange media.

In one experiment, a recombinant protein comprising a biologically active peptide linked to the C-terminus of the Fc moiety of an IgG1 molecule via a linker and having a molecular weight of about 57 kDa and comprising 8 disulfide bonds, was expressed in a non-mammalian expression system, namely *E. coli*, harvested, refolded under appropriate conditions, and captured using cation exchange media.

The growth media in which the cells were growing was centrifuged and the liquid fraction removed, leaving the cells as a paste. The cells were resuspended in water. The cells were lysed by means of multiple passes through a high pressure homogenizer. After the cells were lysed, the lysate was centrifuged to collect the protein, which was expressed in a limited solubility non-native form, namely as inclusion bodies. The protein slurry was washed multiple times by resuspending the slurry in water, mixing, and centrifugation to collect the protein. The concentrated protein was then transferred to a solubilization buffer containing guanidine and DTT. After incubation for one hour, the protein solution was diluted in to a refold buffer containing appropriate levels of arginine, urea, glycerol, cysteine, and cystamine.

In a separate operation, a packed column comprising EMD Fractogel SO_3^- cation exchange resin with dimensions of 1.1 cm internal diameter and 20 cm height, was prepared and equilibrated with 5 column volumes of 30 mM MES; pH 4.5 buffered solution.

An aliquot of a protein comprising an Fc moiety was sampled directly from a refold solution, was diluted 3-fold with water, titrated with 50% hydrochloric acid to ~pH 4.5 and was filtered through a series of depth and/or membrane filter to remove particulates. The conditioned and filtered protein mixture was loaded to approximately 0.96 millimoles total protein/L resin at 60 cm/hr.

After loading, the column was washed with 30 mM MES; pH 4.5, for 3 CV at 60 cm/hr, then washed with an additional 3 CV of 30 mM MES; pH 6.0. The protein of interest was recovered from the resin by gradient elution over 25 CV between 30 mM MES; pH 6.0 and 30 mM MES, 500 mM

NaCl; pH 6.0 at 60 cm/hr. The collected protein in the elution pool was stored at 2-8° C. until the next purification step was carried out.

Purity levels achieved, as determined by SEC and RP-HPLC are shown in FIG. **5**.

Following the separation, the resin media was cleaned in-place by flowing 3 CV of 1 M sodium hydroxide, at 120 cm/hr and held for 60 minutes prior an additional 3CV wash with 1 m sodium hydroxide.

The results of this separation demonstrate that an insoluble protein expressed in a non-mammalian system can be captured and purified from a refold buffer with a variety of separation matrices, including an ion-exchange separation matrix.

Example 4

Re-Usability of Protein A Affinity Resin Used to Isolate a Fc-Containing Protein Directly from a Refold Buffer by Affinity Chromatography

In another aspect of the method, a range of column cleaning methods can be employed in conjunction with the methods described herein, allowing the chromatography resins to be reused to an extent that make the method economically feasible. As described in Examples 2 and 3 for the case of Protein A affinity resins, cleaning protocols have been developed and demonstrated to remove product and non-product contaminants from the resin to allow reuse. The cleaning agents include caustic (e.g. sodium or potassium hydroxide), detergents (e.g. SDS or Triton X-100), denaturants (e.g. urea or guanidine-derivatives), and reductants (e.g. DTT, or thioglycolates). These agents can be used in combination or alone.

In order to demonstrate the reusability of column resins following application of the direct capture methods described, an aliquot of pH adjusted and filtered Fc-containing protein was loaded on new, unused resin and resin that had been previously cycled 94 times to evaluate the cleaning of the Protein A resin and the effect on purification binding and separation of an Fc-containing protein with regard to resin history.

The media was equilibrated with 5 column volumes (CV) of 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution. An aliquot of protein sampled directly from a refold buffer was filtered through a series of depth and/or membrane filter to remove particulates. The conditioned and filtered protein mixture was then loaded on the column to approximately 0.35 millimoles total protein/mL resin at a 6-10 minute residence time. See FIG. **1**, which correlates protein bound and protein loaded as a function of residence time.

After loading, the column was washed with 25 mM Tris, 100 mM sodium chloride; pH 7.4, or similar buffered solution, for 4.5 CV at up to 400 cm/hr. The protein of interest was recovered from the resin by eluting with 100 mM sodium acetate, pH 3.7 at up to 300 cm/hr. Each column was regenerated using 5CV phosphoric acid and 5 CV of an acidic buffered solution containing 50 mM Tris, 10 mM citrate, 6M urea, and 50 mM DTT; pH 7.4.

This procedure was repeated for greater than 100 cycles. Selected samples from this reuse study were submitted for SEC-HPLC analysis. The goal was to track the % MP purity, % HMW and % dimer species from the pools as well as to understand the change of purity level from the load. No major differences were observed between the used columns and new columns.

This Example demonstrates that not only can a complex protein be captured from a complex chemical solution, but that the resin can be cycled repeatedly and cleaned and reused reproducibly over a number of industrially-relevant cycles.

What is claimed is:

1. A method of purifying a protein expressed in a non-native soluble form in a non-mammalian expression system comprising:

(a) lysing a non-mammalian cell in which the protein is expressed in a nonnative soluble form to generate a cell lysate;

(b) contacting the cell lysate with a separation matrix under conditions suitable for the protein to associate with the separation matrix;

(c) washing the separation matrix; and

(d) eluting the protein from the separation matrix.

2. The method of claim **1**, wherein the protein is a complex protein.

3. The method of claim **2**, wherein the complex protein is selected from the group consisting of a multimeric protein, an antibody and an Fc fusion protein.

4. The method of claim **1**, wherein the non-mammalian expression system comprises bacteria or yeast cells.

5. The method of claim **1**, wherein the separation matrix is an affinity resin.

6. The method of claim **1**, wherein the separation matrix is a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin.

7. The method of claim **1**, wherein the cell lysate is filtered before it is contacted with the separation matrix.

8. The method of claim **1**, further comprising refolding the protein to its native form after it is eluted.

9. A method of purifying a protein expressed in a non-native limited solubility form in a non-mammalian expression system comprising:

(a) solubilizing the expressed protein in a solubilization solution comprising one or more of the following:

(i) a denaturant;

(ii) a reductant; and

(iii) a surfactant;

(b) forming a refold solution comprising the solubilization solution and a refold buffer, the refold buffer comprising one or more of the following:

(i) a denaturant;

(ii) an aggregation suppressor;

(iii) a protein stabilizer; and

(iv) a redox component;

(c) applying the refold solution to a separation matrix under conditions suitable for the protein to associate with the matrix;

(d) washing the separation matrix; and

(e) eluting the protein from the separation matrix.

10. The method of claim **9**, wherein the non-native limited solubility form is a component of an inclusion body.

11. The method of claim **9**, wherein the protein is a complex protein.

12. The method of claim **10**, wherein the complex protein is selected from the group consisting of a multimeric protein, an antibody, a peptibody, and an Fc fusion protein.

13. The method of any one of claims **9-12**, wherein the non-mammalian expression system comprises bacteria or yeast cells.

14. The method of any one of claims **9-12**, wherein the denaturant of the solubilization solution or the refold buffer

comprises one or more of urea, guanidinium salts, dimethyl urea, methylurea and ethylurea.

15. The method of claim **9**, wherein the reductant comprises one or more of cysteine, dithiothreitol (DTT), beta-mercaptoethanol and glutathione.

16. The method of claim **9**, wherein the surfactant comprises one or more of sarcosyl and sodium dodecylsulfate.

17. The method of claim **9**, wherein the aggregation suppressor is selected from the group consisting of arginine, proline, polyethylene glycols, nonionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes.

18. The method of claim **9**, wherein the protein stabilizer comprises one or more of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, tris, sodium sulfate, potassium sulfate and osmolytes.

19. The method of claim **9**, wherein the redox component comprises one or more of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol.

20. The method of claim **9**, wherein the separation matrix is:

(i) an affinity resin, selected from the group consisting of Protein A, Protein G, and synthetic mimetic affinity resin; or

(ii) a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin.

21. The method of any one of claim **1** or **9-12**, wherein the protein is isolated after elution from the separation matrix.

22. The method of claim **8**, wherein the protein is isolated after refolding.

23. The method of claim **14**, wherein the reductant comprises one or more of cysteine, dithiothreitol (DTT), beta-mercaptoethanol and glutathione.

24. The method of claim **15**, wherein the surfactant comprises one or more of sarcosyl and sodium dodecylsulfate.

25. The method of claim **16**, wherein the aggregation suppressor is selected from the group consisting of arginine, proline, polyethylene glycols, nonionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes.

26. The method of claim **17**, wherein the protein stabilizer comprises one or more of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, tris, sodium sulfate, potassium sulfate and osmolytes.

27. The method of claim **18**, wherein the redox component comprises one or more of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol.

28. The method of claim **19**, wherein the separation matrix is:

(i) an affinity resin, selected from the group consisting of Protein A, Protein G, and synthetic mimetic affinity resin; or

(ii) a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin.

29. The method of claim **13**, wherein the protein is isolated after elution from the separation matrix.

30. The method of claim **20**, wherein the protein is isolated after elution from the separation matrix.

* * * * *

Exhibit 3

US008952138B2

(12) **United States Patent**
Shultz et al.

(10) **Patent No.: US 8,952,138 B2**
(45) **Date of Patent: *Feb. 10, 2015**

(54) **REFOLDING PROTEINS USING A CHEMICALLY CONTROLLED REDOX STATE**

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(73) Assignee: **Amgen Inc.**, Thousand Oaks, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 403 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **12/820,087**

(22) Filed: **Jun. 21, 2010**

(65) **Prior Publication Data**

US 2010/0324269 A1 Dec. 23, 2010

Related U.S. Application Data

(60) Provisional application No. 61/219,257, filed on Jun. 22, 2009.

(51) **Int. Cl.**
C07K 1/22 (2006.01)
C07K 1/113 (2006.01)

(52) **U.S. Cl.**
CPC ***C07K 1/1133*** (2013.01)
USPC **530/413**

(58) **Field of Classification Search**
None
See application file for complete search history.

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Primary Examiner — Yunsoo Kim
(74) *Attorney, Agent, or Firm* — David B. Ran

(57) **ABSTRACT**

A method of refolding proteins expressed in non-mammalian cells present in concentrations of 2.0 g/L or higher is disclosed. The method comprises identifying the thiol pair ratio and the redox buffer strength to achieve conditions under which efficient folding at concentrations of 2.0 g/L or higher is achieved and can be employed over a range of volumes, including commercial scale.

24 Claims, 8 Drawing Sheets

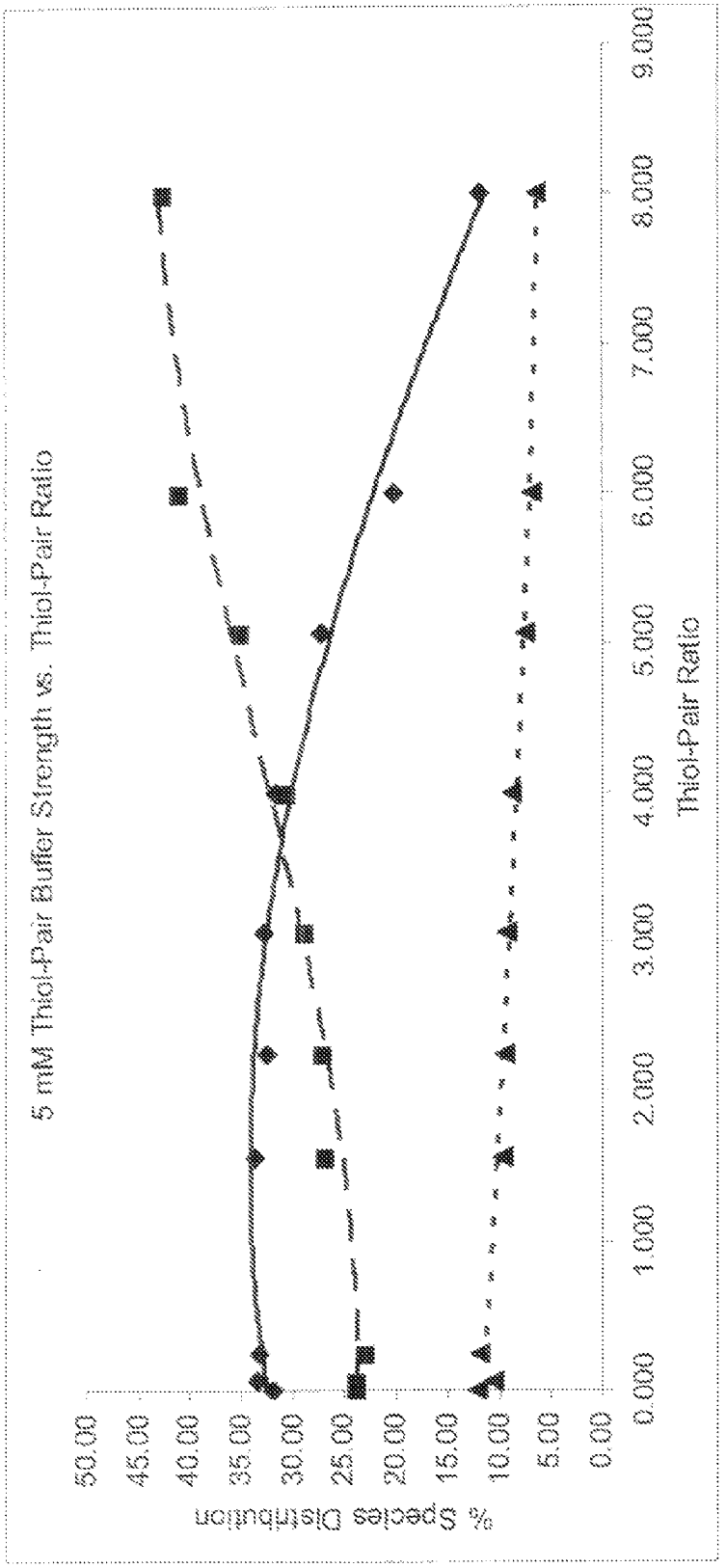

5 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
% Species Distribution
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0.000
1.000
2.000
3.000
4.000
5.000
6.000
7.000
8.000
9.000
Thiol-Pair Ratio

Figure 1a

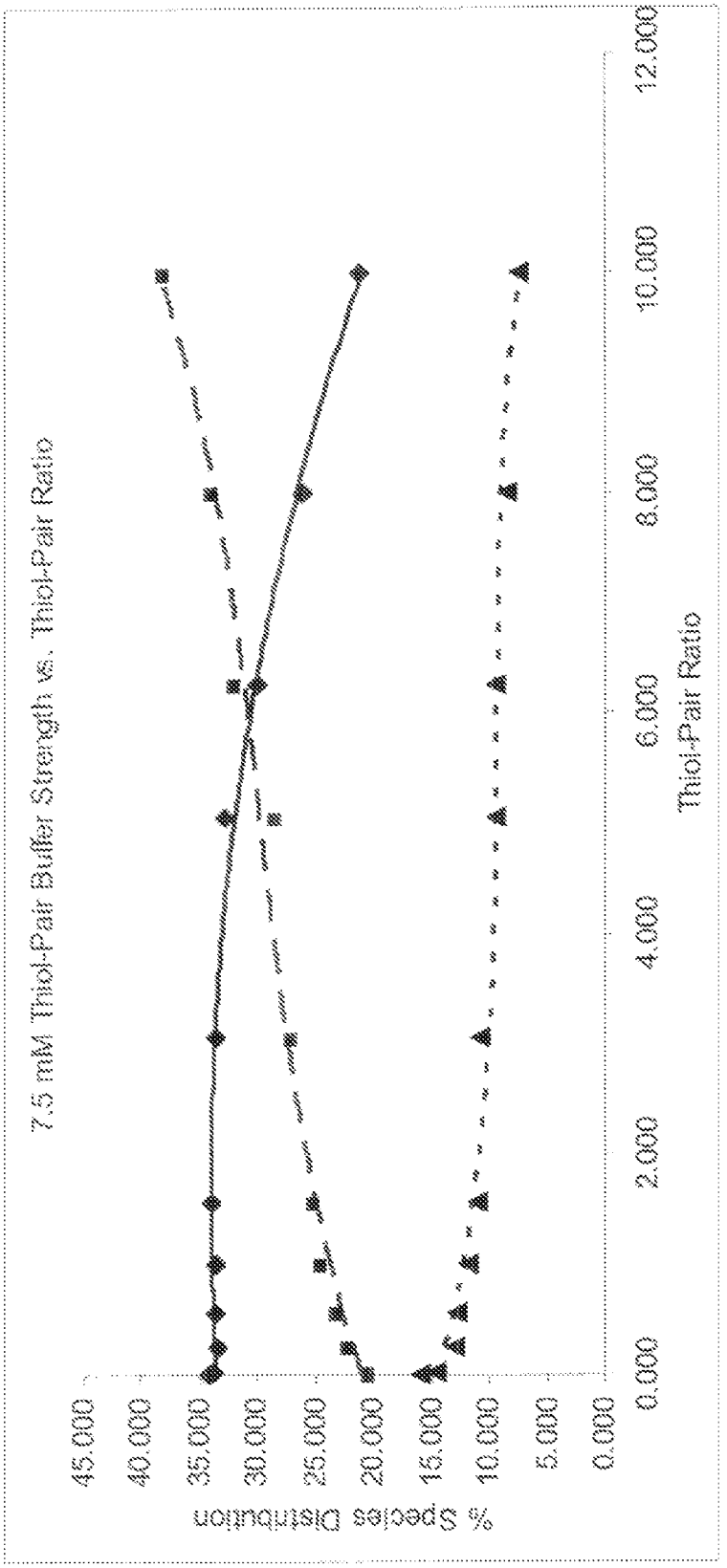

7.5 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
% Species Distribution
45.000
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
0.000
2.000
4.000
6.000
8.000
10.000
12.000
Thiol-Pair Ratio

Figure 1b

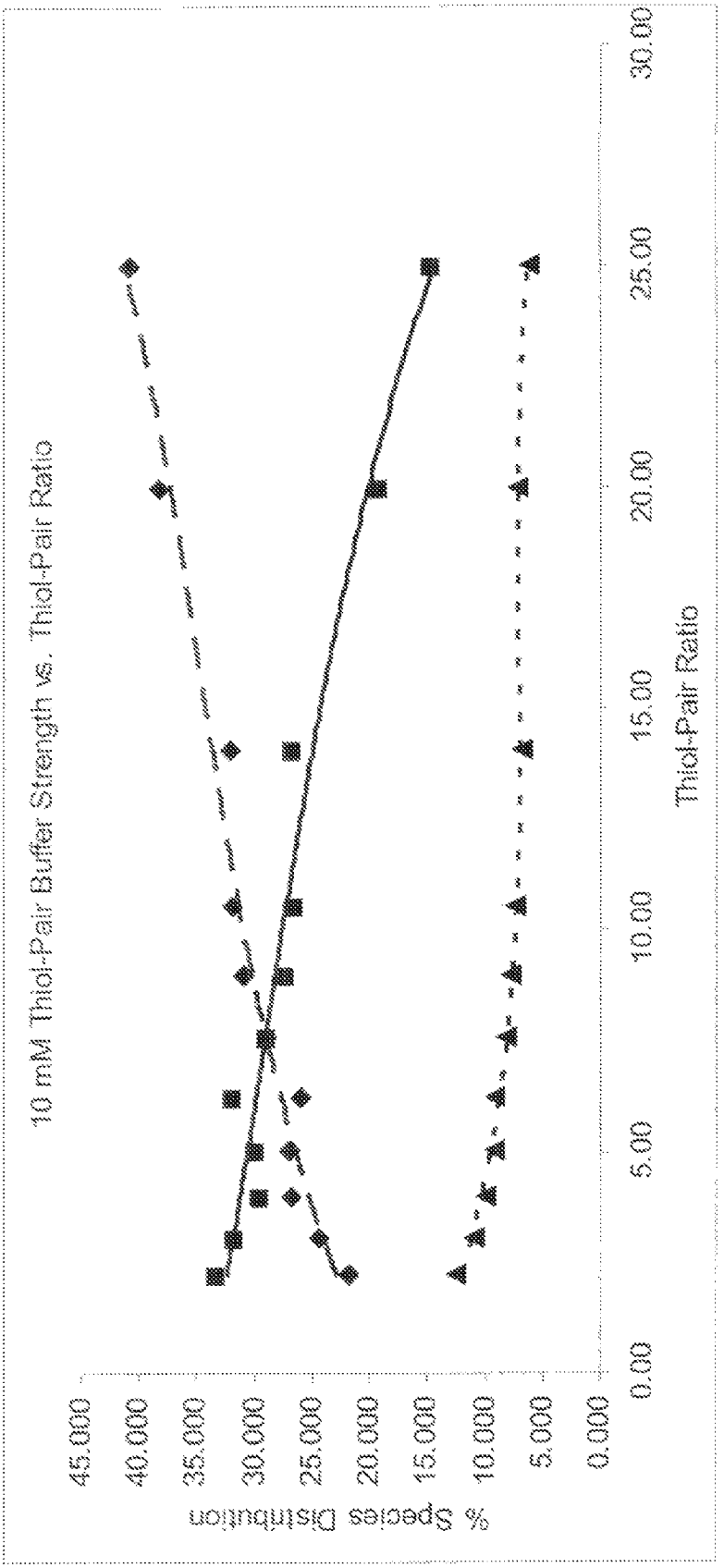
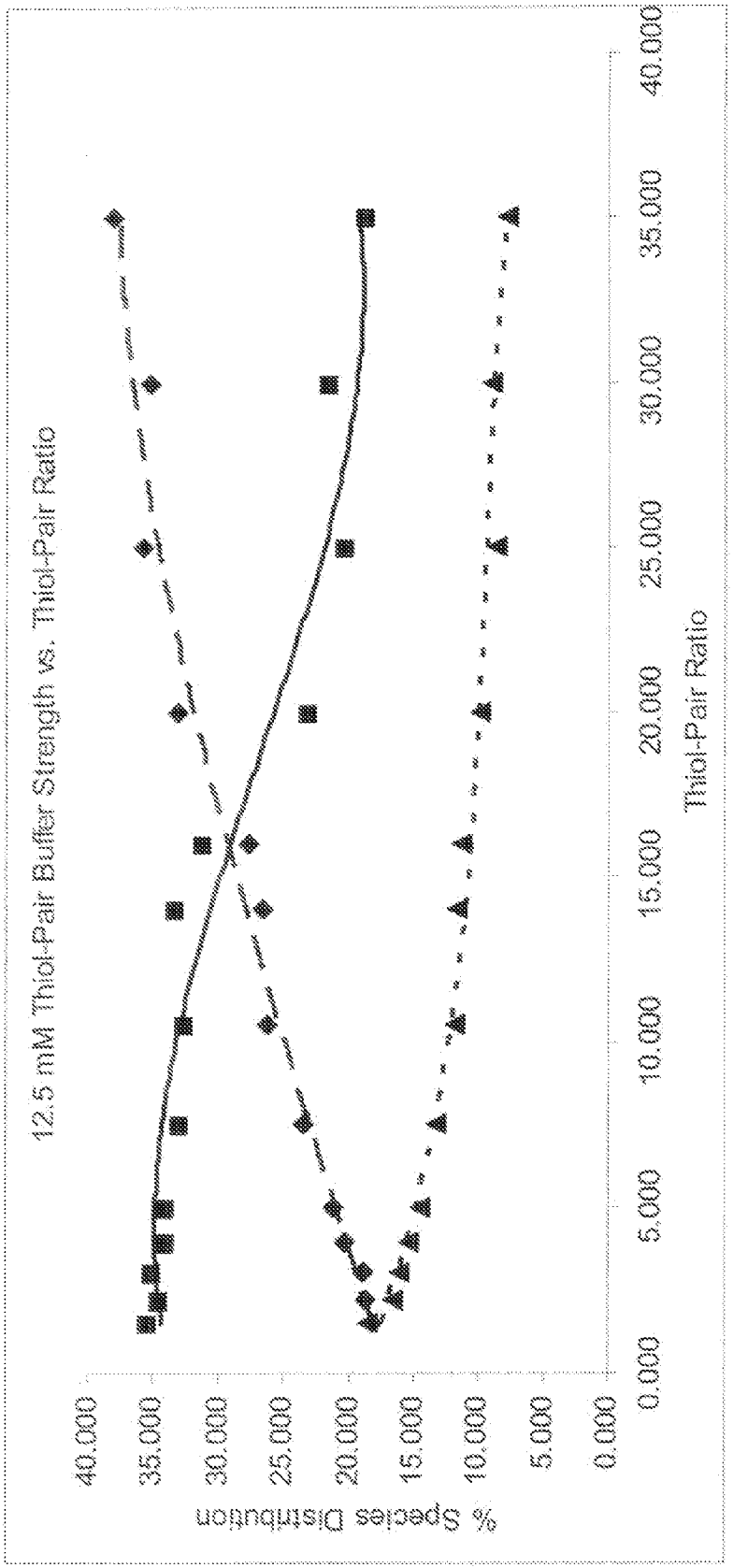

10 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
% Species Distribution
45.000
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
0.00
5.00
10.00
15.00
20.00
25.00
30.00
Thiol-Pair Ratio

Figure 1C


12.5 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
% Species Distribution
Thiol-Pair Ratio
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5.000
10.000
15.000
20.000
25.000
30.000
35.000
40.000

Figure 1d

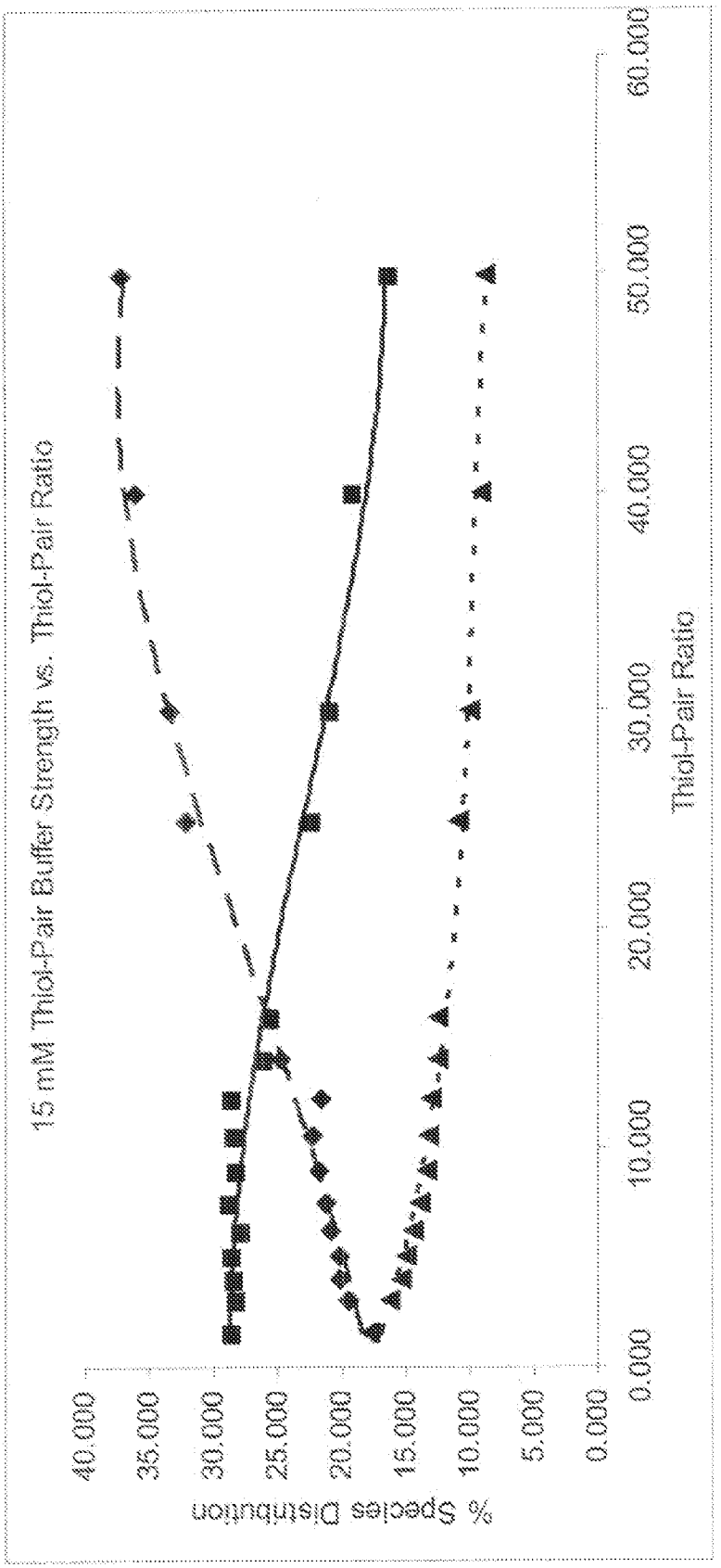
15 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
% Species Distribution
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
0.000
10.000
20.000
30.000
40.000
50.000
60.000
Thiol-Pair Ratio

Figure 1e

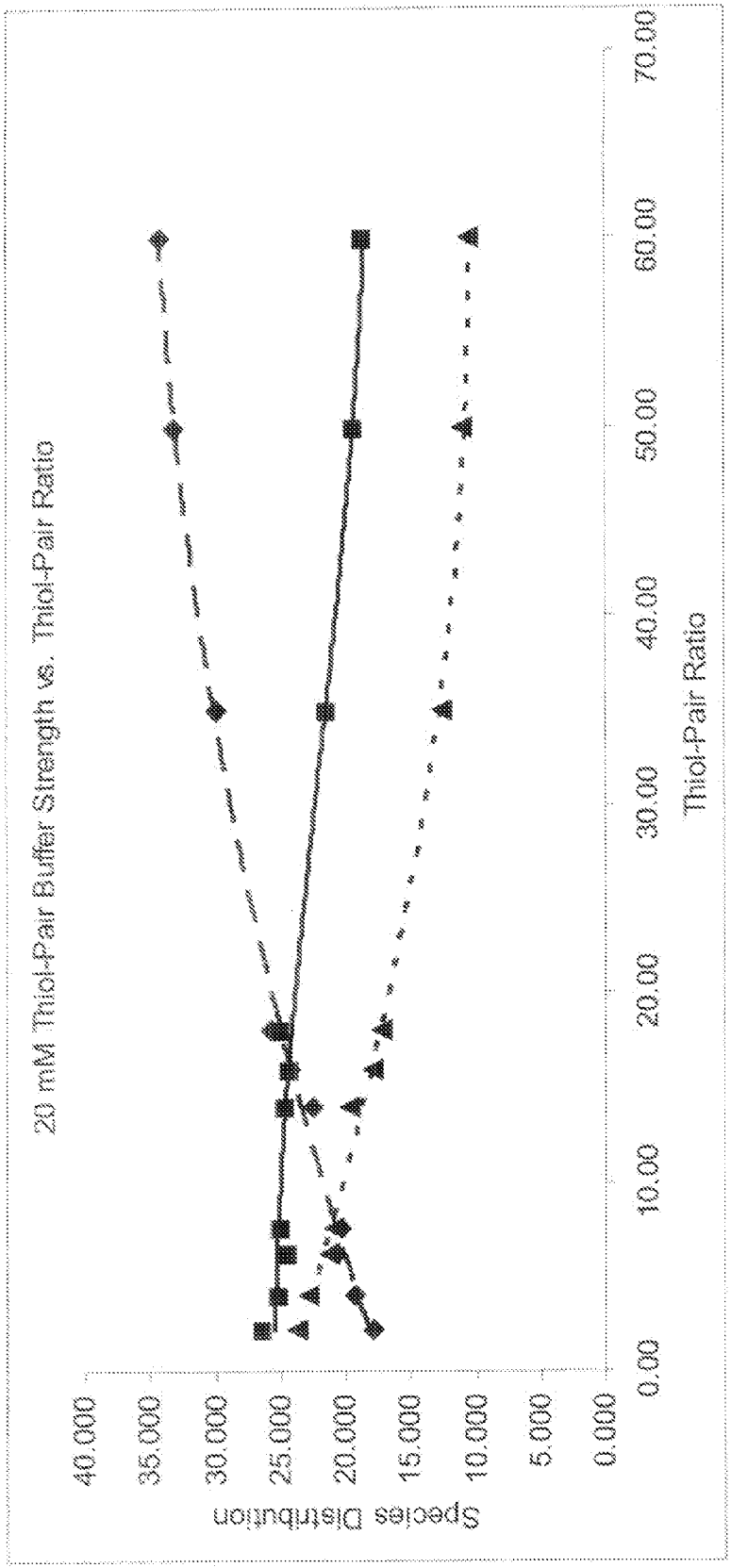
20 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
Species Distribution
Thiol-Pair Ratio
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00

Figure 1f

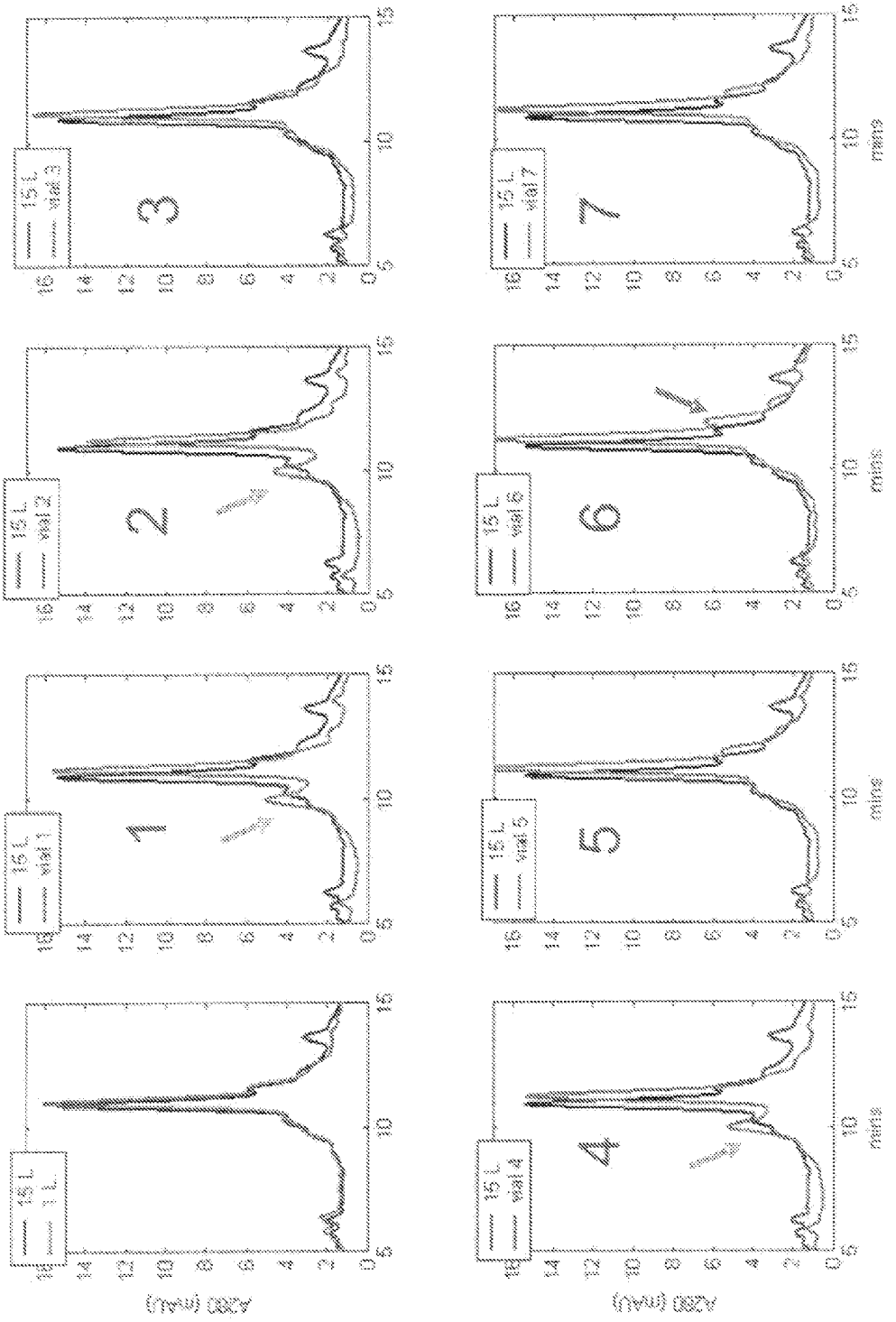

Figure 2

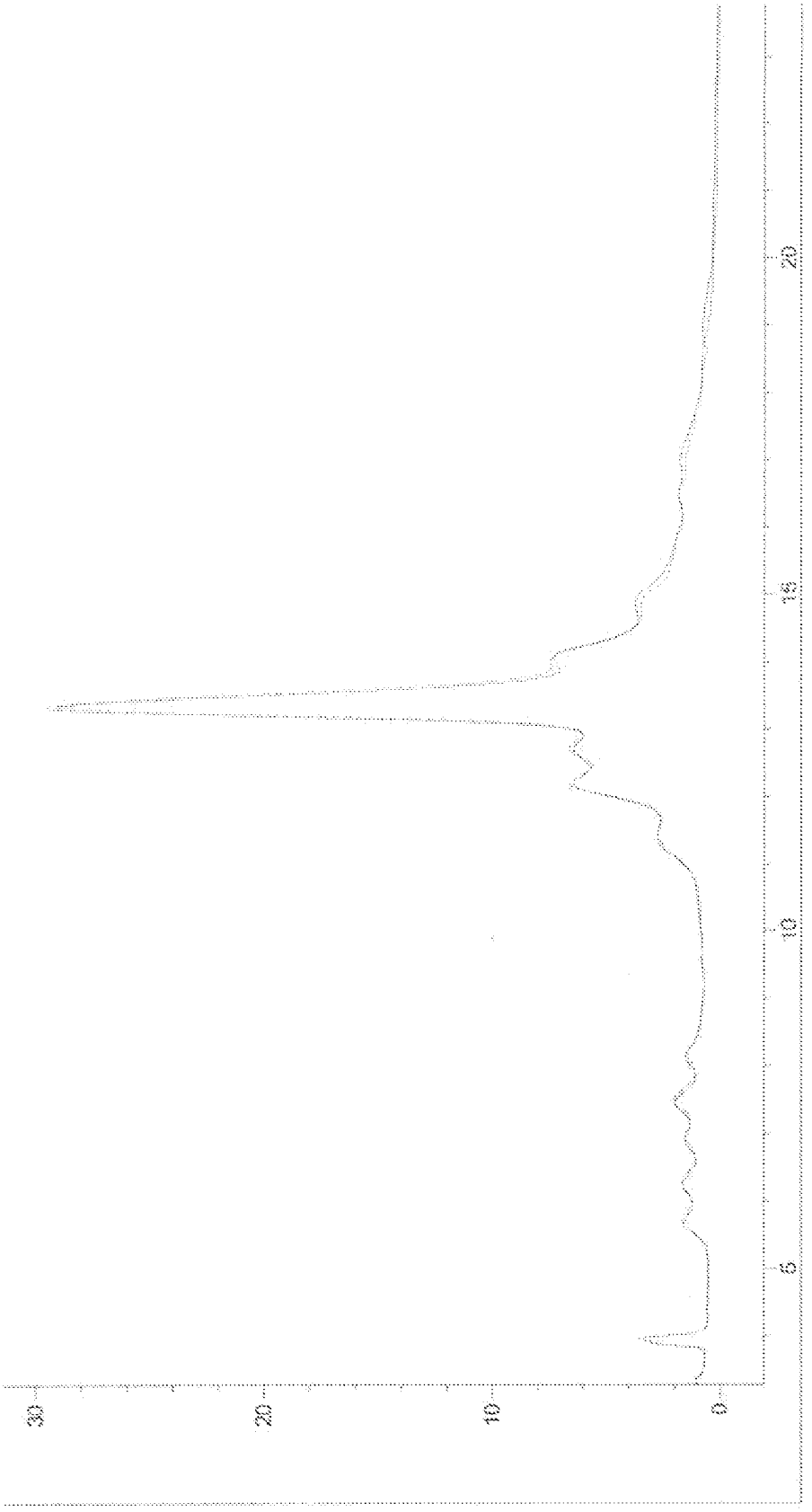

Figure 3

REFOLDING PROTEINS USING A CHEMICALLY CONTROLLED REDOX STATE

This application claims the benefit of U.S. Provisional Application No. 61/219,257 filed Jun. 22, 2009, which is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention generally relates to refolding proteins at high concentrations, and more particularly to refolding proteins in volumes at concentrations of 2.0 g/L and above.

BACKGROUND OF THE INVENTION

Recombinant proteins can be expressed in a variety of expression systems, including non-mammalian cells, such as bacteria and yeast. A difficulty associated with the expression of recombinant proteins in prokaryotic cells, such as bacteria, is the precipitation of the expressed proteins in limited-solubility intracellular precipitates typically referred to as inclusion bodies. Inclusion bodies are formed as a result of the inability of a bacterial host cell to fold recombinant proteins properly at high levels of expression and as a consequence the proteins become insoluble. This is particularly true of prokaryotic expression of large, complex or protein sequences of eukaryotic origin. Formation of incorrectly folded recombinant proteins has, to an extent, limited the commercial utility of bacterial fermentation to produce recombinant large, complex proteins, at high levels of efficiency.

Since the advent of the recombinant expression of proteins at commercially viable levels in non-mammalian expression systems such as bacteria, various methods have been developed for obtaining correctly folded proteins from bacterial inclusion bodies. These methods generally follow the procedure of expressing the protein, which typically precipitates in inclusion bodies, lysing the cells, collecting the inclusion bodies and then solubilizing the inclusion bodies in a solubilization buffer comprising a denaturant or surfactant and optionally a reductant, which unfolds the proteins and disassembles the inclusion bodies into individual protein chains with little to no structure. Subsequently, the protein chains are diluted into or washed with a refolding buffer that supports renaturation to a biologically active form. When cysteine residues are present in the primary amino acid sequence of the protein, it is often necessary to accomplish the refolding in an environment which allows correct formation of disulfide bonds (e.g., a redox system).

Typical refold concentrations for complex molecules, such as molecules comprising two or more disulfides, are less than 2.0 g/L and more typically 0.01-0.5 g/L (Rudolph & Lilie, (1996) *FASEB J.* 10:49-56). Thus, refolding large masses of a complex protein, such as an antibody, peptibody or other Fc fusion protein, at industrial production scales poses significant limitations due to the large volumes required to refold proteins, at these typical product concentration, and is a common problem facing the industry. One factor that limits the refold concentration of these types of proteins is the formation of incorrectly paired disulfide bonds, which may in turn increase the propensity for those forms of the protein to aggregate. Due to the large volumes of material and large pool sizes involved when working with industrial scale protein production, significant time, and resources can be saved by eliminating or simplifying one or more steps in the process.

While protein refolding has previously been demonstrated at higher concentrations, the proteins that were refolded were either significantly smaller in molecular weight, less complex molecules containing only one or two disulfide bonds (see, e.g., Creighton, (1974) *J. Mol. Biol.* 87:563-577). Additionally, the refolding processes for such proteins employed detergent-based refolding chemistries (see, e.g., Stockel et al., (1997) *Eur J Biochem* 248:684-691) or utilized high pressure folding strategies (St John et al., (2001) *J. Biol. Chem.* 276(50):46856-63). More complex molecules, such as antibodies, peptibodies and other large proteins, are generally not amenable to detergent refold conditions and are typically refolded in chaotropic refold solutions. These more complex molecules often have greater than two disulfide bonds, often between 8 and 24 disulfide bonds, and can be multi-chain proteins that form homo- or hetero-dimers.

Until the present disclosure, these types of complex molecules could not be refolded at high concentrations, i.e., concentrations of 2.0 g/L and higher, with any meaningful degree of efficiency on a small scale, and notably not on an industrial scale. The disclosed methods, in contrast, can be performed at high concentrations on a small or large (e.g, industrial) scale to provide properly refolded complex proteins. The ability to refold proteins at high concentrations and at large scales can translate into not only enhanced efficiency of the refold operation itself, but also represents time and cost savings by eliminating the need for additional equipment and personnel. Accordingly, a method of refolding proteins present in high concentrations could translate into higher efficiencies and cost savings to a protein production process.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a series of plots depicting depicting the effect of thiol-pair ratio and redox buffer strength on product-species distribution; FIG. **1***a* depicts the effect of a 5 mM buffer strength; FIG. **1***b* depicts the effect of a 7.5 mM buffer strength; FIG. **1***c* depicts the effect of a 10 mM buffer strength; FIG. **1***d* depicts the effect of a 12.5 mM buffer strength; FIG. **1***e* depicts the effect of a 15 mM buffer strength and FIG. **1***f* depicts the effect of a 20 mM buffer strength.

FIG. **2** is a series of plots depicting the effect of the degree of aeration on the species distribution under fixed thiol-pair ratio and thiol-pair buffer strength.

FIG. **3** is an analytical overlay of a chemically controlled, non-aerobic refold performed at 6 g/L and optimized using an embodiment of the described method performed at 1 L and 2000 L.

SUMMARY OF THE INVENTION

A method of refolding a protein expressed in a non-mammalian expression system and present in a volume at a concentration of 2.0 g/L or greater comprising: (a) contacting the protein with a refold buffer comprising a redox component comprising a final thiol-pair ratio having a range of 0.001 to 100 and a redox buffer strength of 2 mM or greater and one or more of: (i) a denaturant; (ii) an aggregation suppressor; and (iii) a protein stabilizer; to form a refold mixture; (b) incubating the refold mixture; and (c) isolating the protein from the refold mixture.

In various embodiments the redox component has a final thiol-pair ratio greater than or equal to 0.001 but less than or equal to 100, for example within a range of 0.05 to 50, 0.1 to 50, 0.25 to 50, 0.5 to 50, 0.75 to 40, 1.0 to 50 or 1.5 to 50, 2 to 50, 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50 or 40 to 50 and a Thiol-pair buffer strength equal to or greater than 2 mM, for

example greater than or equal to 2.25 mM, 2.5 mM, 2.75 mM, 3 mM, 5 mM, 7.5 mM, 10 mM, or 15 mM, wherein the thiol-pair buffer strength is effectively bounded at a maximum of 100 mM. Restated, in terms of ranges, the thiol buffer strength can be between 2 and 20 mM, for example between 2.25 mM and 20 mM, 2.5 mM and 20 mM, 2.75 mM and 20 mM, 3 mM and 20 mM, 5 mM and 20 mM, 7.5 mM and 20 mM, 10 mM and 20 mM, or 15 mM and 20 mM, to form a mixture.

In one embodiment of a refold buffer, the refold buffer comprises urea, arginine-HCl, cysteine and cystamine in Tris buffer. In a further embodiment the components are present in the refold buffer in proportions described in Example 3.

In another embodiment of a refold buffer, the refold buffer comprises urea, arginine HCl, glycerol, cysteine, and cystamine in Tris buffer. In a further embodiment the components are present in the refold buffer in proportions described in Example 4.

In some embodiments, the protein is initially present in a volume in a non-native limited solubility form, such as an inclusion body. Alternatively, the protein is present in the volume in a soluble form. The protein can be a recombinant protein or it can be an endogenous protein. The protein can be a complex protein such as an antibody or a multimeric protein. In another embodiment, the protein is an Fc-protein conjugate, such as a protein fused or linked to a Fc domain.

The non-mammalian expression system can be a bacterial expression system or a yeast expression system.

The denaturant in the refold buffer can be selected from the group consisting of urea, guanidinium salts, dimethyl urea, methylurea and ethylurea. The protein stabilizer in the refold buffer can be selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes. The aggregation suppressor can be selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes. The thiol-pairs can comprise at least one component selected from the group consisting of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol.

In various embodiments, the purification can comprise contacting the mixture with an affinity separation matrix, such as a Protein A or Protein G resin. Alternatively, the affinity resin can be a mixed mode separation matrix or an ion exchange separation matrix. In various aspects, the incubation can be performed under aerobic conditions or under non-aerobic conditions.

DETAILED DESCRIPTION OF THE INVENTION

The relevant literature suggests that when optimizing various protein refolding operations, the refold buffer thiol-pair ratio has been purposefully varied and as a result the thiol buffer strength was unknowingly varied across a wide range of strengths (see, e.g., Lille, Schwarz & Rudolph, (1998) *Current Opinion in Biotechnology* 9(5):497-501, and Tran-Moseman, Schauer & Clark (1999) *Protein Expression & Purification* 16(1):181-189). In one study, a relationship between the thiol pair ratio and the buffer strength was investigated for lysozyme, a simple, single-chain protein that forms a molten globule. (De Bernardez et al., (1998) *Biotechnol. Prog.* 14:47-54). The De Bernardez work described thiol concentration in terms of a model that considered only the kinetics of a one-way reaction model. However, most complex proteins are governed by reversible thermodynamic equilibria that are not as easily described (see, e.g., Darby et al., (1995) *J. Mol. Biol.* 249:463-477). More complex behavior is expected in the case of large multi-chain proteins containing many disulfide bonds, such as antibodies, peptibodies and other Fc fusion proteins. Until the present disclosure, specific relationships had not been provided for thiol buffer strength, thiol-pair ratio chemistry, and protein concentration with respect to complex proteins that related to the efficiency of protein production. Consequently, the ability to refold proteins in a highly concentrated volume has largely been an inefficient or unachievable goal, leading to bottlenecks in protein production, particularly on the industrial scale.

Prior to the present disclosure a specific controlled investigation of the independent effects of thiol-pair ratio and thiol-pair buffer strength had not been disclosed for complex proteins. As described herein, by controlling the thiol-pair buffer strength, in conjunction with thiol-pair ratio and protein concentration, the efficiency of protein folding operations can be optimized and enhanced and the refolding of proteins at high concentrations, for example 2 g/L or greater, can be achieved.

Thus, in one aspect, the present disclosure relates to the identification and control of redox thiol-pair ratio chemistries that facilitate protein refolding at high protein concentrations, such as concentrations higher than 2.0 g/L. The method can be applied to any type of protein, including simple proteins and complex proteins (e.g., proteins comprising 2-23 disulfide bonds or greater than 250 amino acid residues, or having a MW of greater than 20,000 daltons), including proteins comprising a Fc domain, such as antibodies, peptibodies and other Fc fusion proteins, and can be performed on a laboratory scale (typically milliliter or liter scale), a pilot plant scale (typically hundreds of liters) or an industrial scale (typically thousands of liters). Examples of complex molecules known as peptibodies, and other Fc fusions, are described in U.S. Pat. Nos. 6,660,843, 7,138,370 and 7,511,012.

As described herein, the relationship between thiol buffer strength and redox thiol-pair ratio has been investigated and optimized in order to provide a reproducible method of refolding proteins at concentrations of 2.0 g/L and higher on a variety of scales. A mathematical formula was deduced to allow the precise calculation of the ratios and strengths of individual redox couple components to achieve matrices of buffer thiol-pair ratio and buffer thiol strength. Once this relationship was established, it was possible to systematically demonstrate that thiol buffer strength and the thiol-pair ratio interact to define the distribution of resulting product-related species in a refolding reaction.

The buffer thiol-pair ratio is, however, only one component in determining the total system thiol-pair ratio in the total reaction. Since the cysteine residues in the unfolded protein are reactants as well, the buffer thiol strength needs to vary in proportion with increases in protein concentration to achieve the optimal system thiol-pair ratio. Thus, in addition to demonstrating that buffer thiol strength interacts with the thiol-pair ratio, it has also been shown that the buffer thiol strength relates to the protein concentration in the total reaction as well. Optimization of the buffer thiol strength and the system thiol pair ratio can be tailored to a particular protein, such as a complex protein, to minimize cysteine mispairing yet still facilitate a refold at a high concentration.

I. Definitions

As used herein, the terms “a” and “an” mean one or more unless specifically indicated otherwise.

As used herein, the term “non-mammalian expression system” means a system for expressing proteins in cells derived from an organism other than a mammal, including but not limited to, prokaryotes, including bacteria such as *E. coli*, and yeast. Often a non-mammalian expression system is

employed to express a recombinant protein of interest, while in other instances a protein of interest is an endogenous protein that is expressed by a non-mammalian cell. For purposes of the present disclosure, regardless of whether a protein of interest is endogenous or recombinant, if the protein is expressed in a non-mammalian cell then that cell is a "non-mammalian expression system." Similarly, a "non-mammalian cell" is a cell derived from an organism other than a mammal, examples of which include bacteria or yeast.

As used herein, the term "denaturant" means any compound having the ability to remove some or all of a protein's secondary and tertiary structure when placed in contact with the protein. The term denaturant refers to particular chemical compounds that affect denaturation, as well as solutions comprising a particular compound that affect denaturation. Examples of denaturants that can be employed in the disclosed method include, but are not limited to urea, guanidinium salts, dimethyl urea, methylurea, ethylurea and combinations thereof.

As used herein, the term "aggregation suppressor" means any compound having the ability to disrupt and decrease or eliminate interactions between two or more proteins. Examples of aggregation suppressors can include, but are not limited to, amino acids such as arginine, proline, and glycine; polyols and sugars such as glycerol, sorbitol, sucrose, and trehalose; surfactants such as, polysorbate-20, CHAPS, Triton X-100, and dodecyl maltoside; and combinations thereof.

As used herein, the term "protein stabilizer" means any compound having the ability to change a protein's reaction equilibrium state, such that the native state of the protein is improved or favored. Examples of protein stabilizers can include, but are not limited to, sugars and polyhedric alcohols such as glycerol or sorbitol; polymers such as polyethylene glycol (PEG) and α-cyclodextrin; amino acids salts such as arginine, proline, and glycine; osmolytes and certain Hoffmeister salts such as Tris, sodium sulfate and potassium sulfate; and combinations thereof.

As used herein, the terms "Fc" and "Fc region" are used interchangeably and mean a fragment of an antibody that comprises human or non-human (e.g., murine) C_{H2} and C_{H3} immunoglobulin domains, or which comprises two contiguous regions which are at least 90% identical to human or non-human C_{H2} and C_{H3} immunoglobulin domains. An Fc can but need not have the ability to interact with an Fc receptor. See, e.g., Hasemann & Capra, "Immunoglobulins: Structure and Function," in William E. Paul, ed., *Fundamental Immunology*, Second Edition, 209, 210-218 (1989), which is incorporated by reference herein in its entirety.

As used herein, the terms "protein" and "polypeptide" are used interchangeably and mean any chain of at least five naturally or non-naturally occurring amino acids linked by peptide bonds.

As used herein, the terms "isolated" and "purify" are used interchangeably and mean to reduce by 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95%, or more, the amount of heterogenous elements, for example biological macromolecules such as proteins or DNA, that may be present in a sample comprising a protein of interest. The presence of heterogenous proteins can be assayed by any appropriate method including High-performance Liquid Chromatography (HPLC), gel electrophoresis and staining and/or ELISA assay. The presence of DNA and other nucleic acids can be assayed by any appropriate method including gel electrophoresis and staining and/or assays employing polymerase chain reaction.

As used herein, the term "complex molecule" means any protein that is (a) larger than 20,000 MW, or comprises greater than 250 amino acid residues, and (b) comprises two or more disulfide bonds in its native form. A complex molecule can, but need not, form multimers. Examples of complex molecules include but are not limited to, antibodies, peptibodies and other chimeric molecules comprising an Fc domain and other large proteins. Examples of complex molecules known as peptibodies, and other Fc fusions, are described in U.S. Pat. Nos. 6,660,843, 7,138,370 and 7,511,012.

As used herein, the term "peptibody" refers to a polypeptide comprising one or more bioactive peptides joined together, optionally via linkers, with an Fc domain. See U.S. Pat. Nos. 6,660,843, 7,138,370 and 7,511,012 for examples of peptibodies.

As used herein, the term "refolding" means a process of reintroducing secondary and tertiary structure to a protein that has had some or all of its native secondary or tertiary structure removed, either in vitro or in vivo, e.g., as a result of expression conditions or intentional denaturation and/or reduction. Thus, a refolded protein is a protein that has had some or all of its native secondary or tertiary structure reintroduced.

As used herein, the term "buffer thiol-pair ratio" is defined by the relationship of the reduced and oxidized redox species used in the refold buffer as defined in Equation 1:

$$\text{Definition of Buffer Thiol-Pair Ratio } (TPR) \qquad \text{Equation 1}$$

$$\text{Buffer } TPR = \frac{[\text{reductant}]^2}{[\text{oxidant}]} = \frac{[\text{cysteine}]^2}{[\text{cystamine}]}.$$

As used herein, the terms "Buffer Thiol Strength", "Thiol-Pair Buffer Strength", and "Thiol-pair Strength" are used interchangeably and are defined in Equation 2, namely as the total mono-equivalent thiol concentration, wherein the total concentration is the sum of the reduced species and twice the concentration of the oxidized species.

$$\text{Definition of Buffer Thiol-Pair Buffer Strength/Thiol Buffer Strength (BS) Thiol-Pair Buffer Strength} = 2[\text{oxidant}] + [\text{reductant}] = 2[\text{cystamine}] + [\text{cysteine}] \qquad \text{Equation 2.}$$

The relationship between the thiol-pair ratio and thiol-pair buffer strength is described in equations 3 and 4.

$$\text{Calculation of the Reduced Redox Species with Regard to a Defined Redox Buffer Strength } (BS) \text{ and buffer Redox Potential} \qquad \text{Equation 3}$$

$$\text{Concentration of Reduced Redox Component} = \frac{\left(\sqrt{bufferTPR^2 + 8 * bufferTPR * BS}\right) - bufferTPR}{4}.$$

$$\text{Calculation of the Oxidized Redox Species with Regard to a Defined Redox Buffer Strength } (BS) \text{ and Buffer Redox Potential} \qquad \text{Equation 4}$$

$$\text{Concentration of Oxidized Redox Component} = \frac{(\text{Concentration of Reduced Redox Component})^2}{TPR}.$$

As used herein, the term "redox component" means any thiol-reactive chemical or solution comprising such a chemical that facilitates a reversible thiol exchange with another thiol or the cysteine residues of a protein. Examples of such compounds include, but are not limited to, glutathione-re-

duced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine, beta-mercaptoethanol and combinations thereof.

As used herein, the term "solubilization" means a process in which salts, ions, denaturants, detergents, reductants and/or other organic molecules are added to a solution comprising a protein of interest, thereby removing some or all of a protein's secondary and/or tertiary structure and dissolving the protein into the solvent. This process can include the use of elevated temperatures, typically 10-50° C., but more typically 15-25° C., and/or alkaline pH, such as pH 7-12. Solubilization can also be accomplished by the addition of acids, such as 70% formic acid (see, e.g., Cowley & Mackin (1997) *FEBS Lett* 402:124-130).

A "solubilized protein" is a protein in which some or all of the protein's secondary and/or tertiary structure has been removed.

A "solublization pool" is a volume of solution comprising a solubilized protein of interest as well as the salts, ions, denaturants, detergents, reductants and/or other organic molecules selected to solubilize the protein.

As used herein, the term "non-aerobic condition" means any reaction or incubation condition that is performed without the intentional aeration of the mixture by mechanical or chemical means. Under non-aerobic conditions oxygen can be present, as long as it is naturally present and was not introduced into the system with the intention of adding oxygen to the system. Non-aerobic conditions can be achieved by, for example, limiting oxygen transfer to a reaction solution by limiting headspace pressure, the absence of, or limited exposure to, air or oxygen contained in the holding vessel, air or oxygen overlay, the lack of special accommodations to account for mass transfer during process scaling, or the absence of gas sparging or mixing to encourage the presence of oxygen in the reaction system. Non-aerobic conditions can also be achieved by intentionally limiting or removing oxygen from the system via chemical treatment, headspace overlays or pressurization with inert gases or vacuums, or by sparging with gases such as argon or nitrogen, results in the reduction of oxygen concentration in the reaction mixture.

As used herein, the terms "non-native" and "non-native form" are used interchangeably and when used in the context of a protein of interest, such as a protein comprising a Fc domain, mean that the protein lacks at least one formed structure attribute found in a form of the protein that is biologically active in an appropriate in vivo or in vitro assay designed to assess the protein's biological activity. Examples of structural features that can be lacking in a non-native form of a protein can include, but are not limited to, a disulfide bond, quaternary structure, disrupted secondary or tertiary structure or a state that makes the protein biologically inactive in an appropriate assay. A protein in a non-native form can but need not form aggregates.

As used herein, the term "non-native limited solubility form" when used in the context of a protein of interest, such as a protein comprising a Fc domain, means any form or state in which the protein lacks at least one formed structural feature found in a form of the protein that (a) is biologically active in an appropriate in vivo or in vitro assay designed to assess the protein's biological activity and/or (b) forms aggregates that require treatment, such as chemical treatment, to become soluble. The term specifically includes proteins existing in inclusion bodies, such as those sometimes found when a recombinant protein is expressed in a non-mammalian expression system.

II. Theory

Refolding microbial-derived molecules present in a pool at concentrations of 2.0 g/L or higher is advantageous for a variety of reasons, primarily because of the associated reduction in reaction volumes and increases in process throughput. From a process scaling standpoint, it is advantageous to refold under conditions that do not require aerobic conditions; such conditions can be achieved, for example, by constant or intermittent sparging, the implementation of air or oxygen headspace overlays, by pressurizing the headspace, or by employing high efficiency mixing. Since the oxygen concentration in the system is related to mass transfer, the scaling of the refold reaction becomes considerably more difficult as factors such as tank geometry, volume, and mixing change. Furthermore, oxygen may not be a direct reactant in the formation of disulfide bonds in the protein, making a direct link to the mass transfer coefficient unlikely. This further complicates scaling of the reaction. Therefore, non-aerobic, chemically controlled redox systems are preferred for refolding proteins. Examples of such conditions are provided herein.

The optimal refold chemistry for a given protein represents a careful balance that maximizes the folded/oxidized state while minimizing undesirable product species, such as aggregates, unformed disulfide bridges (e.g., reduced cysteine pairs), incorrect disulfide pairings (which can lead to misfolds), oxidized amino acid residues, deamidated amino acid residues, incorrect secondary structure, and product-related adducts (e.g., cysteine or cysteamine adducts). One factor that is important in achieving this balance is the redox-state of the refold system. The redox-state is affected by many factors, including, but not limited to, the number of cysteine residues contained in the protein, the ratio and concentration of the redox couple chemicals in the refold solution (e.g., cysteine, cystine, cystamine, cysteamine, glutathione-reduced and glutathione-oxidized), the concentration of reductant carried over from the solubilization buffer (e.g., DTT, glutathione and beta-mercaptoethanol), the level of heavy metals in the mixture, and the concentration of oxygen in the solution.

Thiol-pair ratio and thiol-pair buffer strength are defined in Equations 1 and 2, infra, using cysteine and cystamine as an example reductant and oxidant, respectively. These quantities, coupled with protein concentration and reductant carryover from the solubilization, can be factors in achieving a balance between the thiol-pair ratio and the thiol-pair buffer strength.

Turning to FIG. **1**, this figure depicts the effect of thiol-pair ratio and thiol buffer strength on the distribution of product-related species, as visualized by reversed phase-HPLC analysis, for a complex dimeric protein. In FIGS. **1***a*-**1***f*, the dotted lines represent protein species with oxidized amino acid residues, single chain species, and stable mixed disulfide intermediates, the dashed lines represent mis-paired or incorrectly formed disulfide protein species and protein species with partially unformed disulfide linkages. The solid lines represent properly folded protein species. FIGS. **1***a*-**1***f* demonstrate that at a constant 6 g/L protein concentration, as the thiol-pair buffer strength is increased, the thiol-pair ratio required to achieve a comparable species distribution must also increase. For example, as shown in FIG. **1**, if the buffer strength is increased to 10 mM, from 5 mM, the balanced thiol-pair ratio would be about 2-fold higher, to achieve a comparable species distribution. This is largely due to increased buffering of the reductant carried over from the solubilization, on the total system thiol-pair ratio. At lower redox buffer strengths, the overall system becomes much more difficult to control. The protein concentration and number of cysteines contained in the protein sequence also relate to the minimum required thiol-pair buffer strength required to control the system. Below a certain point, which will vary from protein to protein,

the protein thiol concentration can overwhelm the redox couple chemistry and lead to irreproducible results.

In the results depicted in FIG. **1**, when the thiol-pair ratio of the refolding solution is intentionally set to be more reducing, the resultant product distribution shifts to produce more of the reduced product species (dashed lines). When the Thiol-Pair Ratio of the refolding solution is intentionally set to be lower, or more oxidizing, the resultant product distribution shifts to produce more oxidized residues, single chain forms, and stable mixed disulfide intermediate species (dotted lines). The ability to select an optimal Thiol-Pair Ratio and Thiol-pair Buffer Strength allows for the optimization of the yield of a desired folded protein form. This optimized yield can be achieved by maximizing the mass or yield of desired folded protein species in the refolding pool or by purposefully shifting the resultant undesired product-related species to a form that is most readily removed in the subsequent purification steps and thusly leads to an overall benefit to process yield or purity.

Optimization of the redox component Thiol-pair Ratios and Thiol-pair Buffer Strengths can be performed for each protein. A matrix or series of multifactorial matrices can be evaluated to optimize the refolding reaction for conditions that optimize yield and distributions of desired species. An optimization screen can be set up to systematically evaluate redox chemistries, Thiol-pair ratios, Thiol-pair Buffer Strengths, incubation times, protein concentration and pH in a full or partial factorial matrix, with each component varied over a range of at least three concentration or pH levels with all other parameters kept constant. The completed reactions can be evaluated by RP-HPLC and SE-HPLC analysis for yield and product quality using standard multivariate statistical tools.

III. Method Of Refolding A Protein Expressed In A Non-Mammalian Expression System And Present In A Volume At A Concentration Of 2.0 G/L Or Greater

The disclosed refold method is particularly useful for refolding proteins expressed in non-mammalian expression systems. As noted herein, non-mammalian cells can be engineered to produce recombinant proteins that are expressed intracellularly in either a soluble or a completely insoluble or non-native limited solubility form. Often the cells will deposit the recombinant proteins into large insoluble or limited solubility aggregates called inclusion bodies. However, certain cell growth conditions (e.g., temperature or pH) can be modified to drive the cells to produce a recombinant protein in the form of intracellular, soluble monomers. As an alternative to producing proteins in insoluble inclusion bodies, proteins can be expressed as soluble proteins, including proteins comprising an Fc region, which can be captured directly from cell lysate by affinity chromatography. Capturing directly from lysate allows for the refolding of relatively pure protein and avoids the very intensive harvesting and separation process that is required in inclusion body processes. The refolding method, however, is not limited to samples that have been affinity purified and can be applied to any sample comprising a protein that was expressed in a non-mammalian expression system, such as a protein found in a volume of cell lysate (i.e., a protein that has not been purified in any way).

In one aspect, the present disclosure relates to a method of refolding a protein expressed in a non-mammalian expression system in a soluble form and present in a volume at a concentration of 2.0 g/L or greater, such as a protein that has been purified by affinity chromatography from the cell lysate of non-mammalian cells in which the protein was expressed. Although the volume can be derived from any stage of a protein purification process, in one example the volume is an affinity chromatography elution pool (e.g., a Protein A elution pool). In another example, the volume is situated in a process stream. The method is not confined to Fc-containing proteins, however, and can be applied to any kind of peptide or protein that is expressed in a soluble form and captured from non-mammalian-derived cell lysate. The isolated soluble protein is often released from non-mammalian cells in a reduced form and therefore can be prepared for refolding by addition of a denaturant, such as a chaotrope. Further combination with protein stabilizers, aggregation suppressors and redox components, at an optimized Thiol-pair ration and Thiol-pair Buffer Strength, allows for refolding at concentrations of 1-40 g/L, for example at concentrations of 10-20 g/L.

In one particular embodiment of the method, a protein is expressed in a non-mammalian expression system, and is released from the expressing cell by high pressure lysis. The protein is then captured from the lysate by Protein A affinity chromatography and is present in a volume at a concentration of 10 g/L or greater. The protein is then contacted with a refold buffer comprising a denaturant, an aggregation suppressor, a protein stabilizer and a redox component, wherein the redox component has a final thiol-pair ratio (as defined herein) having a range of 0.001 to 100, for example within a range of 0.05 to 50, 0.1 to 50, 0.25 to 50, 0.5 to 50, 0.75 to 40, 1.0 to 50 or 1.5 to 50, 2 to 50, 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50 or 40 to 50 and a Thiol-pair buffer strength (as defined herein) equal to or greater than 2 mM, for example greater than or equal to 2.25 mM, 2.5, 2.75 mM, 3 mM, 5 mM, 7.5 mM, 10 mM, or 15 mM, wherein the thiol-pair buffer strength is effectively bounded at a maximum of 100 mM. Restated, in terms of ranges, the thiol buffer strength is between 2 and 20 mM, for example between 2.25 mM and 20 mM, 2.5 mM and 20 mM, 2.75 mM and 20 mM, 3 mM and 20 mM, 5 mM and 20 mM, 7.5 mM and 20 mM, 10 mM and 20 mM, or 15 mM and 20 mM.

In another aspect, the present disclosure relates to a method of refolding a protein expressed in a non-mammalian expression system in an insoluble or limited-solubility form, such as in the form of inclusion bodies. When the protein is disposed in inclusion bodies, the inclusion bodies can be harvested from lysed cells, washed, concentrated and refolded.

Optimization of the refold buffer can be performed for each protein and each final protein concentration level using the novel method provided herein. As shown in the Examples, good results can be obtained when refolding a protein comprising an Fc region when the refold buffer contains a denaturant (e.g., urea or other chaotrope, organic solvent or strong detergent), aggregation suppressors (e.g., a mild detergent, arginine or low concentrations of PEG), protein stabilizers (e.g., glycerol, sucrose or other osmolyte, salts) and redox components (e.g., cysteine, cystamine, glutathione). The optimal thiol-pair ratio and redox buffer strength can be determined using an experimental matrix of thiol-pair ratio (which can have a range of 0.001 to 100, for example within a range of 0.05 to 50, 0.1 to 50, 0.25 to 50, 0.5 to 50, 0.75 to 40, 1.0 to 50 or 1.5 to 50, 2 to 50, 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50 or 40 to 50) versus thiol-pair buffer strength (which can be greater than 2 mM, for example greater than or equal to 2.25 mM, 2.5, 2.75 mM, 3 mM, 5 mM, 7.5 mM, 10 mM, or 15 mM, wherein the thiol-pair buffer strength is effectively bounded at a maximum of 100 mM. Restated, in terms of ranges, the thiol buffer strength is between 2 and 20 mM, for example between 2.25 mM and 20 mM, 2.5 mM and 20 mM, 2.75 mM and 20 mM, 3 mM and 20 mM, 5 mM and 20 mM,

7.5 mM and 20 mM, 10 mM and 20 mM, or 15 mM and 20 mM, depending on the protein concentration and the concentration of reductant used to solubilize the inclusion bodies). Conditions can be optimized using the novel methods described in Example 2.

In one particular embodiment of the method, a protein is expressed in a non-mammalian expression system and is present in a volume at a concentration of 2.0 g/L or greater. The protein is contacted with a refold buffer comprising a denaturant, an aggregation suppressor, a protein stabilizer and a redox component, wherein the redox component has a final thiol-pair ratio (as defined herein) having a range of 0.001 to 100, for example within a range of 0.05 to 50, 0.1 to 50, 0.25 to 50, 0.5 to 50, 0.75 to 40, 1.0 to 50 or 1.5 to 50, 2 to 50, 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50 or 40 to 50, and a Thiol-pair buffer strength (as defined herein) equal to or greater than 2 mM, for example greater than or equal to 2.25 mM, 2.5 mM, 2.75 mM, 3 mM, 5 mM, 7.5 mM, 10 mM, or 15 mM, wherein the thiol-pair buffer strength is effectively bounded at a maximum of 100 mM. Restated, in terms of ranges, the thiol buffer strength is between 2 and 20 mM, for example between 2.25 mM and 20 mM, 2.5 mM and 20 mM, 2.75 mM and 20 mM, 3 mM and 20 mM, 5 mM and 20 mM, 7.5 mM and 20 mM, 10 mM and 20 mM, or 15 mM and 20 mM.to form a mixture. A wide range of denaturant types may be employed in the refold buffer. Examples of some common denaturants that can be employed in the refold buffer include urea, guanidinium, dimethyl urea, methylurea, or ethylurea. The specific concentration of the denaturant can be determined by routine optimization, as described herein.

A wide range of protein stabilizers or aggregation suppressors can be employed in the refold buffer. Examples of some common aggregation suppressors that can be useful in the refold buffer include arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate, other osmolytes, or similar compounds. The specific concentration of the aggregation suppressor can be determined by routine optimization, as described herein.

A redox component of the refold buffer can be of any composition, with the caveat that the redox component has a final thiol-pair ratio in a range of 0.001 to 100, for example within a range of 0.05 to 50, 0.1 to 50, 0.25 to 50, 0.5 to 50, 0.75 to 40, 1.0 to 50 or 1.5 to 50, 2 to 50, 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50 or 40 to 50, and a Thiol-pair buffer strength of greater than or equal to 2 mM, for example greater than or equal to 2.25 mM, 2.5, 2.75 mM, 3 mM, 5 mM, 7.5 mM, 10 mM, or 15 mM, wherein the thiol-pair buffer strength is effectively bounded at a maximum of 100 mM. Restated, in terms of ranges, the thiol buffer strength is between 2 and 20 mM, for example between 2.25 mM and 20 mM, 2.5 mM and 20 mM, 2.75 mM and 20 mM, 3 mM and 20 mM, 5 mM and 20 mM, 7.5 mM and 20 mM, 10 mM and 20 mM, or 15 mM and 20 mM. Methods of identifying a suitable redox component, i.e., determining appropriate thiol-pair ratios and redox buffer strengths, are known and/or are provided herein. Examples of specific thiol pairs that can form the redox component can include one or more of reduced glutathione, oxidized glutathione, cysteine, cystine, cysteamine, cystamine, and beta-mercaptoethanol. Thus, a thiol-pair can comprise, for example, reduced glutathione and oxidized glutathione. Another example of a thiol pair is cysteine and cystamine. The redox component can be optimized as described herein.

After the protein has been contacted with a redox component having the recited thiol pair ratio and redox buffer strength to form a refold mixture, the refold mixture is then incubated for a desired period of time. The incubation can be performed under non-aerobic conditions, as defined herein. Non-aerobic conditions need not be completely free of oxygen, only that no additional oxygen other than that present in the initial system is purposefully introduced. The incubation period is variable and is selected such that a stable refold mixture can be achieved with the desired analytical properties. An incubation period can be, for example, 1 hour, 4 hours, 12 hours, 24 hours, 48 hours, 72 hours, or longer.

Due to the sensitivity of high concentration refolds to the level of oxygen present in the system and the tendency for oxygen mass transfer to be greater at small-scale, a methodology and/or apparatus can be developed to control the oxygen levels and maintain non-aerobic conditions for the incubation step. In one embodiment, the procedure can comprise the preparation, dispensing and mixing of all refold components under a blanket of inert gas, such as nitrogen or argon, to avoid entraining levels of oxygen into the reaction. This approach is particularly helpful in identifying an acceptable thiol-pair ratio. In another embodiment useful at scales of 15 liters or less, the headspace of the refold reactor containing the protein and refold buffer can be purged with an inert gas or a mixture of inert gas and air or oxygen, and the reaction vessel sealed and mixed at a low rotational speed for the duration of the incubation time.

Following the incubation, the protein is isolated from the refold mixture. The isolation can be achieved using any known protein purification method. If the protein comprises a Fc domain, for example, a Protein A column provides an appropriate method of separation of the protein from the refold excipients. In other embodiments, various column chromatography strategies can be employed and will depend on the nature of the protein being isolated. Examples include HIC, AEX, CEX and SEC chromatography. Non-chromatographic separations can also be considered, such as precipitation with a salt, acid or with a polymer such as PEG (see, e.g., US 20080214795). Another alternative method for isolating the protein from the refold components can include dialysis or diafiltration with a tangential-flow filtration system.

In another exemplary refolding operation, inclusion bodies obtained from a non-mammalian expression system are solubilized in the range of 10 to 100 grams of protein per liter and more typically from 20-40 g/L for approximately 10-300 min. The solubilized inclusion bodies are then diluted to achieve reduction of the denaturants and reductants in the solution to a level that allows the protein to refold. The dilution results in protein concentration in the range of 1 to 15 g/L in a refold buffer containing urea, glycerol or sucrose, arginine, and the redox pair (e.g., cysteine and cystamine). In one embodiment the final composition is 1-4 M urea, 5-40% glycerol or sucrose, 25-500 mM arginine, 0.1-10 mM cysteine and 0.1-10 mM cystamine. The solution is then mixed during incubation over a time that can span from 1 hour to 4 days.

As noted herein, the disclosed method is particularly useful for proteins expressed in bacterial expression systems, and more particularly in bacterial systems in which the protein is expressed in the form of inclusion bodies within the bacterial cell. The protein can be a complex protein, i.e., a protein that (a) is larger than 20,000 MW, or comprises greater than 250 amino acid residues, and (b) comprises two or more disulfide bonds in its native form. When the protein is expressed in an inclusion body it is likely that any disulfide bond found in the protein's native form will be misformed or not formed at all. The disclosed method is applicable to these and other forms of a protein of interest. Specific examples of proteins that can be considered for refolding using the disclosed methods include antibodies, which are traditionally very difficult to

refold at high concentrations using typical refold methods due to their relatively large size and number of disulfide bonds. The method can also be employed to refold other Fc-containing molecules such as peptibodies, and more generally to refold any fusion protein comprising an Fc domain fused to another protein.

Another aspect of the disclosed method is its scalability, which allows the method to be practiced on any scale, from bench scale to industrial or commercial scale. Indeed, the disclosed method will find particular application at the commercial scale, where it can be employed to efficiently refold large quantities of protein.

The present disclosure will now be illustrated by reference to the following examples, which set forth certain embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as restricting the invention in any way.

EXAMPLES

The Examples presented herein demonstrate that thiol-pair ratio and redox buffer strength is a significant consideration in achieving an efficient refolding reaction that is insensitive to environmental influences and aeration. This insensitivity is a consideration for the ease of scaling and on an industrial or commercial scale, the transfer of the process from plant to plant.

The Examples also demonstrate that at typical refolding reaction concentrations (0.01-2.0 g/L); the sensitivity to external aeration is relatively muted. However, at concentrations of about 2 g/L and above, the sensitivity of the refold reaction to the thiol-pair ratio and redox buffer strength is increased and nearly all of the chemical components, especially the redox components, may need to be adjusted to accommodate for changes in the protein concentration in the reaction.

Example 1

Expression of Recombinant Protein

In one experiment, recombinant proteins comprising an Fc moiety were expressed in a non-mammalian expression system, namely *E coli*, and driven to form cytoplasmic deposits in the form of inclusion bodies. For each protein refolded the following procedure was followed.

After the completion of the expression phase, the cell broth was centrifuged and the liquid fraction removed, leaving the cells as a paste. The cells were resuspended in water to approximately 60% of the original volume. The cells were then lysed by means of three passes through a high pressure homogenizer. After the cells were lysed, the lysate was centrifuged in a disc-stack centrifuge to collect the protein in the solid fraction, which was expressed in a limited solubility non-native form, namely as inclusion bodies. The protein slurry was washed multiple times by repeatedly resuspending the captured solids slurry in water to between 50% and 80% of the original fermentation broth volume, mixing, and centrifugation to collect the protein in the solid fraction. The final washed inclusion bodies were captured and stored frozen.

Example 2

Identification of Refold Conditions/Redox Components

Multiple complex, microbial-derived proteins were evaluated. Each protein was solubilized in an appropriate level of guanidine and/or urea, typically at levels the equivalent of 4-6 M guanidine or 4-9 M urea, or combinations of both denaturants, which fully denatured the protein. The protein was reduced with DTT, 5-20 mM, at pH 8.5, and incubated at room temperature for approximately 1 hour.

Identification of the refold buffer was performed for each protein. A multifactorial matrix or a series of multifactorial matrices were evaluated to identify the refolding reaction for conditions that optimize yield and minimize aggregate formation. An identification screen was set up to systematically evaluate urea, arginine, glycerol and pH in a full factorial matrix, with each component varied over a range of at least three concentration or pH levels with all other parameters kept constant. The completed reactions were evaluated by RP-HPLC and SE-HPLC analysis for yield and product quality using standard multivariate statistical tools. A subset of the conditions having the desired behavior was then further evaluated in subsequent screens that evaluated a range of pH, thiol-pair ratio, thiol-pair buffer strength, and potentially further excipient levels in a factorial screen. Secondary interactions were also evaluated using standard multivariate statistical tools.

Best results, as determined by reversed-phase and size exclusion HPLC analysis, were observed using a refold buffer containing a denaturant (e.g., urea, dimethyl urea or other chaotrope at non-denaturing levels at levels between 1 and 4 M), an aggregation suppressor (e.g., arginine at levels between 5 and 500 mM), a protein stabilizer (e.g., glycerol or sucrose at levels between 5 and 40% w/v) and a redox component (e.g., cysteine or cystamine). The thiol-pair ratio and redox buffer strength were determined using an experimental matrix of thiol-pair ratio (0.1 to 100, more typically 1 to 25) versus buffer strength (typically 2 mM to 20 mM, depending on the protein concentration, the number of cysteine residues in the protein, and the concentration of reductant used to solubilize the inclusion bodies).

Individual reactions were formed with varying levels of cysteine and cystamine that would allow for a controlled matrix of thiol-pair ratio at various thiol-pair buffer strengths. The relationships were calculated using Equations 3 and 4. Each condition was screened under both aerobic and non-aerobic conditions, utilizing the techniques described herein. Optimum conditions were selected to meet a stable balance of yield, desired distribution of folding species, insensitivity to environmental oxidants (e.g., air), and insensitivity to normal variation in DTT carry-over from the solubilization step.

Example 3

High Concentration Refolding of Non-Native Soluble Protein Form Captured from Cell Lysate

In one experiment, a recombinant protein comprising a plurality of polypeptides joined to an Fc moiety was expressed in *E. coli* as an intracellular soluble peptide chain, lysed from harvested and washed cells, isolated from the lysate by affinity chromatography, and then refolded at a concentration of approximately 12 g/L, as described herein.

After the completion of the expression phase, an aliquot of whole fermentation broth was centrifuged and the liquid fraction removed, leaving the cells as a paste. The cells were resuspended in water to approximately 60% of the original volume. The cells were then lysed by means of three passes through a high pressure homogenizer. After the cells were lysed, the lysate pool was mixed in the presence of air for 8-72 hours to allow for dimerization of the peptide chains. Following the dimerization process, the peptide chain of interest was

isolated from the lysate pool using a Protein A affinity chromatography column. The Protein A column elution pool was mixed at a ratio of 8 parts Protein A elution material to 2 parts of a refold buffer containing urea (10 M), arginine-HCl (2.5 M), Tris at pH 8.5 (1050 mM), and cysteine (10 mM, 5 mM, or 4 mM) and cystamine (4 mM). The diluted mixture was titrated to pH 8.5 and incubated at approximately 5° C. under nitrogen until a stable pool was achieved (~24 hours.) Yields of desired product of approximately 30-80% were obtained a depending on the redox condition evaluated.

In order to emulate the non-anaerobic conditions similar to those typically present in very large-scale protein production processes several steps were taken. When reaction volumes were less than approximately 15 L the refold vessel headspace was purged with nitrogen to limit the effect oxygen could have in the system. The vessel was then sealed and incubation began.

When reaction volumes were more than approximately 15 L but less than 500 L, the refold buffer was prepared and allowed to equilibrate at approximately 5° C. to achieve a stable oxygen level in the solution (typically 50% to 70% dissolved oxygen, relative to air saturation). Once the refold mixture was formed, the vessel headspace was purged with nitrogen to limit any additional effect oxygen could have in the system, the vessel was sealed and incubation period initiated.

Example 4

High Concentration Refolding from Inclusion Bodies

In one experiment, a recombinant protein comprising a biologically active peptide linked to the C-terminus of the Fc moiety of an IgG1 molecule via a linker and having a molecular weight of about 57 kDa and comprising 8 disulfide bonds, was expressed in *E. coli* as inclusion bodies, harvested, washed, concentrated, solubilized, and refolded at a concentration of 6 g/L as described herein.

An aliquot of frozen concentrated inclusion bodies were thawed to room temperature and mixed with an appropriate amount of guanidine and/or urea to generate a denaturant level equivalent to 4-6 M guanidine, which fully denatures the protein. The protein was then reduced with DTT, at 5-20 mM, at pH 8.5, and incubated at room temperature for approximately 1 hour. After the inclusion bodies were dissolved, denatured and reduced, they were diluted into a refold buffer containing urea (1-5 M), arginine-HCl (5-500 mM), glycerol (10-30% w/v), and the identified levels of cysteine and cystamine as determined by the procedure described in Example 2. The final component concentrations are 4 M urea, 150 mM arginine HCl, 20.9% (w/v) glycerol, 2.03 mM cysteine, and 2.75 mM cystamine. The level of dilution was chosen to balance the dilution of the denaturants from the solubilization, maintain the thermodynamic stability of the molecule during refolding, and maintain the highest possible protein concentration in the refold mixture. The diluted mixture was titrated to an alkaline pH (between pH 8 and pH 10) and incubated at 5° C. under non-aerobic conditions until a stable pool was achieved (12-72 hours), as determined by relevant analytical measurements. The resulting process was demonstrated to show stable scalablity from 1 L-scale to 2000 L-scale (see FIG. **3**). Yields of desired product of approximately 27-35% were obtained at both scales. The distribution of product related impurities was also maintained within a tight variance (see FIG. **3**).

Oxygen mass transfer at small-scale is readily achieved and should be inhibited in order to emulate the relatively poorer mass transfer observed at large-scale, where the volume of refold solution is large relative to the volume of air and surface area present at the surface of a large-scale vessel. Thus, in order to emulate the non-anaerobic conditions similar to those typically present in very large-scale protein production processes several steps were taken. When reaction volumes were less than approximately 15 L the refold buffer was sparged with nitrogen to strip oxygen from the solution, the components were dispensed under a blanket of nitrogen and once the refold mixture was formed, the vessel headspace was purged with nitrogen to limit the effect oxygen could have in the system. The vessel was then sealed and incubation began.

When reaction volumes were more than approximately 15 L but less than 500L, the refold buffer was prepared and allowed to equilibrate at approximately 5° C. to achieve a stable oxygen level in the solution (typically 50% to 70% dissolved oxygen, relative to air saturation). Once the refold mixture was formed, the vessel headspace was purged with nitrogen to limit any addition effect oxygen could have in the system, the vessel was sealed and the incubation period was initiated.

At scales greater than 500 L the refold buffer was prepared and allowed to equilibrate at approximately 5° C. to achieve a stable oxygen level in the solution (typically 50% to 70% dissolved oxygen, relative to air saturation). Once the refold mixture was formed, the vessel was sealed and the incubation period was initiated.

The protein concentration of the refold mixture was 6 g/L, which is a four-fold enhancement over the recovery of 1.5 g/L obtained using a method other than the method described in this Example. Overall annual process productivity, in one specific manufacturing facility, was calculated to be increased by >930% due to increased volumetric efficiency in the existing facility tanks.

Example 5

Effect of Thiol-Pair Oxidation State on Disulfide Pairings

FIGS. **1***a*-**1***f* demonstrate that as the thiol-pair ratio is forced to a more oxidizing state (lower thiol-pair ratio), a higher proportion of product species have oxidized amino acid residues and mixed disulfide forms. As the thiol-pair ratio is driven to a more reductive state (higher thiol-pair ratio), this results in lower levels of oxidized amino acid variant species and higher levels of product species with incorrect disulfide pairings or unformed disulfide bonds. As the overall thiol-pair buffer strength is modified, the corresponding optimal thiol-pair ratio is shifted. This effect is similar to how buffer strength modulates the sensitivity of pH to acid and base additions in a buffered solution.

An optimal balance of species was attainable. As shown in FIGS. **1***a*-**1***f*, there is a clear relationship between thiol-pair buffer strength and thiol-pair ratio that can be identified to maintain the optimal species balance and thus facilitate efficient refolding of low solubility proteins. The ability to control product variant species, such as incorrectly disulfide-bonded species and misfolded species, via modulation of the thiol-pair ratio and thiol-pair buffer strength, enables efficient, effective and reliable subsequent purification processes.

Example 6

Effect of Non-Aerobic Conditions on Refolding Efficiency

FIGS. **2** and **3** demonstrate that when the thiol-pair buffer strength is selected appropriately, taking into account the protein concentration and number of cysteine residues in the protein, the sensitivity to external influences, such as oxygen, is significantly reduced. This allows for a non-aerobic refolding condition that is significantly easier to transfer between scales and reactor configurations.

FIG. **2** compares the RP-HPLC analytical species distribution between a 15 L-scale refold and a 20 mL-scale refold under several environmental conditions. For Condition 1 (the trace labeled "1" in FIG. **1**), the solubilization chemicals and solutions were dispensed in air and the refold mixture was incubated in air. In Condition 2 solubilization chemicals and solutions were dispensed in air and incubated under nitrogen headspace. In Conditions 3-7 solubilization chemicals and solutions were dispensed under nitrogen overlay conditions and in conditions 3, 5, 6, and 7 solubilization chemicals and solutions were incubated under nitrogen. In Condition 7, the refold solution was also stripped of nitrogen prior to combination with the solubilization solution. In Condition 4 the solubilization chemicals and solutions were incubated under ambient air conditions.

The results shown in FIG. **2** demonstrate that the conditions under which the solubilization chemicals and solutions were dispensed or incubated in the presence of air (i.e., Conditions 1, 2, and 4) do not achieve results that are comparable to the larger-scale control. In Conditions 1, 2 and 4, increased formation of oxidized species (pre-peaks) are observed. The pre-peaks are indicated by arrows in the panels for Conditions 1, 2 and 4.

FIG. **3** compares the RP-HPLC analytical results of an identified condition, achieved as described in Example 2, at 1 L-scale and 2000 L-scale. In this figure, essentially no difference in the distribution of species is detectable. Taken together, FIGS. **2** and **3** demonstrate that when aeration is carefully controlled, the small-scale refold reactions are more predictive of those expected upon scale-up of the refold reaction, facilitating the implementation of large-scale protein refolding processes.

What is claimed is:

1. A method of refolding a protein expressed in a non-mammalian expression system and present in a volume at a concentration of 2.0 g/L or greater comprising:

(a) contacting the protein with a refold buffer comprising a redox component comprising a final thiol-pair ratio having a range of 0.001 to 100 and a redox buffer strength of 2 mM or greater and one or more of:

(i) a denaturant;

(ii) an aggregation suppressor; and

(iii) a protein stabilizer;

to form a refold mixture;

(b) incubating the refold mixture; and

(c) isolating the protein from the refold mixture.

2. The method of claim **1**, wherein the final thiol-pair ratio is selected from the group consisting of 0.05 to 50, 0.1 to 50, 0.25 to 50, 0.5 to 50, 0.75 to 40, 1.0 to 50 and 1.5 to 50, 2 to 50, 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50 or 40 to 50.

3. The method of claim **1**, wherein the thiol-pair buffer strength is selected from the group consisting of greater than or equal to 2.25 mM, 2.5 mM, 2.75 mM, 3 mM, 5 mM, 7.5 mM, 10 mM and 15 mM.

4. The method of claim **1**, wherein the protein is present in the volume in a non-native limited solubility form.

5. The method of claim **4**, wherein the non-native limited solubility form is an inclusion body.

6. The method of claim **1**, wherein the protein is present in the volume in a soluble form.

7. The method of claim **1**, wherein the protein is recombinant.

8. The method of claim **1**, wherein the protein is an endogenous protein.

9. The method of claim **1**, wherein the protein is an antibody.

10. The method of claim **1**, wherein the protein is a complex protein.

11. The method of claim **1**, wherein the protein is a multimeric protein.

12. The method of claim **1**, wherein the protein is an Fc-protein conjugate.

13. The method of claim **1**, wherein the non-mammalian expression system is one of a bacterial expression system and a yeast expression system.

14. The method of claim **1**, wherein the denaturant is selected from the group consisting of urea, guanidinium salts, dimethyl urea, methylurea and ethylurea.

15. The method of claim **1**, wherein the protein stabilizer is selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes.

16. The method of claim **1**, wherein the aggregation suppressor is selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes.

17. The method of claim **1**, wherein the thiol-pairs comprise at least one component selected from the group consisting of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol.

18. The method of claim **1**, wherein the incubation is performed under non-aerobic conditions.

19. The method of claim **1**, wherein the isolation comprises contacting the mixture with an affinity separation matrix.

20. The method of claim **19**, wherein the affinity separation matrix is a Protein A resin.

21. The method of claim **19**, wherein the affinity resin is a mixed mode separation matrix.

22. The method of claim **1**, wherein the isolating comprises contacting the mixture with an ion exchange separation matrix.

23. The method of claim **1**, wherein the isolating further comprises a filtration step.

24. The method of claim **23**, wherein the filtration step comprises depth filtration.

* * * * *

Exhibit 4

US009856287B2

(12) **United States Patent**
Shultz et al.

(10) **Patent No.: US 9,856,287 B2**
(45) **Date of Patent: *Jan. 2, 2018**

(54) **REFOLDING PROTEINS USING A CHEMICALLY CONTROLLED REDOX STATE**

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(72) Inventors: **Joseph Edward Shultz**, Binningen (CH); **Roger Hart**, Needham, MA (US); **Ronald Nixon Keener, III**, Newbury Park, CA (US)

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **15/422,327**

(22) Filed: **Feb. 1, 2017**

(65) **Prior Publication Data**

US 2017/0145049 A1 May 25, 2017

Related U.S. Application Data

(60) Continuation of application No. 14/793,590, filed on Jul. 7, 2015, which is a continuation of application No. 14/611,037, filed on Jan. 30, 2015, which is a division of application No. 12/820,087, filed on Jun. 21, 2010, now Pat. No. 8,952,138.

(60) Provisional application No. 61/219,257, filed on Jun. 22, 2009.

(51) **Int. Cl.**

C07K 14/00	(2006.01)
C07K 1/113	(2006.01)
C07K 1/14	(2006.01)
C07K 16/00	(2006.01)

(52) **U.S. Cl.**

CPC ***C07K 1/1136*** (2013.01); ***C07K 1/1133*** (2013.01); ***C07K 1/14*** (2013.01); ***C07K 14/00*** (2013.01); ***C07K 16/00*** (2013.01); *C07K 2319/30* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

A method of refolding proteins expressed in non-mammalian cells present in concentrations of 2.0 g/L or higher is disclosed. The method comprises identifying the thiol pair ratio and the redox buffer strength to achieve conditions under which efficient folding at concentrations of 2.0 g/L or higher is achieved and can be employed over a range of volumes, including commercial scale.

30 Claims, 8 Drawing Sheets

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Reply to Patent Owner Response; United States Patent and Trademark Office Before the Patent Trial and Appeal Board, *Apotex Inc. and Apotex Corp.* v. *Amgen Inc. and Amgen Manufacturing Limited*, Inter Partes Review No. IPR2016-01542 U.S. Pat. No. 8,952,138, pp. 1-36; Aug. 21, 2017.

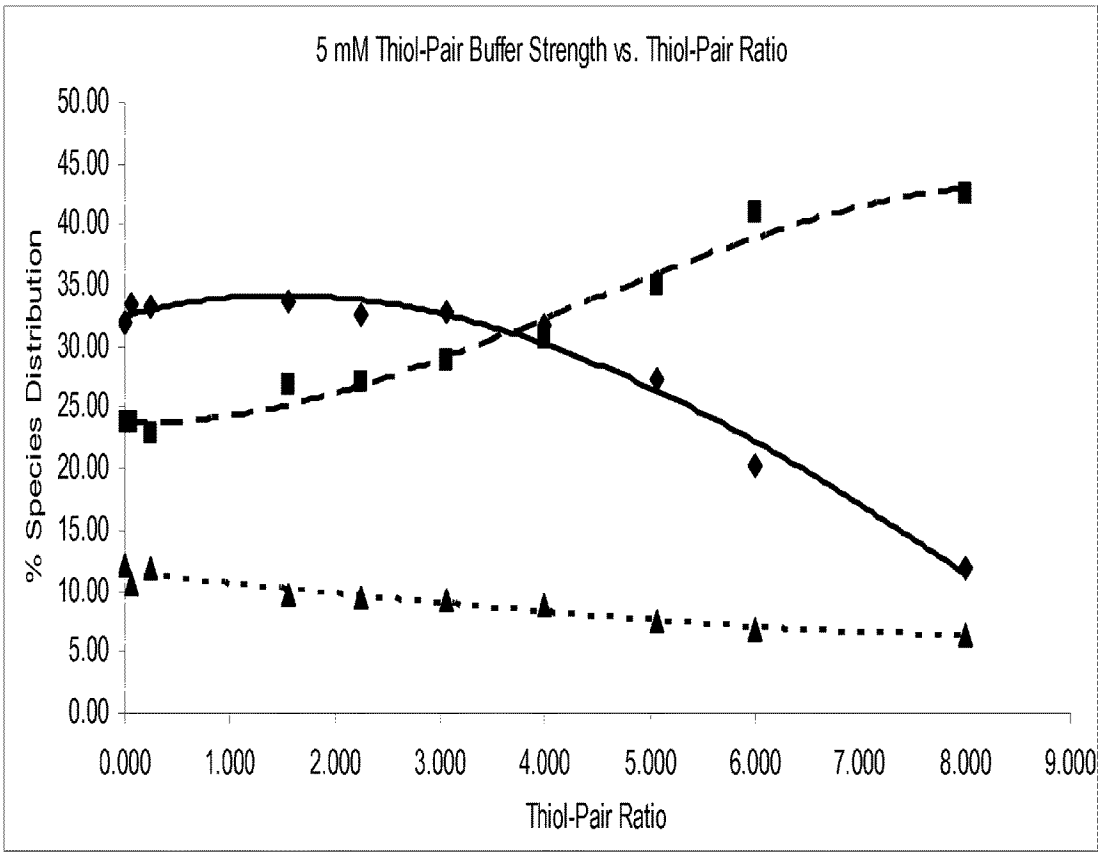
5 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
% Species Distribution
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0.000
1.000
2.000
3.000
4.000
5.000
6.000
7.000
8.000
9.000
Thiol-Pair Ratio

Figure 1a

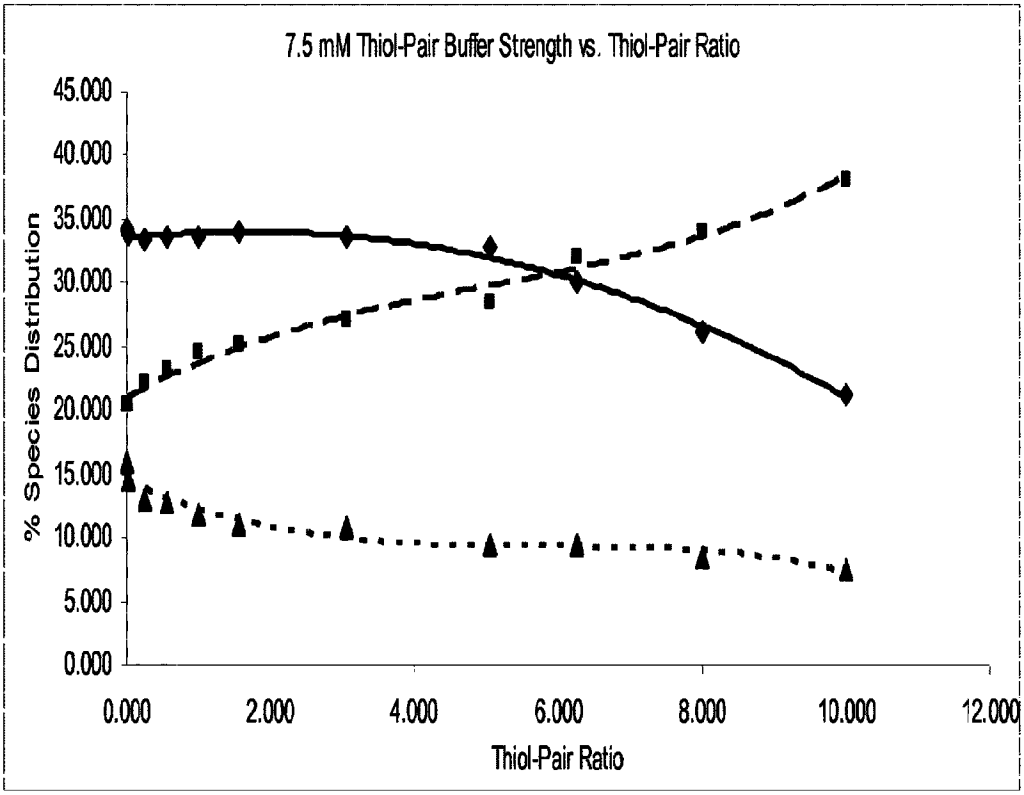
7.5 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
% Species Distribution
45.000
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
0.000
2.000
4.000
6.000
8.000
10.000
12.000
Thiol-Pair Ratio

Figure 1b

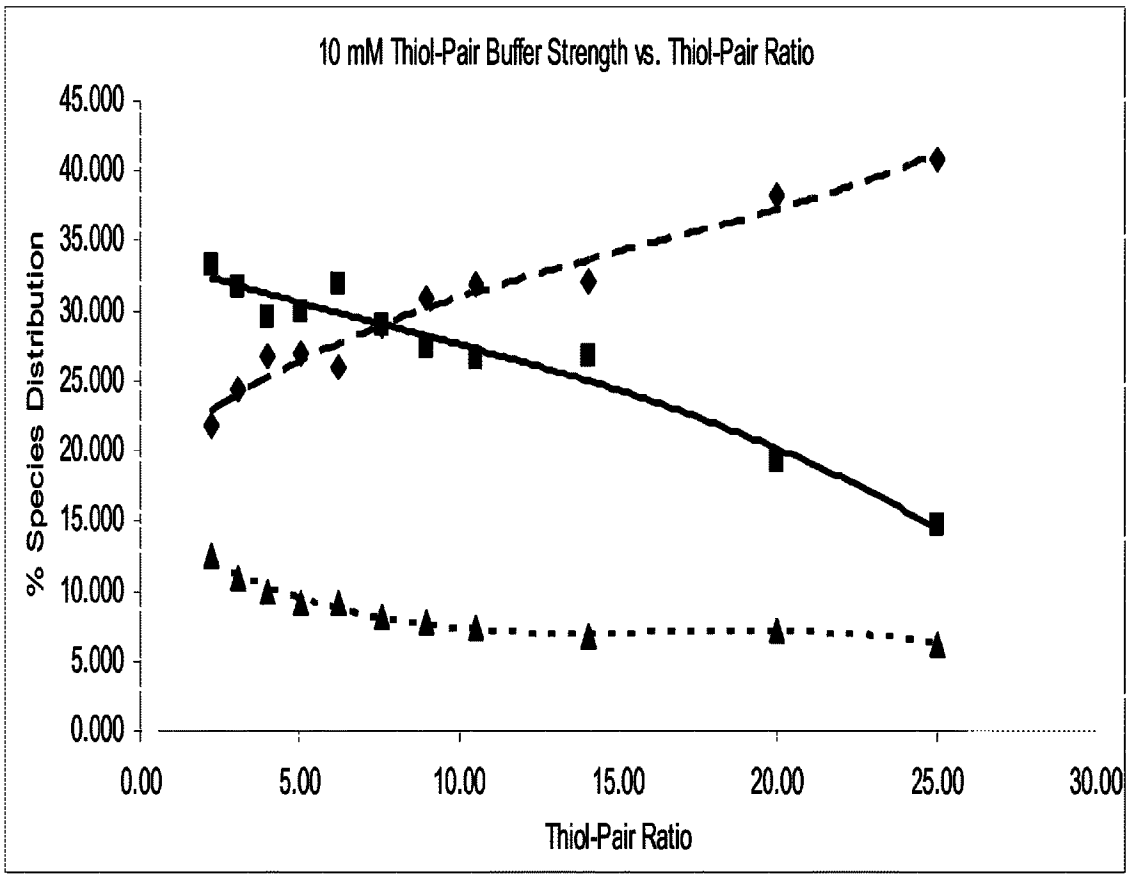
10 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
% Species Distribution
45.000
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
0.00
5.00
10.00
15.00
20.00
25.00
30.00
Thiol-Pair Ratio

Figure 1c

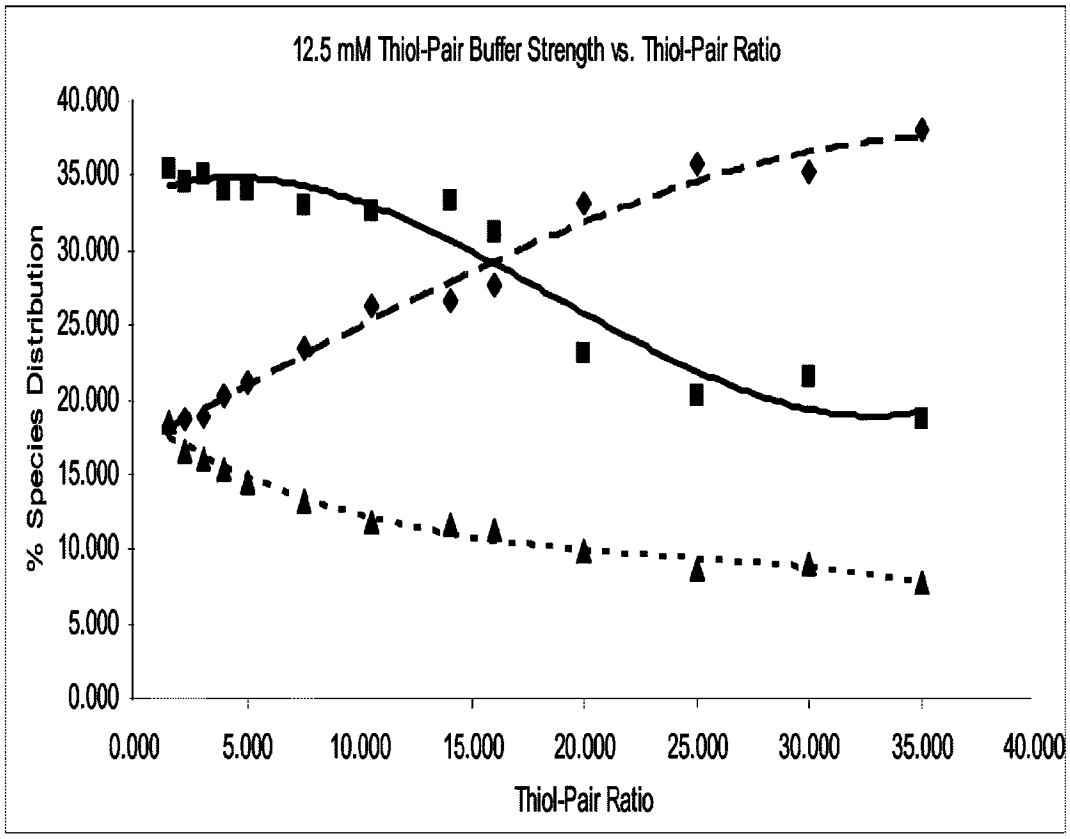
12.5 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
% Species Distribution
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
0.000
5.000
10.000
15.000
20.000
25.000
30.000
35.000
40.000
Thiol-Pair Ratio

Figure 1d

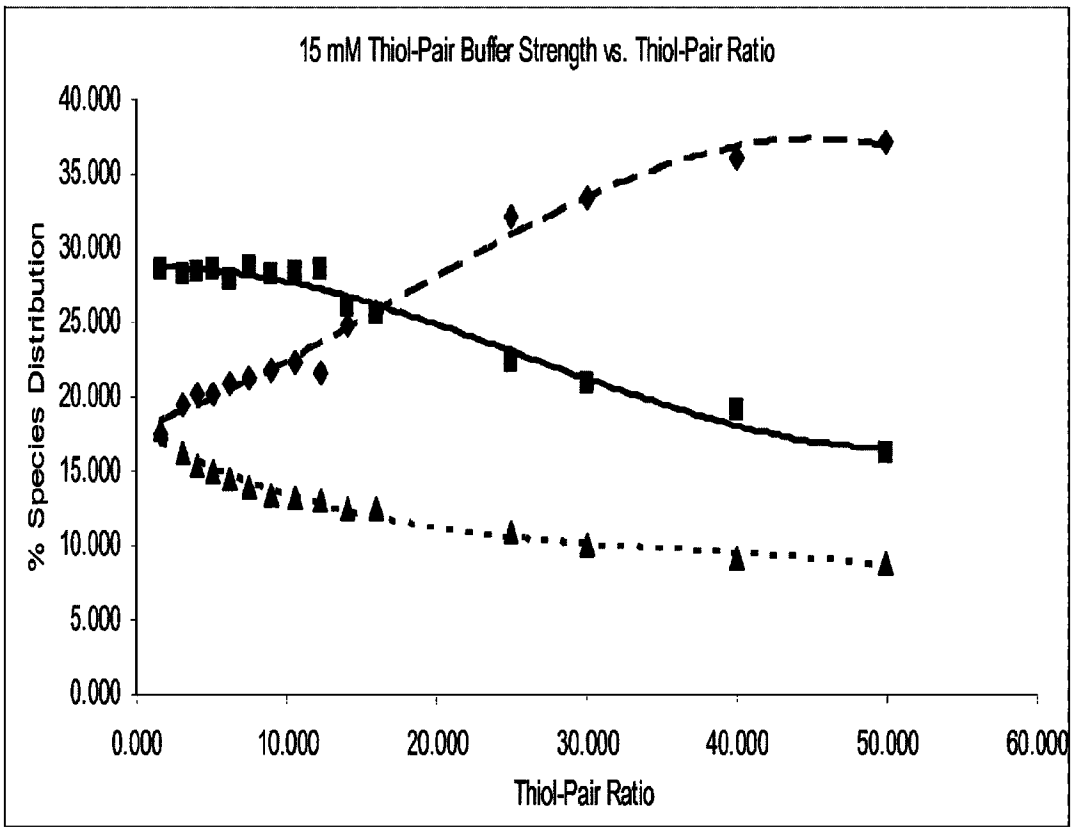
15 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
% Species Distribution
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
0.000
10.000
20.000
30.000
40.000
50.000
60.000
Thiol-Pair Ratio

Figure 1e

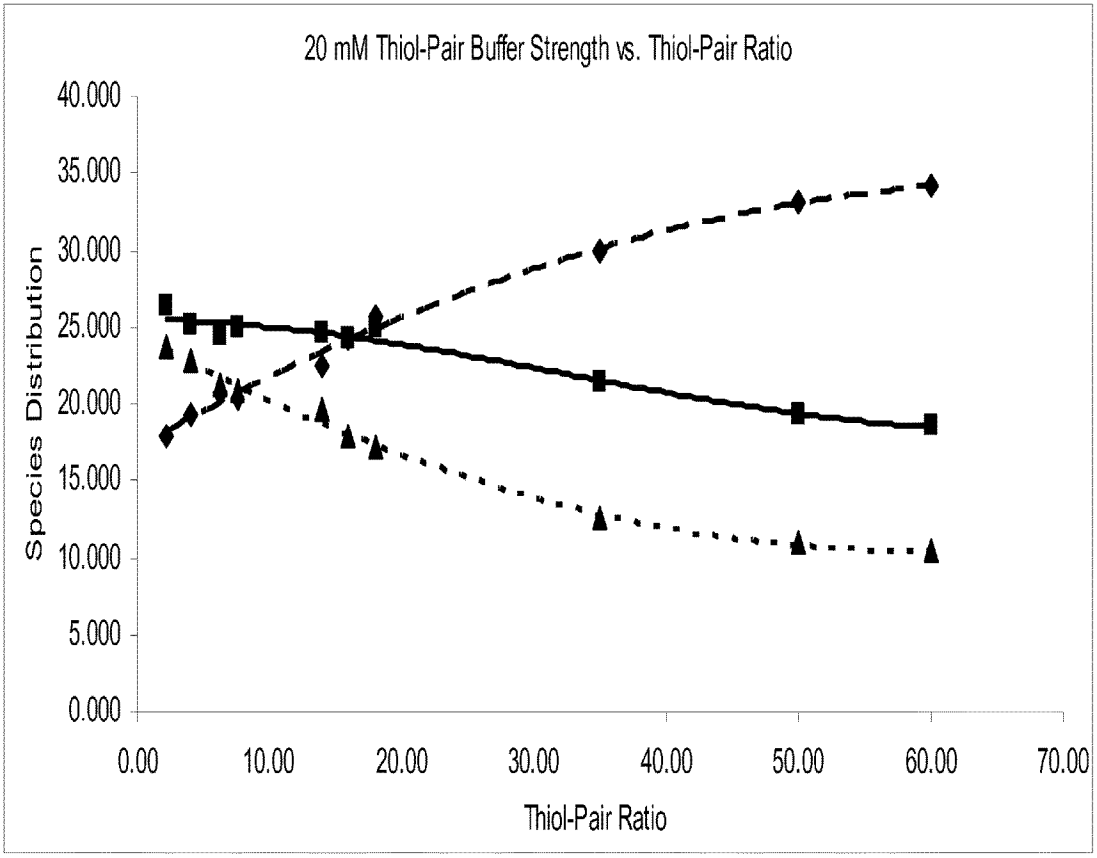
20 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
Species Distribution
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
Thiol-Pair Ratio

Figure 1f

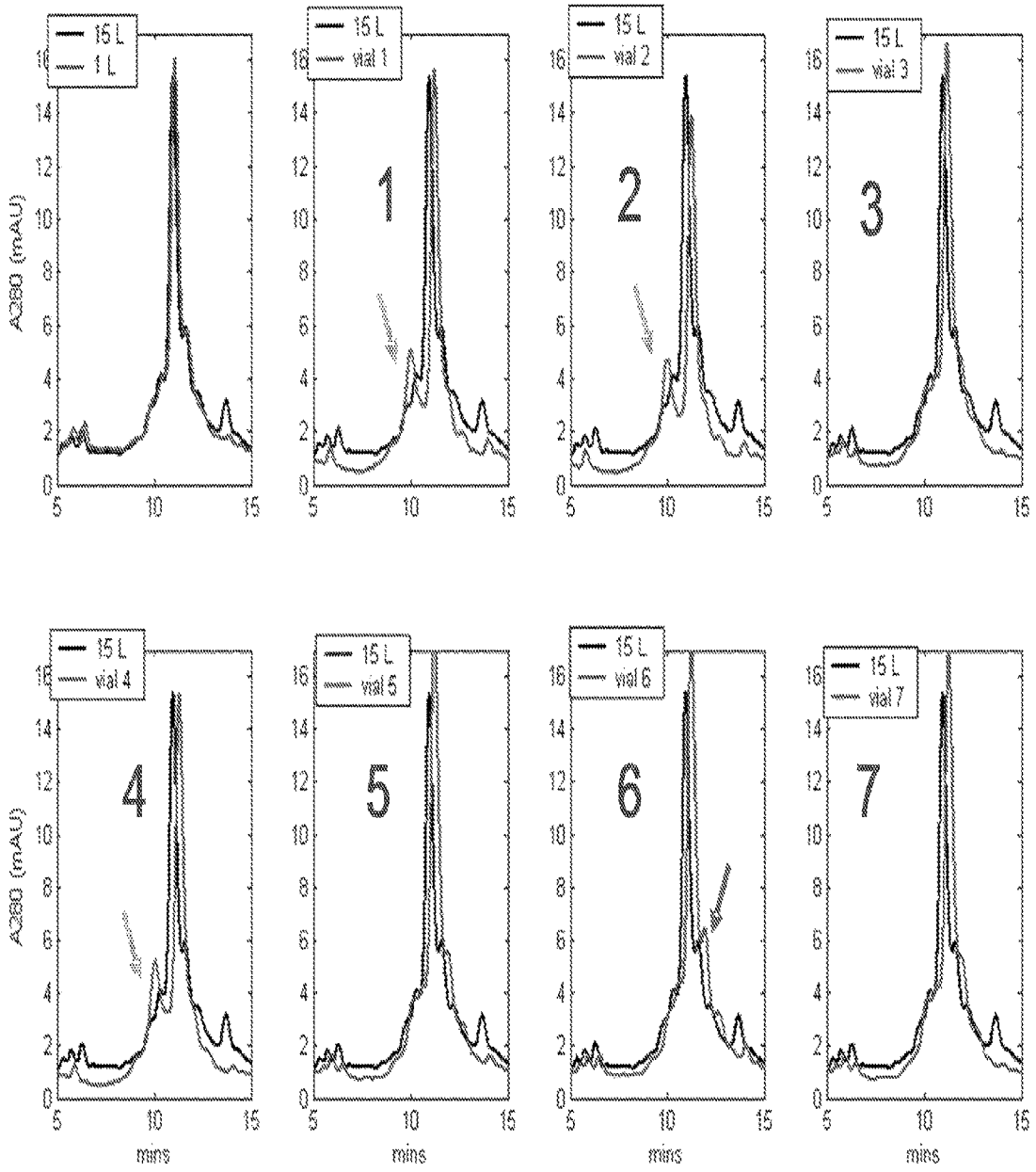
15 L
1 L
vial 1
vial 2
vial 3
vial 4
vial 5
vial 6
vial 7
1
2
3
4
5
6
7
A280 (mAU)
mins

Figure 2

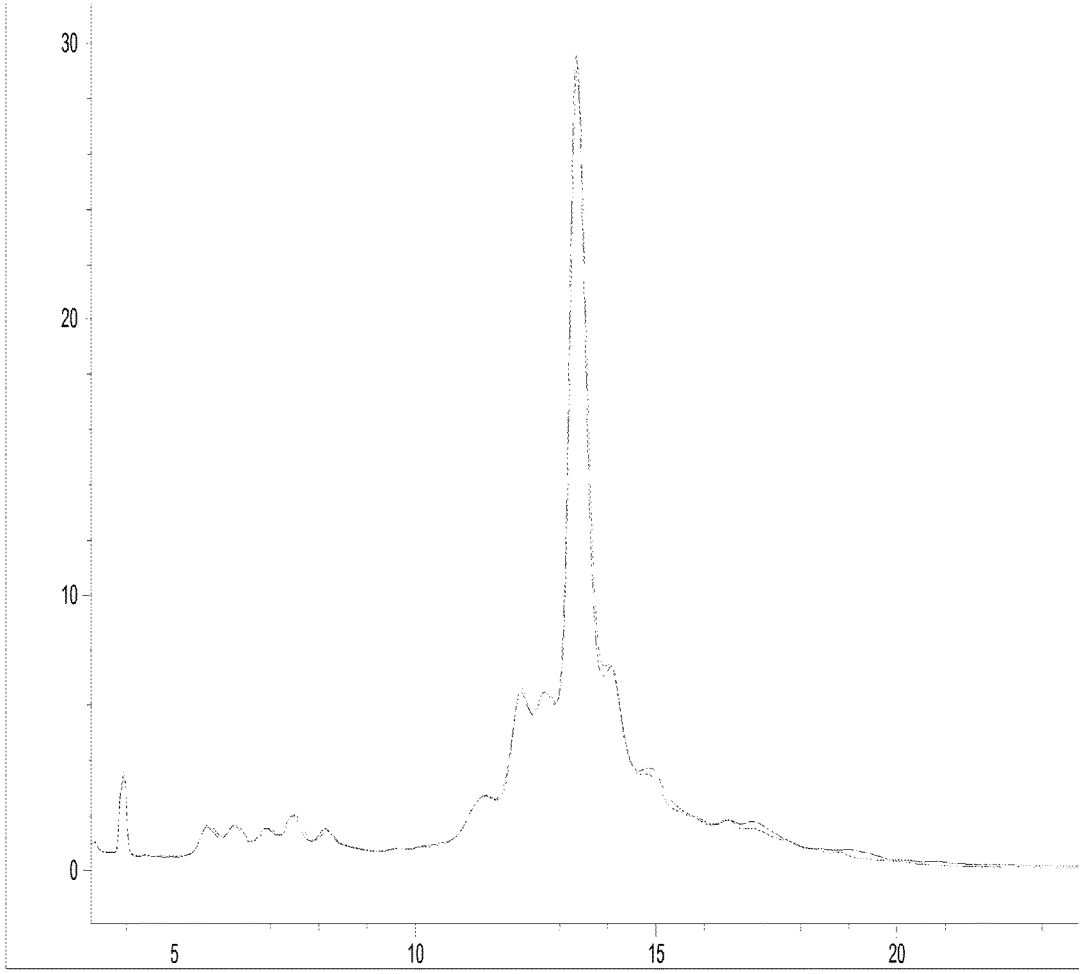

Figure 3

REFOLDING PROTEINS USING A CHEMICALLY CONTROLLED REDOX STATE

This application is a continuation of pending U.S. patent application Ser. No. 14/793,590, filed on Jul. 7, 2015; which is a continuation of pending U.S. patent application Ser. No. 14/611,037, filed on Jan. 30, 2015; which is a divisional of U.S. patent application Ser. No. 12/820,087, filed on Jun. 21, 2010, now U.S. Pat. No. 8,952,138; which claims the benefit of U.S. Provisional Application No. 61/219,257 filed Jun. 22, 2009, which is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention generally relates to refolding proteins at high concentrations, and more particularly to refolding proteins in volumes at concentrations of 2.0 g/L and above.

BACKGROUND OF THE INVENTION

Recombinant proteins can be expressed in a variety of expression systems, including non-mammalian cells, such as bacteria and yeast. A difficulty associated with the expression of recombinant proteins in prokaryotic cells, such as bacteria, is the precipitation of the expressed proteins in limited-solubility intracellular precipitates typically referred to as inclusion bodies. Inclusion bodies are formed as a result of the inability of a bacterial host cell to fold recombinant proteins properly at high levels of expression and as a consequence the proteins become insoluble. This is particularly true of prokaryotic expression of large, complex or protein sequences of eukaryotic origin. Formation of incorrectly folded recombinant proteins has, to an extent, limited the commercial utility of bacterial fermentation to produce recombinant large, complex proteins, at high levels of efficiency.

Since the advent of the recombinant expression of proteins at commercially viable levels in non-mammalian expression systems such as bacteria, various methods have been developed for obtaining correctly folded proteins from bacterial inclusion bodies. These methods generally follow the procedure of expressing the protein, which typically precipitates in inclusion bodies, lysing the cells, collecting the inclusion bodies and then solubilizing the inclusion bodies in a solubilization buffer comprising a denaturant or surfactant and optionally a reductant, which unfolds the proteins and disassembles the inclusion bodies into individual protein chains with little to no structure. Subsequently, the protein chains are diluted into or washed with a refolding buffer that supports renaturation to a biologically active form. When cysteine residues are present in the primary amino acid sequence of the protein, it is often necessary to accomplish the refolding in an environment which allows correct formation of disulfide bonds (e.g., a redox system).

Typical refold concentrations for complex molecules, such as molecules comprising two or more disulfides, are less than 2.0 g/L and more typically 0.01-0.5 g/L (Rudolph & Lilie, (1996) *FASEB J.* 10:49-56). Thus, refolding large masses of a complex protein, such as an antibody, peptibody or other Fe fusion protein, at industrial production scales poses significant limitations due to the large volumes required to refold proteins, at these typical product concentration, and is a common problem facing the industry. One factor that limits the refold concentration of these types of proteins is the formation of incorrectly paired disulfide bonds, which may in turn increase the propensity for those forms of the protein to aggregate. Due to the large volumes of material and large pool sizes involved when working with industrial scale protein production, significant time, and resources can be saved by eliminating or simplifying one or more steps in the process.

While protein refolding has previously been demonstrated at higher concentrations, the proteins that were refolded were either significantly smaller in molecular weight, less complex molecules containing only one or two disulfide bonds (see, e.g., Creighton, (1974) *J. Mol. Biol.* 87:563-577). Additionally, the refolding processes for such proteins employed detergent-based refolding chemistries (see, e.g., Stockel et al., (1997) *Eur J Biochem* 248:684-691) or utilized high pressure folding strategies (St John et al., (2001) *J. Biol. Chem.* 276(50):46856-63). More complex molecules, such as antibodies, peptibodies and other large proteins, are generally not amenable to detergent refold conditions and are typically refolded in chaotropic refold solutions. These more complex molecules often have greater than two disulfide bonds, often between 8 and 24 disulfide bonds, and can be multi-chain proteins that form homo- or hetero-dimers.

Until the present disclosure, these types of complex molecules could not be refolded at high concentrations, i.e., concentrations of 2.0 g/L and higher, with any meaningful degree of efficiency on a small scale, and notably not on an industrial scale. The disclosed methods, in contrast, can be performed at high concentrations on a small or large (e.g, industrial) scale to provide properly refolded complex proteins. The ability to refold proteins at high concentrations and at large scales can translate into not only enhanced efficiency of the refold operation itself, but also represents time and cost savings by eliminating the need for additional equipment and personnel. Accordingly, a method of refolding proteins present in high concentrations could translate into higher efficiencies and cost savings to a protein production process.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1***a*-**1***f* are a series of plots depicting the effect of thiol-pair ratio and redox buffer strength on product-species distribution; FIG. **1***a* depicts the effect of a 5 mM buffer strength; FIG. **1***b* depicts the effect of a 7.5 mM buffer strength; FIG. **1***c* depicts the effect of a 10 mM buffer strength; FIG. **1***d* depicts the effect of a 12.5 mM buffer strength; FIG. **1***e* depicts the effect of a 15 mM buffer strength; and FIG. **1***f* depicts the effect of a 20 mM buffer strength.

FIG. **2** is a series of plots depicting the effect of the degree of aeration on the species distribution under fixed thiol-pair ratio and thiol-pair buffer strength.

FIG. **3** is an analytical overlay of a chemically controlled, non-aerobic refold performed at 6 g/L and optimized using an embodiment of the described method performed at 1 L and 2000 L.

SUMMARY OF THE INVENTION

A method of refolding a protein expressed in a non-mammalian expression system and present in a volume at a concentration of 2.0 g/L or greater comprising: (a) contacting the protein with a refold buffer comprising a redox component comprising a final thiol-pair ratio having a range of 0.001 to 100 and a redox buffer strength of 2 mM or

greater and one or more of: (i) a denaturant; (ii) an aggregation suppressor; and (iii) a protein stabilizer, to form a refold mixture; (b) incubating the refold mixture; and (c) isolating the protein from the refold mixture.

In various embodiments the redox component has a final thiol-pair ratio greater than or equal to 0.001 but less than or equal to 100, for example within a range of 0.05 to 50, 0.1 to 50, 0.25 to 50, 0.5 to 50, 0.75 to 40, 1.0 to 50 or 1.5 to 50, 2 to 50, 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50 or 40 to 50 and a Thiol-pair buffer strength equal to or greater than 2 mM, for example greater than or equal to 2.25 mM, 2.5 mM, 2.75 mM, 3 mM, 5 mM, 7.5 mM, 10 mM, or 15 mM, wherein the thiol-pair buffer strength is effectively bounded at a maximum of 100 mM. Restated, in terms of ranges, the thiol buffer strength can be between 2 and 20 mM, for example between 2.25 mM and 20 mM, 2.5 mM and 20 mM, 2.75 mM and 20 mM, 3 mM and 20 mM, 5 mM and 20 mM, 7.5 mM and 20 mM, 10 mM and 20 mM, or 15 mM and 20 mM, to form a mixture.

In one embodiment of a refold buffer, the refold buffer comprises urea, arginine-HCl, cysteine and cystamine in Tris buffer. In a further embodiment the components are present in the refold buffer in proportions described in Example 3.

In another embodiment of a refold buffer, the refold buffer comprises urea, arginine HCl, glycerol, cysteine, and cystamine in Tris buffer. In a further embodiment the components are present in the refold buffer in proportions described in Example 4.

In some embodiments, the protein is initially present in a volume in a non-native limited solubility form, such as an inclusion body. Alternatively, the protein is present in the volume in a soluble form. The protein can be a recombinant protein or it can be an endogenous protein. The protein can be a complex protein such as an antibody or a multimeric protein. In another embodiment, the protein is an Fc-protein conjugate, such as a protein fused or linked to a Fc domain.

The non-mammalian expression system can be a bacterial expression system or a yeast expression system.

The denaturant in the refold buffer can be selected from the group consisting of urea, guanidinium salts, dimethyl urea, methylurea and ethylurea. The protein stabilizer in the refold buffer can be selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes. The aggregation suppressor can be selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes. The thiol-pairs can comprise at least one component selected from the group consisting of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol.

In various embodiments, the purification can comprise contacting the mixture with an affinity separation matrix, such as a Protein A or Protein G resin. Alternatively, the affinity resin can be a mixed mode separation matrix or an ion exchange separation matrix. In various aspects, the incubation can be performed under aerobic conditions or under non-aerobic conditions.

DETAILED DESCRIPTION OF THE INVENTION

The relevant literature suggests that when optimizing various protein refolding operations, the refold buffer thiol-pair ratio has been purposefully varied and as a result the thiol buffer strength was unknowingly varied across a wide range of strengths (see, e.g., Lilie, Schwarz & Rudolph, (1998) *Current Opinion in Biotechnology* 9(5):497-501, and Tran-Moseman, Schauer & Clark (1999) *Protein Expression & Purification* 16(1):181-189). In one study, a relationship between the thiol pair ratio and the buffer strength was investigated for lysozyme, a simple, single-chain protein that forms a molten globule. (De Bernardez et al., (1998) *Biotechnol. Prog.* 14:47-54). The De Bernardez work described thiol concentration in terms of a model that considered only the kinetics of a one-way reaction model. However, most complex proteins are governed by reversible thermodynamic equilibria that are not as easily described (see, e.g., Darby et al., (1995) *J. Mol. Biol.* 249:463-477). More complex behavior is expected in the case of large multi-chain proteins containing many disulfide bonds, such as antibodies, peptibodies and other Fc fusion proteins. Until the present disclosure, specific relationships had not been provided for thiol buffer strength, thiol-pair ratio chemistry, and protein concentration with respect to complex proteins that related to the efficiency of protein production. Consequently, the ability to refold proteins in a highly concentrated volume has largely been an inefficient or unachievable goal, leading to bottlenecks in protein production, particularly on the industrial scale.

Prior to the present disclosure a specific controlled investigation of the independent effects of thiol-pair ratio and thiol-pair buffer strength had not been disclosed for complex proteins. As described herein, by controlling the thiol-pair buffer strength, in conjunction with thiol-pair ratio and protein concentration, the efficiency of protein folding operations can be optimized and enhanced and the refolding of proteins at high concentrations, for example 2 g/L or greater, can be achieved.

Thus, in one aspect, the present disclosure relates to the identification and control of redox thiol-pair ratio chemistries that facilitate protein refolding at high protein concentrations, such as concentrations higher than 2.0 g/L. The method can be applied to any type of protein, including simple proteins and complex proteins (e.g., proteins comprising 2-23 disulfide bonds or greater than 250 amino acid residues, or having a MW of greater than 20,000 daltons), including proteins comprising a Fc domain, such as antibodies, peptibodies and other Fc fusion proteins, and can be performed on a laboratory scale (typically milliliter or liter scale), a pilot plant scale (typically hundreds of liters) or an industrial scale (typically thousands of liters). Examples of complex molecules known as peptibodies, and other Fc fusions, are described in U.S. Pat. No. 6,660,843, U.S. Pat. No. 7,138,370 and U.S. Pat. No. 7,511,012.

As described herein, the relationship between thiol buffer strength and redox thiol-pair ratio has been investigated and optimized in order to provide a reproducible method of refolding proteins at concentrations of 2.0 g/L and higher on a variety of scales. A mathematical formula was deduced to allow the precise calculation of the ratios and strengths of individual redox couple components to achieve matrices of buffer thiol-pair ratio and buffer thiol strength. Once this relationship was established, it was possible to systematically demonstrate that thiol buffer strength and the thiol-pair ratio interact to define the distribution of resulting product-related species in a refolding reaction.

The buffer thiol-pair ratio is, however, only one component in determining the total system thiol-pair ratio in the total reaction. Since the cysteine residues in the unfolded protein are reactants as well, the buffer thiol strength needs

to vary in proportion with increases in protein concentration to achieve the optimal system thiol-pair ratio. Thus, in addition to demonstrating that buffer thiol strength interacts with the thiol-pair ratio, it has also been shown that the buffer thiol strength relates to the protein concentration in the total reaction as well. Optimization of the buffer thiol strength and the system thiol pair ratio can be tailored to a particular protein, such as a complex protein, to minimize cysteine mispairing yet still facilitate a refold at a high concentration.

I. Definitions

As used herein, the terms "a" and "an" mean one or more unless specifically indicated otherwise.

As used herein, the term "non-mammalian expression system" means a system for expressing proteins in cells derived from an organism other than a mammal, including but not limited to, prokaryotes, including bacteria such as *E. coli*, and yeast. Often a non-mammalian expression system is employed to express a recombinant protein of interest, while in other instances a protein of interest is an endogenous protein that is expressed by a non-mammalian cell. For purposes of the present disclosure, regardless of whether a protein of interest is endogenous or recombinant, if the protein is expressed in a non-mammalian cell then that cell is a "non-mammalian expression system." Similarly, a "non-mammalian cell" is a cell derived from an organism other than a mammal, examples of which include bacteria or yeast.

As used herein, the term "denaturant" means any compound having the ability to remove some or all of a protein's secondary and tertiary structure when placed in contact with the protein. The term denaturant refers to particular chemical compounds that affect denaturation, as well as solutions comprising a particular compound that affect denaturation. Examples of denaturants that can be employed in the disclosed method include, but are not limited to urea, guanidinium salts, dimethyl urea, methylurea, ethylurea and combinations thereof.

As used herein, the term "aggregation suppressor" means any compound having the ability to disrupt and decrease or eliminate interactions between two or more proteins. Examples of aggregation suppressors can include, but are not limited to, amino acids such as arginine, proline, and glycine; polyols and sugars such as glycerol, sorbitol, sucrose, and trehalose; surfactants such as, polysorbate-20, CHAPS, Triton X-100, and dodecyl maltoside; and combinations thereof.

As used herein, the term "protein stabilizer" means any compound having the ability to change a protein's reaction equilibrium state, such that the native state of the protein is improved or favored. Examples of protein stabilizers can include, but are not limited to, sugars and polyhedric alcohols such as glycerol or sorbitol; polymers such as polyethylene glycol (PEG) and α-cyclodextrin; amino acids salts such as arginine, proline, and glycine; osmolytes and certain Hoffmeister salts such as Tris, sodium sulfate and potassium sulfate; and combinations thereof.

As used herein, the terms "Fc" and "Fe region" are used interchangeably and mean a fragment of an antibody that comprises human or non-human (e.g., murine) C_{H2} and C_{H3} immunoglobulin domains, or which comprises two contiguous regions which are at least 90% identical to human or non-human C_{H2} and C_{H3} immunoglobulin domains. An Fc can but need not have the ability to interact with an Fc receptor. See, e.g., Hasemann & Capra, "Immunoglobulins: Structure and Function," in William E. Paul, ed., *Fundamental Immunology*, Second Edition, 209, 210-218 (1989), which is incorporated by reference herein in its entirety.

As used herein, the terms "protein" and "polypeptide" are used interchangeably and mean any chain of at least five naturally or non-naturally occurring amino acids linked by peptide bonds.

As used herein, the terms "isolated" and "purify" are used interchangeably and mean to reduce by 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95%, or more, the amount of heterogenous elements, for example biological macromolecules such as proteins or DNA, that may be present in a sample comprising a protein of interest. The presence of heterogenous proteins can be assayed by any appropriate method including High-performance Liquid Chromatography (HPLC), gel electrophoresis and staining and/or ELISA assay. The presence of DNA and other nucleic acids can be assayed by any appropriate method including gel electrophoresis and staining and/or assays employing polymerase chain reaction.

As used herein, the term "complex molecule" means any protein that is (a) larger than 20,000 MW, or comprises greater than 250 amino acid residues, and (b) comprises two or more disulfide bonds in its native form. A complex molecule can, but need not, form multimers. Examples of complex molecules include but are not limited to, antibodies, peptibodies and other chimeric molecules comprising an Fc domain and other large proteins. Examples of complex molecules known as peptibodies, and other Fc fusions, are described in U.S. Pat. No. 6,660,843, U.S. Pat. No. 7,138,370 and U.S. Pat. No. 7,511,012.

As used herein, the term "peptibody" refers to a polypeptide comprising one or more bioactive peptides joined together, optionally via linkers, with an Fc domain. See U.S. Pat. No. 6,660,843, U.S. Pat. No. 7,138,370 and U.S. Pat. No. 7,511,012 for examples of peptibodies.

As used herein, the term "refolding" means a process of reintroducing secondary and tertiary structure to a protein that has had some or all of its native secondary or tertiary structure removed, either in vitro or in vivo, e.g., as a result of expression conditions or intentional denaturation and/or reduction. Thus, a refolded protein is a protein that has had some or all of its native secondary or tertiary structure reintroduced.

As used herein, the term "buffer thiol-pair ratio" is defined by the relationship of the reduced and oxidized redox species used in the refold buffer as defined in Equation 1:

Definition of Buffer Thiol-Pair Ratio (*TPR*) Equation 1

$$\text{Buffer } TPR = \frac{[\text{reductant}]^2}{[\text{oxidant}]} = \frac{[\text{cysteine}]^2}{[\text{cystamine}]}.$$

As used herein, the terms "Buffer Thiol Strength", "Thiol-Pair Buffer Strength", and "Thiol-pair Strength" are used interchangeably and are defined in Equation 2, namely as the total mono-equivalent thiol concentration, wherein the total concentration is the sum of the reduced species and twice the concentration of the oxidized species.

Definition of Buffer Thiol-Pair Buffer Strength/Thiol Buffer Strength (BS)

$$\text{Thiol-Pair Buffer Strength} = 2[\text{oxidant}] + [\text{reductant}] = 2[\text{cystamine}] + [\text{cysteine}] \quad \text{Equation 2.}$$

The relationship between the thiol-pair ratio and thiol-pair buffer strength is described in equations 3 and 4.

Calculation of the Reduced Redox Species with Regard to a Defined Redox Buffer Strength (*BS*) and buffer Redox Potential — Equation 3

$$\text{Concentration of Reduced Redox Component} = \frac{\left(\sqrt{bufferTPR^2 + 8 * bufferTPR * BS}\right) - bufferTPR}{4}.$$

Calculation of the Oxidized Redox Species with Regard to a Defined Redox Buffer Strength (*BS*) and Buffer Redox Potential — Equation 4

$$\text{Concentration of Oxidized Redox Component} = \frac{(\text{Concentration of Reduced Redox Component})^2}{TPR}.$$

As used herein, the term "redox component" means any thiol-reactive chemical or solution comprising such a chemical that facilitates a reversible thiol exchange with another thiol or the cysteine residues of a protein. Examples of such compounds include, but are not limited to, glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine, beta-mercaptoethanol and combinations thereof.

As used herein, the term "solubilization" means a process in which salts, ions, denaturants, detergents, reductants and/or other organic molecules are added to a solution comprising a protein of interest, thereby removing some or all of a protein's secondary and/or tertiary structure and dissolving the protein into the solvent. This process can include the use of elevated temperatures, typically 10-50° C., but more typically 15-25° C., and/or alkaline pH, such as pH 7-12. Solubilization can also be accomplished by the addition of acids, such as 70% formic acid (see, e.g., Cowley & Mackin (1997) *FEBS Lett* 402:124-130).

A "solubilized protein" is a protein in which some or all of the protein's secondary and/or tertiary structure has been removed.

A "solublization pool" is a volume of solution comprising a solubilized protein of interest as well as the salts, ions, denaturants, detergents, reductants and/or other organic molecules selected to solubilize the protein.

As used herein, the term "non-aerobic condition" means any reaction or incubation condition that is performed without the intentional aeration of the mixture by mechanical or chemical means. Under non-aerobic conditions oxygen can be present, as long as it is naturally present and was not introduced into the system with the intention of adding oxygen to the system. Non-aerobic conditions can be achieved by, for example, limiting oxygen transfer to a reaction solution by limiting headspace pressure, the absence of, or limited exposure to, air or oxygen contained in the holding vessel, air or oxygen overlay, the lack of special accommodations to account for mass transfer during process scaling, or the absence of gas sparging or mixing to encourage the presence of oxygen in the reaction system. Non-aerobic conditions can also be achieved by intentionally limiting or removing oxygen from the system via chemical treatment, headspace overlays or pressurization with inert gases or vacuums, or by sparging with gases such as argon or nitrogen, results in the reduction of oxygen concentration in the reaction mixture.

As used herein, the terms "non-native" and "non-native form" are used interchangeably and when used in the context of a protein of interest, such as a protein comprising a Fc domain, mean that the protein lacks at least one formed structure attribute found in a form of the protein that is biologically active in an appropriate in vivo or in vitro assay designed to assess the protein's biological activity. Examples of structural features that can be lacking in a non-native form of a protein can include, but are not limited to, a disulfide bond, quaternary structure, disrupted secondary or tertiary structure or a state that makes the protein biologically inactive in an appropriate assay. A protein in a non-native form can but need not form aggregates.

As used herein, the term "non-native limited solubility form" when used in the context of a protein of interest, such as a protein comprising a Fc domain, means any form or state in which the protein lacks at least one formed structural feature found in a form of the protein that (a) is biologically active in an appropriate in vivo or in vitro assay designed to assess the protein's biological activity and/or (b) forms aggregates that require treatment, such as chemical treatment, to become soluble. The term specifically includes proteins existing in inclusion bodies, such as those sometimes found when a recombinant protein is expressed in a non-mammalian expression system.

II. Theory

Refolding microbial-derived molecules present in a pool at concentrations of 2.0 g/L or higher is advantageous for a variety of reasons, primarily because of the associated reduction in reaction volumes and increases in process throughput. From a process scaling standpoint, it is advantageous to refold under conditions that do not require aerobic conditions; such conditions can be achieved, for example, by constant or intermittent sparging, the implementation of air or oxygen headspace overlays, by pressurizing the headspace, or by employing high efficiency mixing. Since the oxygen concentration in the system is related to mass transfer, the scaling of the refold reaction becomes considerably more difficult as factors such as tank geometry, volume, and mixing change. Furthermore, oxygen may not be a direct reactant in the formation of disulfide bonds in the protein, making a direct link to the mass transfer coefficient unlikely. This further complicates scaling of the reaction. Therefore, non-aerobic, chemically controlled redox systems are preferred for refolding proteins. Examples of such conditions are provided herein.

The optimal refold chemistry for a given protein represents a careful balance that maximizes the folded/oxidized state while minimizing undesirable product species, such as aggregates, unformed disulfide bridges (e.g., reduced cysteine pairs), incorrect disulfide pairings (which can lead to misfolds), oxidized amino acid residues, deamidated amino acid residues, incorrect secondary structure, and product-related adducts (e.g., cysteine or cysteamine adducts). One factor that is important in achieving this balance is the redox-state of the refold system. The redox-state is affected by many factors, including, but not limited to, the number of cysteine residues contained in the protein, the ratio and concentration of the redox couple chemicals in the refold solution (e.g., cysteine, cystine, cystamine, cysteamine, glutathione-reduced and glutathione-oxidized), the concentration of reductant carried over from the solubilization buffer (e.g., DTT, glutathione and beta-mercaptoethanol), the level of heavy metals in the mixture, and the concentration of oxygen in the solution.

Thiol-pair ratio and thiol-pair buffer strength are defined in Equations 1 and 2, infra, using cysteine and cystamine as

an example reductant and oxidant, respectively. These quantities, coupled with protein concentration and reductant carry-over from the solubilization, can be factors in achieving a balance between the thiol-pair ratio and the thiol-pair buffer strength.

Turning to FIGS. **1***a*-**1***f*, these figures depict the effect of thiol-pair ratio and thiol buffer strength on the distribution of product-related species, as visualized by reversed phase-HPLC analysis, for a complex dimeric protein. In FIGS. **1***a*-**1***f*, the dotted lines represent protein species with oxidized amino acid residues, single chain species, and stable mixed disulfide intermediates, the dashed lines represent mis-paired or incorrectly formed disulfide protein species and protein species with partially unformed disulfide linkages. The solid lines represent properly folded protein species. FIGS. **1***a*-**1***f* demonstrate that at a constant 6 g/L protein concentration, as the thiol-pair buffer strength is increased, the thiol-pair ratio required to achieve a comparable species distribution must also increase. For example, as shown in FIGS. **1***a*-**1***f*, if the buffer strength is increased to 10 mM, from 5 mM, the balanced thiol-pair ratio would be about 2-fold higher, to achieve a comparable species distribution. This is largely due to increased buffering of the reductant carried over from the solubilization, on the total system thiol-pair ratio. At lower redox buffer strengths, the overall system becomes much more difficult to control. The protein concentration and number of cysteines contained in the protein sequence also relate to the minimum required thiol-pair buffer strength required to control the system. Below a certain point, which will vary from protein to protein, the protein thiol concentration can overwhelm the redox couple chemistry and lead to irreproducible results.

In the results depicted in FIGS. **1***a*-**1***f*, when the thiol-pair ratio of the refolding solution is intentionally set to be more reducing, the resultant product distribution shifts to produce more of the reduced product species (dashed lines). When the Thiol-Pair Ratio of the refolding solution is intentionally set to be lower, or more oxidizing, the resultant product distribution shifts to produce more oxidized residues, single chain forms, and stable mixed disulfide intermediate species (dotted lines). The ability to select an optimal Thiol-Pair Ratio and Thiol-pair Buffer Strength allows for the optimization of the yield of a desired folded protein form. This optimized yield can be achieved by maximizing the mass or yield of desired folded protein species in the refolding pool or by purposefully shifting the resultant undesired product-related species to a form that is most readily removed in the subsequent purification steps and thusly leads to an overall benefit to process yield or purity.

Optimization of the redox component Thiol-pair Ratios and Thiol-pair Buffer Strengths can be performed for each protein. A matrix or series of multifactorial matrices can be evaluated to optimize the refolding reaction for conditions that optimize yield and distributions of desired species. An optimization screen can be set up to systematically evaluate redox chemistries, Thiol-pair ratios, Thiol-pair Buffer Strengths, incubation times, protein concentration and pH in a full or partial factorial matrix, with each component varied over a range of at least three concentration or pH levels with all other parameters kept constant. The completed reactions can be evaluated by RP-HPLC and SE-HPLC analysis for yield and product quality using standard multivariate statistical tools.

III. Method of Refolding a Protein Expressed in a Non-Mammalian Expression System and Present in a Volume at a Concentration of 2.0 g/L or Greater

The disclosed refold method is particularly useful for refolding proteins expressed in non-mammalian expression systems. As noted herein, non-mammalian cells can be engineered to produce recombinant proteins that are expressed intracellularly in either a soluble or a completely insoluble or non-native limited solubility form. Often the cells will deposit the recombinant proteins into large insoluble or limited solubility aggregates called inclusion bodies. However, certain cell growth conditions (e.g., temperature or pH) can be modified to drive the cells to produce a recombinant protein in the form of intracellular, soluble monomers. As an alternative to producing proteins in insoluble inclusion bodies, proteins can be expressed as soluble proteins, including proteins comprising an Fc region, which can be captured directly from cell lysate by affinity chromatography. Capturing directly from lysate allows for the refolding of relatively pure protein and avoids the very intensive harvesting and separation process that is required in inclusion body processes. The refolding method, however, is not limited to samples that have been affinity purified and can be applied to any sample comprising a protein that was expressed in a non-mammalian expression system, such as a protein found in a volume of cell lysate (i.e., a protein that has not been purified in any way).

In one aspect, the present disclosure relates to a method of refolding a protein expressed in a non-mammalian expression system in a soluble form and present in a volume at a concentration of 2.0 g/L or greater, such as a protein that has been purified by affinity chromatography from the cell lysate of non-mammalian cells in which the protein was expressed. Although the volume can be derived from any stage of a protein purification process, in one example the volume is an affinity chromatography elution pool (e.g., a Protein A elution pool). In another example, the volume is situated in a process stream. The method is not confined to Fc-containing proteins, however, and can be applied to any kind of peptide or protein that is expressed in a soluble form and captured from non-mammalian-derived cell lysate. The isolated soluble protein is often released from non-mammalian cells in a reduced form and therefore can be prepared for refolding by addition of a denaturant, such as a chaotrope. Further combination with protein stabilizers, aggregation suppressors and redox components, at an optimized Thiol-pair ration and Thiol-pair Buffer Strength, allows for refolding at concentrations of 1-40 g/L, for example at concentrations of 10-20 g/L.

In one particular embodiment of the method, a protein is expressed in a non-mammalian expression system, and is released from the expressing cell by high pressure lysis. The protein is then captured from the lysate by Protein A affinity chromatography and is present in a volume at a concentration of 10 g/L or greater. The protein is then contacted with a refold buffer comprising a denaturant, an aggregation suppressor, a protein stabilizer and a redox component, wherein the redox component has a final thiol-pair ratio (as defined herein) having a range of 0.001 to 100, for example within a range of 0.05 to 50, 0.1 to 50, 0.25 to 50, 0.5 to 50, 0.75 to 40, 1.0 to 50 or 1.5 to 50, 2 to 50, 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50 or 40 to 50 and a Thiol-pair buffer strength (as defined herein) equal to or greater than 2 mM, for example greater than or equal to 2.25 mM, 2.5, 2.75 mM, 3 mM, 5 mM, 7.5 mM, 10 mM, or 15 mM, wherein the thiol-pair buffer strength is effectively bounded at a maximum of 100 mM. Restated, in terms of ranges, the thiol buffer strength is between 2 and 20 mM, for example between 2.25 mM and 20 mM, 2.5 mM and 20 mM, 2.75 mM and 20 mM, 3 mM and 20 mM, 5 mM and 20 mM, 7.5 mM and 20 mM, 10 mM and 20 mM, or 15 mM and 20 mM.

In another aspect, the present disclosure relates to a method of refolding a protein expressed in a non-mammalian expression system in an insoluble or limited-solubility form, such as in the form of inclusion bodies. When the protein is disposed in inclusion bodies, the inclusion bodies can be harvested from lysed cells, washed, concentrated and refolded.

Optimization of the refold buffer can be performed for each protein and each final protein concentration level using the novel method provided herein. As shown in the Examples, good results can be obtained when refolding a protein comprising an Fc region when the refold buffer contains a denaturant (e.g., urea or other chaotrope, organic solvent or strong detergent), aggregation suppressors (e.g., a mild detergent, arginine or low concentrations of PEG), protein stabilizers (e.g., glycerol, sucrose or other osmolyte, salts) and redox components (e.g., cysteine, cystamine, glutathione). The optimal thiol-pair ratio and redox buffer strength can be determined using an experimental matrix of thiol-pair ratio (which can have a range of 0.001 to 100, for example within a range of 0.05 to 50, 0.1 to 50, 0.25 to 50, 0.5 to 50, 0.75 to 40, 1.0 to 50 or 1.5 to 50, 2 to 50, 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50 or 40 to 50) versus thiol-pair buffer strength (which can be greater than 2 mM, for example greater than or equal to 2.25 mM, 2.5, 2.75 mM, 3 mM, 5 mM, 7.5 mM, 10 mM, or 15 mM, wherein the thiol-pair buffer strength is effectively bounded at a maximum of 100 mM. Restated, in terms of ranges, the thiol buffer strength is between 2 and 20 mM, for example between 2.25 mM and 20 mM, 2.5 mM and 20 mM, 2.75 mM and 20 mM, 3 mM and 20 mM, 5 mM and 20 mM, 7.5 mM and 20 mM, 10 mM and 20 mM, or 15 mM and 20 mM, depending on the protein concentration and the concentration of reductant used to solubilize the inclusion bodies). Conditions can be optimized using the novel methods described in Example 2.

In one particular embodiment of the method, a protein is expressed in a non-mammalian expression system and is present in a volume at a concentration of 2.0 g/L or greater. The protein is contacted with a refold buffer comprising a denaturant, an aggregation suppressor, a protein stabilizer and a redox component, wherein the redox component has a final thiol-pair ratio (as defined herein) having a range of 0.001 to 100, for example within a range of 0.05 to 50, 0.1 to 50, 0.25 to 50, 0.5 to 50, 0.75 to 40, 1.0 to 50 or 1.5 to 50, 2 to 50, 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50 or 40 to 50, and a Thiol-pair buffer strength (as defined herein) equal to or greater than 2 mM, for example greater than or equal to 2.25 mM, 2.5 mM, 2.75 mM, 3 mM, 5 mM, 7.5 mM, 10 mM, or 15 mM, wherein the thiol-pair buffer strength is effectively bounded at a maximum of 100 mM. Restated, in terms of ranges, the thiol buffer strength is between 2 and 20 mM, for example between 2.25 mM and 20 mM, 2.5 mM and 20 mM, 2.75 mM and 20 mM, 3 mM and 20 mM, 5 mM and 20 mM, 7.5 mM and 20 mM, 10 mM and 20 mM, or 15 mM and 20 mM, to form a mixture. A wide range of denaturant types may be employed in the refold buffer. Examples of some common denaturants that can be employed in the refold buffer include urea, guanidinium, dimethyl urea, methylurea, or ethylurea. The specific concentration of the denaturant can be determined by routine optimization, as described herein.

A wide range of protein stabilizers or aggregation suppressors can be employed in the refold buffer. Examples of some common aggregation suppressors that can be useful in the refold buffer include arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate, other osmolytes, or similar compounds. The specific concentration of the aggregation suppressor can be determined by routine optimization, as described herein.

A redox component of the refold buffer can be of any composition, with the caveat that the redox component has a final thiol-pair ratio in a range of 0.001 to 100, for example within a range of 0.05 to 50, 0.1 to 50, 0.25 to 50, 0.5 to 50, 0.75 to 40, 1.0 to 50 or 1.5 to 50, 2 to 50, 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50 or 40 to 50, and a Thiol-pair buffer strength of greater than or equal to 2 mM, for example greater than or equal to 2.25 mM, 2.5, 2.75 mM, 3 mM, 5 mM, 7.5 mM, 10 mM, or 15 mM, wherein the thiol-pair buffer strength is effectively bounded at a maximum of 100 mM. Restated, in terms of ranges, the thiol buffer strength is between 2 and 20 mM, for example between 2.25 mM and 20 mM, 2.5 mM and 20 mM, 2.75 mM and 20 mM, 3 mM and 20 mM, 5 mM and 20 mM, 7.5 mM and 20 mM, 10 mM and 20 mM, or 15 mM and 20 mM. Methods of identifying a suitable redox component, i.e., determining appropriate thiol-pair ratios and redox buffer strengths, are known and/or are provided herein. Examples of specific thiol pairs that can form the redox component can include one or more of reduced glutathione, oxidized glutathione, cysteine, cystine, cysteamine, cystamine, and beta-mercaptoethanol. Thus, a thiol-pair can comprise, for example, reduced glutathione and oxidized glutathione. Another example of a thiol pair is cysteine and cystamine. The redox component can be optimized as described herein.

After the protein has been contacted with a redox component having the recited thiol pair ratio and redox buffer strength to form a refold mixture, the refold mixture is then incubated for a desired period of time. The incubation can be performed under non-aerobic conditions, as defined herein. Non-aerobic conditions need not be completely free of oxygen, only that no additional oxygen other than that present in the initial system is purposefully introduced. The incubation period is variable and is selected such that a stable refold mixture can be achieved with the desired analytical properties. An incubation period can be, for example, 1 hour, 4 hours, 12 hours, 24 hours, 48 hours, 72 hours, or longer.

Due to the sensitivity of high concentration refolds to the level of oxygen present in the system and the tendency for oxygen mass transfer to be greater at small-scale, a methodology and/or apparatus can be developed to control the oxygen levels and maintain non-aerobic conditions for the incubation step. In one embodiment, the procedure can comprise the preparation, dispensing and mixing of all refold components under a blanket of inert gas, such as nitrogen or argon, to avoid entraining levels of oxygen into the reaction. This approach is particularly helpful in identifying an acceptable thiol-pair ratio. In another embodiment useful at scales of 15 liters or less, the headspace of the refold reactor containing the protein and refold buffer can be purged with an inert gas or a mixture of inert gas and air or oxygen, and the reaction vessel sealed and mixed at a low rotational speed for the duration of the incubation time.

Following the incubation, the protein is isolated from the refold mixture. The isolation can be achieved using any known protein purification method. If the protein comprises a Fc domain, for example, a Protein A column provides an appropriate method of separation of the protein from the refold excipients. In other embodiments, various column chromatography strategies can be employed and will depend on the nature of the protein being isolated. Examples include

HIC, AEX, CEX and SEC chromatography. Non-chromatographic separations can also be considered, such as precipitation with a salt, acid or with a polymer such as PEG (see, e.g., US 20080214795). Another alternative method for isolating the protein from the refold components can include dialysis or diafiltration with a tangential-flow filtration system.

In another exemplary refolding operation, inclusion bodies obtained from a non-mammalian expression system are solubilized in the range of 10 to 100 grams of protein per liter and more typically from 20-40 g/L for approximately 10-300 min. The solubilized inclusion bodies are then diluted to achieve reduction of the denaturants and reductants in the solution to a level that allows the protein to refold. The dilution results in protein concentration in the range of 1 to 15 g/L in a refold buffer containing urea, glycerol or sucrose, arginine, and the redox pair (e.g., cysteine and cystamine). In one embodiment the final composition is 1-4 M urea, 5-40% glycerol or sucrose, 25-500 mM arginine, 0.1-10 mM cysteine and 0.1-10 mM cystamine. The solution is then mixed during incubation over a time that can span from 1 hour to 4 days.

As noted herein, the disclosed method is particularly useful for proteins expressed in bacterial expression systems, and more particularly in bacterial systems in which the protein is expressed in the form of inclusion bodies within the bacterial cell. The protein can be a complex protein, i.e., a protein that (a) is larger than 20,000 MW, or comprises greater than 250 amino acid residues, and (b) comprises two or more disulfide bonds in its native form. When the protein is expressed in an inclusion body it is likely that any disulfide bond found in the protein's native form will be misformed or not formed at all. The disclosed method is applicable to these and other forms of a protein of interest. Specific examples of proteins that can be considered for refolding using the disclosed methods include antibodies, which are traditionally very difficult to refold at high concentrations using typical refold methods due to their relatively large size and number of disulfide bonds. The method can also be employed to refold other Fc-containing molecules such as peptibodies, and more generally to refold any fusion protein comprising an Fc domain fused to another protein.

Another aspect of the disclosed method is its scalability, which allows the method to be practiced on any scale, from bench scale to industrial or commercial scale. Indeed, the disclosed method will find particular application at the commercial scale, where it can be employed to efficiently refold large quantities of protein.

The present disclosure will now be illustrated by reference to the following examples, which set forth certain embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as restricting the invention in any way.

EXAMPLES

The Examples presented herein demonstrate that thiol-pair ratio and redox buffer strength is a significant consideration in achieving an efficient refolding reaction that is insensitive to environmental influences and aeration. This insensitivity is a consideration for the ease of scaling and on an industrial or commercial scale, the transfer of the process from plant to plant.

The Examples also demonstrate that at typical refolding reaction concentrations (0.01-2.0 g/L); the sensitivity to external aeration is relatively muted. However, at concentrations of about 2 g/L and above, the sensitivity of the refold reaction to the thiol-pair ratio and redox buffer strength is increased and nearly all of the chemical components, especially the redox components, may need to be adjusted to accommodate for changes in the protein concentration in the reaction.

Example 1

Expression of Recombinant Protein

In one experiment, recombinant proteins comprising an Fc moiety were expressed in a non-mammalian expression system, namely *E. coli*, and driven to form cytoplasmic deposits in the form of inclusion bodies. For each protein refolded the following procedure was followed.

After the completion of the expression phase, the cell broth was centrifuged and the liquid fraction removed, leaving the cells as a paste. The cells were resuspended in water to approximately 60% of the original volume. The cells were then lysed by means of three passes through a high pressure homogenizer. After the cells were lysed, the lysate was centrifuged in a disc-stack centrifuge to collect the protein in the solid fraction, which was expressed in a limited solubility non-native form, namely as inclusion bodies. The protein slurry was washed multiple times by repeatedly resuspending the captured solids slurry in water to between 50% and 80% of the original fermentation broth volume, mixing, and centrifugation to collect the protein in the solid fraction. The final washed inclusion bodies were captured and stored frozen.

Example 2

Identification of Refold Conditions/Redox Components

Multiple complex, microbial-derived proteins were evaluated. Each protein was solubilized in an appropriate level of guanidine and/or urea, typically at levels the equivalent of 4-6 M guanidine or 4-9 M urea, or combinations of both denaturants, which fully denatured the protein. The protein was reduced with DTT, 5-20 mM, at pH 8.5, and incubated at room temperature for approximately 1 hour.

Identification of the refold buffer was performed for each protein. A multifactorial matrix or a series of multifactorial matrices were evaluated to identify the refolding reaction for conditions that optimize yield and minimize aggregate formation. An identification screen was set up to systematically evaluate urea, arginine, glycerol and pH in a full factorial matrix, with each component varied over a range of at least three concentration or pH levels with all other parameters kept constant. The completed reactions were evaluated by RP-HPLC and SE-HPLC analysis for yield and product quality using standard multivariate statistical tools. A subset of the conditions having the desired behavior was then further evaluated in subsequent screens that evaluated a range of pH, thiol-pair ratio, thiol-pair buffer strength, and potentially further excipient levels in a factorial screen. Secondary interactions were also evaluated using standard multivariate statistical tools.

Best results, as determined by reversed-phase and size exclusion HPLC analysis, were observed using a refold buffer containing a denaturant (e.g., urea, dimethyl urea or other chaotrope at non-denaturing levels at levels between 1 and 4 M), an aggregation suppressor (e.g., arginine at levels between 5 and 500 mM), a protein stabilizer (e.g., glycerol

or sucrose at levels between 5 and 40% w/v) and a redox component (e.g., cysteine or cystamine). The thiol-pair ratio and redox buffer strength were determined using an experimental matrix of thiol-pair ratio (0.1 to 100, more typically 1 to 25) versus buffer strength (typically 2 mM to 20 mM, depending on the protein concentration, the number of cysteine residues in the protein, and the concentration of reductant used to solubilize the inclusion bodies).

Individual reactions were formed with varying levels of cysteine and cystamine that would allow for a controlled matrix of thiol-pair ratio at various thiol-pair buffer strengths. The relationships were calculated using Equations 3 and 4. Each condition was screened under both aerobic and non-aerobic conditions, utilizing the techniques described herein. Optimum conditions were selected to meet a stable balance of yield, desired distribution of folding species, insensitivity to environmental oxidants (e.g., air), and insensitivity to normal variation in DTT carry-over from the solubilization step.

Example 3

High Concentration Refolding of Non-Native Soluble Protein Form Captured from Cell Lysate

In one experiment, a recombinant protein comprising a plurality of polypeptides joined to an Fc moiety was expressed in *E. coli* as an intracellular soluble peptide chain, lysed from harvested and washed cells, isolated from the lysate by affinity chromatography, and then refolded at a concentration of approximately 12 g/L, as described herein.

After the completion of the expression phase, an aliquot of whole fermentation broth was centrifuged and the liquid fraction removed, leaving the cells as a paste. The cells were resuspended in water to approximately 60% of the original volume. The cells were then lysed by means of three passes through a high pressure homogenizer. After the cells were lysed, the lysate pool was mixed in the presence of air for 8-72 hours to allow for dimerization of the peptide chains. Following the dimerization process, the peptide chain of interest was isolated from the lysate pool using a Protein A affinity chromatography column. The Protein A column elution pool was mixed at a ratio of 8 parts Protein A elution material to 2 parts of a refold buffer containing urea (10 M), arginine-HCl (2.5 M), Tris at pH 8.5 (1050 mM), and cysteine (10 mM, 5 mM, or 4 mM) and cystamine (4 mM). The diluted mixture was titrated to pH 8.5 and incubated at approximately 5° C. under nitrogen until a stable pool was achieved (~24 hours.) Yields of desired product of approximately 30-80% were obtained a depending on the redox condition evaluated.

In order to emulate the non-anaerobic conditions similar to those typically present in very large-scale protein production processes several steps were taken. When reaction volumes were less than approximately 15 L the refold vessel headspace was purged with nitrogen to limit the effect oxygen could have in the system. The vessel was then sealed and incubation began.

When reaction volumes were more than approximately 15 L but less than 500 L, the refold buffer was prepared and allowed to equilibrate at approximately 5° C. to achieve a stable oxygen level in the solution (typically 50% to 70% dissolved oxygen, relative to air saturation). Once the refold mixture was formed, the vessel headspace was purged with nitrogen to limit any additional effect oxygen could have in the system, the vessel was sealed and incubation period initiated.

Example 4

High Concentration Refolding from Inclusion Bodies

In one experiment, a recombinant protein comprising a biologically active peptide linked to the C-terminus of the Fc moiety of an IgG 1 molecule via a linker and having a molecular weight of about 57 kDa and comprising 8 disulfide bonds, was expressed in *E. coli* as inclusion bodies, harvested, washed, concentrated, solubilized, and refolded at a concentration of 6 g/L as described herein.

An aliquot of frozen concentrated inclusion bodies were thawed to room temperature and mixed with an appropriate amount of guanidine and/or urea to generate a denaturant level equivalent to 4-6 M guanidine, which fully denatures the protein. The protein was then reduced with DTT, at 5-20 mM, at pH 8.5, and incubated at room temperature for approximately 1 hour. After the inclusion bodies were dissolved, denatured and reduced, they were diluted into a refold buffer containing urea (1-5 M), arginine-HCl (5-500 mM), glycerol (10-30% w/v), and the identified levels of cysteine and cystamine as determined by the procedure described in Example 2. The final component concentrations are 4 M urea, 150 mM arginine HCl, 20.9% (w/v) glycerol, 2.03 mM cysteine, and 2.75 mM cystamine. The level of dilution was chosen to balance the dilution of the denaturants from the solubilization, maintain the thermodynamic stability of the molecule during refolding, and maintain the highest possible protein concentration in the refold mixture. The diluted mixture was titrated to an alkaline pH (between pH 8 and pH 10) and incubated at 5° C. under non-aerobic conditions until a stable pool was achieved (12-72 hours), as determined by relevant analytical measurements. The resulting process was demonstrated to show stable scalablity from 1 L-scale to 2000 L-scale (see FIG. **3**). Yields of desired product of approximately 27-35% were obtained at both scales. The distribution of product related impurities was also maintained within a tight variance (see FIG. **3**).

Oxygen mass transfer at small-scale is readily achieved and should be inhibited in order to emulate the relatively poorer mass transfer observed at large-scale, where the volume of refold solution is large relative to the volume of air and surface area present at the surface of a large-scale vessel. Thus, in order to emulate the non-anaerobic conditions similar to those typically present in very large-scale protein production processes several steps were taken. When reaction volumes were less than approximately 15 L the refold buffer was sparged with nitrogen to strip oxygen from the solution, the components were dispensed under a blanket of nitrogen and once the refold mixture was formed, the vessel headspace was purged with nitrogen to limit the effect oxygen could have in the system. The vessel was then sealed and incubation began.

When reaction volumes were more than approximately 15 L but less than 500 L, the refold buffer was prepared and allowed to equilibrate at approximately 5° C. to achieve a stable oxygen level in the solution (typically 50% to 70% dissolved oxygen, relative to air saturation). Once the refold mixture was formed, the vessel headspace was purged with nitrogen to limit any addition effect oxygen could have in the system, the vessel was sealed and the incubation period was initiated.

At scales greater than 500 L the refold buffer was prepared and allowed to equilibrate at approximately 5° C. to achieve a stable oxygen level in the solution (typically 50% to 70% dissolved oxygen, relative to air saturation). Once the refold mixture was formed, the vessel was sealed and the incubation period was initiated.

The protein concentration of the refold mixture was 6 g/L, which is a four-fold enhancement over the recovery of 1.5 g/L obtained using a method other than the method described in this Example. Overall annual process productivity, in one specific manufacturing facility, was calculated to be increased by >930% due to increased volumetric efficiency in the existing facility tanks.

Example 5

Effect of Thiol-Pair Oxidation State on Disulfide Pairings

FIGS. **1***a*-**1***f* demonstrate that as the thiol-pair ratio is forced to a more oxidizing state (lower thiol-pair ratio), a higher proportion of product species have oxidized amino acid residues and mixed disulfide forms. As the thiol-pair ratio is driven to a more reductive state (higher thiol-pair ratio), this results in lower levels of oxidized amino acid variant species and higher levels of product species with incorrect disulfide pairings or unformed disulfide bonds. As the overall thiol-pair buffer strength is modified, the corresponding optimal thiol-pair ratio is shifted. This effect is similar to how buffer strength modulates the sensitivity of pH to acid and base additions in a buffered solution.

An optimal balance of species was attainable. As shown in FIGS. **1***a*-**1***f*, there is a clear relationship between thiol-pair buffer strength and thiol-pair ratio that can be identified to maintain the optimal species balance and thus facilitate efficient refolding of low solubility proteins. The ability to control product variant species, such as incorrectly disulfide-bonded species and misfolded species, via modulation of the thiol-pair ratio and thiol-pair buffer strength, enables efficient, effective and reliable subsequent purification processes.

Example 6

Effect of Non-Aerobic Conditions on Refolding Efficiency

FIGS. **2** and **3** demonstrate that when the thiol-pair buffer strength is selected appropriately, taking into account the protein concentration and number of cysteine residues in the protein, the sensitivity to external influences, such as oxygen, is significantly reduced. This allows for a non-aerobic refolding condition that is significantly easier to transfer between scales and reactor configurations.

FIG. **2** compares the RP-HPLC analytical species distribution between a 15 L-scale refold and a 20 mL-scale refold under several environmental conditions. For Condition 1 (the trace labeled "1" in FIG. **2**), the solubilization chemicals and solutions were dispensed in air and the refold mixture was incubated in air. In Condition 2 solubilization chemicals and solutions were dispensed in air and incubated under nitrogen headspace. In Conditions 3-7 solubilization chemicals and solutions were dispensed under nitrogen overlay conditions and in conditions 3, 5, 6, and 7 solubilization chemicals and solutions were incubated under nitrogen. In Condition 7, the refold solution was also stripped of nitrogen prior to combination with the solubilization solution. In Condition 4 the solubilization chemicals and solutions were incubated under ambient air conditions.

The results shown in FIG. **2** demonstrate that the conditions under which the solubilization chemicals and solutions were dispensed or incubated in the presence of air (i.e., Conditions 1, 2, and 4) do not achieve results that are comparable to the larger-scale control. In Conditions 1, 2 and 4, increased formation of oxidized species (pre-peaks) are observed. The pre-peaks are indicated by arrows in the panels for Conditions 1, 2 and 4.

FIG. **3** compares the RP-HPLC analytical results of an identified condition, achieved as described in Example 2, at 1 L-scale and 2000 L-scale. In this figure, essentially no difference in the distribution of species is detectable. Taken together, FIGS. **2** and **3** demonstrate that when aeration is carefully controlled, the small-scale refold reactions are more predictive of those expected upon scale-up of the refold reaction, facilitating the implementation of large-scale protein refolding processes.

What is claimed is:

1. A method of refolding proteins expressed in a non-mammalian expression system, the method comprising:

contacting the proteins with a preparation that supports the renaturation of at least one of the proteins to a biologically active form, to form a refold mixture, the preparation comprising:

at least one ingredient selected from the group consisting of a denaturant, an aggregation suppressor and a protein stabilizer;

an amount of oxidant; and

an amount of reductant,

wherein the amounts of the oxidant and the reductant are related through a thiol-pair ratio and a thiol-pair buffer strength,

wherein the thiol-pair ratio is in the range of 0.001-100; and

wherein the thiol-pair buffer strength maintains the solubility of the preparation; and

incubating the refold mixture so that at least about 25% of the proteins are properly refolded.

2. The method of claim **1**, wherein the refold mixture has a protein concentration in a range of 1-40 g/L.

3. The method of claim **1**, wherein the refold mixture has a protein concentration of 2.0 g/L or greater.

4. The method of claim **1**, wherein the thiol-pair buffer strength is 2 mM or greater.

5. The method of claim **1**, wherein the thiol-pair buffer strength is increased proportionally to an increase in a total protein concentration in the refold mixture.

6. The method of claim **1**, wherein the thiol-pair buffer strength is decreased proportionally to a decrease in a total protein concentration in the refold mixture.

7. The method of claim **1**, wherein the at least one of the proteins is a complex protein.

8. The method of claim **1**, wherein the thiol-pair ratio is calculated according to the following equation:

$$\frac{[\text{the reductant}]^2}{[\text{the oxidant}]}.$$

9. The method of claim **1**, wherein the thiol-pair buffer strength is calculated according to the following equation:

$$2[\text{the oxidant}]+[\text{the reductant}].$$

10. A method of refolding proteins expressed in a non-mammalian expression system, the method comprising:
contacting the proteins with a preparation that supports the renaturation of at least one of the proteins to a biologically active form, to form a refold mixture, the preparation comprising:
at least one ingredient selected from the group consisting of a denaturant, an aggregation suppressor and a protein stabilizer;
an amount of oxidant; and
an amount of reductant,
wherein the amounts of the oxidant and the reductant are related through a thiol-pair ratio and a thiol-pair buffer strength,
wherein the thiol-pair ratio is in the range of 0.001-100; and
wherein the thiol-pair buffer strength maintains the solubility of the preparation; and
incubating the refold mixture so that about 30-80% of the proteins are properly refolded.

11. The method of claim 10, wherein the refold mixture has a protein concentration in a range of 1-40 g/L.

12. The method of claim 10, wherein the thiol-pair buffer strength is 2 mM or greater.

13. The method of claim 10, wherein the refold mixture has a protein concentration of 2.0 g/L or greater.

14. The method of claim 1, wherein:
the thiol-pair ratio is calculated according to the following equation:

$$\frac{[\text{the reductant}]^2}{[\text{the oxidant}]};$$

and
the thiol-pair buffer strength is calculated according to the following equation:

$$2[\text{the oxidant}]+[\text{the reductant}].$$

15. The method of claim 10, wherein:
the thiol-pair ratio is calculated according to the following equation:

$$\frac{[\text{the reductant}]^2}{[\text{the oxidant}]};$$

and
the thiol-pair buffer strength is calculated according to the following equation:

$$2[\text{the oxidant}]+[\text{the reductant}].$$

16. A method of refolding proteins expressed in a non-mammalian expression system, the method comprising:
preparing a solution comprising:
the proteins;
at least one ingredient selected from the group consisting of a denaturant, an aggregation suppressor and a protein stabilizer;
an amount of oxidant; and
an amount of reductant,
wherein the amounts of the oxidant and the reductant are related through a thiol-pair ratio and a thiol-pair buffer strength,
wherein the thiol-pair ratio is in the range of 0.001-100, and
wherein the thiol-pair buffer strength maintains the solubility of the solution; and
incubating the solution so that at least about 25% of the proteins are properly refolded.

17. The method of claim 16, wherein the solution has a protein concentration in a range of 1-40 g/L.

18. The method of claim 16, wherein the solution has a protein concentration of 2.0 g/L or greater.

19. The method of claim 16, wherein the thiol-pair buffer strength is 2 mM or greater.

20. The method of claim 16, wherein the thiol-pair buffer strength is increased proportionally to an increase in a total protein concentration in the solution.

21. The method of claim 16, wherein the thiol-pair buffer strength is decreased proportionally to a decrease in a total protein concentration in the solution.

22. The method of claim 16, wherein the at least one of the proteins is a complex protein.

23. The method of claim 16, wherein the thiol-pair ratio is calculated according to the following equation:

$$\frac{[\text{the reductant}]^2}{[\text{the oxidant}]}.$$

24. The method of claim 16, wherein the thiol-pair buffer strength is calculated according to the following equation:

$$2[\text{the oxidant}]+[\text{the reductant}].$$

25. The method of claim 16, wherein:
the thiol-pair ratio is calculated according to the following equation:

$$\frac{[\text{the reductant}]^2}{[\text{the oxidant}]};$$

and
the thiol-pair buffer strength is calculated according to the following equation:

$$2[\text{the oxidant}]+[\text{the reductant}].$$

26. A method of refolding proteins expressed in a non-mammalian expression system, the method comprising:
preparing a solution comprising:
the proteins;
at least one ingredient selected from the group consisting of a denaturant, an aggregation suppressor and a protein stabilizer;
an amount of oxidant; and
an amount of reductant,
wherein the amounts of the oxidant and the reductant are related through a thiol-pair ratio and a thiol-pair buffer strength,
wherein the thiol-pair ratio is in the range of 0.001-100, and
wherein the thiol-pair buffer strength maintains the solubility of the solution; and
incubating the solution so that about 30-80% of the proteins are properly refolded.

27. The method of claim 26, wherein the solution has a protein concentration in a range of 1-40 g/L.

28. The method of claim 26, wherein the solution has a protein concentration of 2.0 g/L or greater.

29. The method of claim 26, wherein the thiol-pair buffer strength is 2 mM or greater.

30. The method of claim **26**, wherein:

the thiol-pair ratio is calculated according to the following equation:

$$\frac{[\text{the reductant}]^2}{[\text{the oxidant}]};$$

and

the thiol-pair buffer strength is calculated according to the following equation:

$$2[\text{the oxidant}]+[\text{the reductant}].$$

* * * * *

Exhibit 5

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/599,336	01/16/2015	Joseph Edward SHULTZ	A-1441-US-DIV	3003

30174 7590 10/02/2015

AMGEN INC.
Law - Patent Operations, M/S 28-2-C
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EXAMINER	
GANGLE, BRIAN J	

ART UNIT	PAPER NUMBER
1645	

NOTIFICATION DATE	DELIVERY MODE
10/02/2015	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

pair_amgen@firsttofile.com
PatentOps@amgen.com

Office Action Summary	Application No. 14/599,336	Applicant(s) SHULTZ ET AL.	
	Examiner Brian J. Gangle	Art Unit 1645	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *9/2/2015*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-20* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-20* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *1/16/2015* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

The preliminary amendment filed on 9/2/2015 is acknowledged. Claim 20 is amended. Claims 21-29 are cancelled. Claims 1-20 are pending and are currently under examination.

Information Disclosure Statement

The information disclosure statement filed on 9/2/2015 has been considered. A signed copy is enclosed. The lined through documents were not considered because no copy of the reference has been provided.

Claim Objections

Claim 15 is objected to because of the following informalities: The claim contains the acronym DTT. While acronyms are permissible shorthand in the claims, the first recitation should include the full recitation followed by the acronym in parentheses. Appropriate correction is required.

Double Patenting

A rejection based on double patenting of the "same invention" type finds its support in the language of 35 U.S.C. 101 which states that "whoever invents or discovers any new and useful process... may obtain a patent therefor..." (Emphasis added). Thus, the term "same invention," in this context, means an invention drawn to identical subject matter. See *Miller v. Eagle Mfg. Co.*, 151 U.S. 186 (1894); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Ockert*, 245 F.2d 467, 114 USPQ 330 (CCPA 1957).

A statutory type (35 U.S.C. 101) double patenting rejection can be overcome by canceling or amending the claims that are directed to the same invention so they are no longer coextensive in scope. The filing of a terminal disclaimer cannot overcome a double patenting rejection based upon 35 U.S.C. 101.

Claim 5 is rejected under 35 U.S.C. 101 as claiming the same invention as that of claim 1 of prior U.S. Patent No. 8,940,878. This is a statutory double patenting rejection.

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection

is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 1-4 and 6-20 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-24 of U.S. Patent No. 8,952,138.

Although the conflicting claims are not identical, they are not patentably distinct from each other because both claim sets are drawn to purifying a protein expressed in a non-native soluble form. All of the limitations of the instant claims are found within the patented claims.

Claims 1-7 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-24 of U.S. Patent No. 8,952,138.

Although the conflicting claims are not identical, they are not patentably distinct from each other because both claim sets are drawn to purifying a protein expressed in a non-native soluble form. Though the copending claims do not address lysing of the cells, one would necessarily lyse the cells in order to accomplish the method. In addition, while the copending claims do recite a soluble protein, they do not specifically state that it is in a non-native form. However, as the point of the method is to refold the protein, the protein must necessarily not have been folded previously and would thus be in a non-native form.

Claims 1-7 and 9-20 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-24 of U.S. Patent No. 8,952,138 in view of Fischer *et al*. (Biotechnol. Bioengin., 41:3-13, 1993; IDS filed 5/13/2011).

Although the conflicting claims are not identical, they are not patentably distinct from each other because both claim sets are drawn to purifying a protein expressed in a non-native soluble form. Though the copending claims do not address lysing of the cells, one would necessarily lyse the cells in order to accomplish the method. In addition, while the copending claims do recite a soluble protein, they do not specifically state that it is in a non-native form. However, as the point of the method is to refold the protein, the protein must necessarily not have been folded previously and would thus be in a non-native form. The copending claims do not recite a step of solubilizing the expressed protein in a solubilization solution. However, this step is a necessary step in order to solubilize proteins that are in a non-native limited solubility form, such as an inclusion body. Fischer *et al*, state that inactive pellets are solubilized in guanidine or urea prior to being refolded (see page 3, column 2, paragraph 2).

Claims 1-7 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claim 1 of copending Application No. 14/611,037. Although the conflicting claims are not identical, they are not patentably distinct from each other because both claim sets are drawn to purifying a protein expressed in a non-native soluble form. Though the copending claims do not address lysing of the cells, one would necessarily lyse the cells in order to accomplish the method. In addition, while the copending claims do recite a soluble protein, they do not specifically state that it is in a non-native form. However, as the point of the method is to refold the protein, the protein must necessarily not have been folded previously and would thus be in a non-native form.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Claims 1-7 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 25-54 of copending Application No.

14/793,590. Although the conflicting claims are not identical, they are not patentably distinct from each other because both claim sets are drawn to purifying a protein expressed in a non-native soluble form. Though the copending claims do not address lysing of the cells, one would necessarily lyse the cells in order to accomplish the method. In addition, while the copending claims do recite a soluble protein, they do not specifically state that it is in a non-native form. However, as the point of the method is to refold the protein, the protein must necessarily not have been folded previously and would thus be in a non-native form.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claim 14 is rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 14 recites the limitation "the denaturant" in line 1. There is insufficient antecedent basis for this limitation in the claim. The parent claim recited two denaturants and it is not clear which one claim 14 is referring to.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1-4 and 6-7 are rejected under 35 U.S.C. 102(b) as being anticipated by Pavlinkova *et al.* (Nuclear Med. Biol., 26:27-34, 1999; IDS filed 9/2/2015).

The instant claims are drawn to methods of purifying proteins expressed in a non-native soluble form in a non-mammalian expression system comprising lysing the cells, contacting the lysate with a separation matrix, washing the matrix, and eluting the protein from the matrix.

Pavlinkova *et al.* disclose a method where an scFv antibody was expressed in *E. coli*, the cells were lysed, filtered, and applied to an ion exchange resin before being eluted (see page 28, column 1, paragraph 2). ScFv are non-native proteins in that they lack disulfide bonds found in native antibodies.

Claims 1-5 and 8 are rejected under 35 U.S.C. 102(b) as being anticipated by Ronnmark *et al.* (J. Immunologic. Meth., 261:199-211, 2002; IDS filed 9/2/2015).

The instant claims are drawn to methods of purifying proteins expressed in a non-native soluble form in a non-mammalian expression system comprising lysing the cells, contacting the lysate with a separation matrix, washing the matrix, and eluting the protein from the matrix.

Ronnmark *et al.* disclose a method of producing affibody-Fc chimera proteins (see page 200, final paragraph). The soluble proteins were expressed *E. coli* cells, which were then lysed by osmotic shock, followed by purification by protein A chromatography (which requires washing and elution of the proteins), and then refolded under reducing conditions (see section 2.4 and page 205, column 1).

Claims 9-15 and 17-20 are rejected under 35 U.S.C. 102(b) as being anticipated by Oliner *et al.* (US Patent 7,138,370, 2006; IDS filed 9/2/2015).

The instant claims are drawn to methods of purifying proteins expressed in a non-native limited solubility form in a non-mammalian expression system comprising expressing the protein in a non-mammalian cell, lysing the cell, solubilizing the protein in a solubilization solution, forming a refold solution, applying the refold solution to a separation matrix, washing the matrix, and eluting the protein.

Oliner *et al.* disclose a method of producing Fc-fusion proteins by expressing them in *E. coli* cells, lysing the cells (which contain the proteins in inclusion bodies), solubilizing the proteins with a solution containing guanidine and DTT, followed by forming a refolding solution by diluting the solubilization solution into a solution containing urea, arginine and cysteine (an

alternate solution also contained glycerol and cystamine), which was then filtered and loaded onto an ion exchange chromatography column, followed by elution to remove the protein (see column 76, lines 31-65). Oliner *et al.* also disclose that protein A affinity chromatography can be used to purify the proteins (see column 53, lines 32-45).

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* v. *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 9-19 are rejected under 35 U.S.C. 103(a) as being unpatentable over Oliner *et al.* (US Patent 7,138,370, 2006; IDS filed 9/2/2015) in view of Fischer *et al.* (Biotechnol. Bioengin., 41:3-13, 1993; IDS filed 9/2/2015).

The instant claims are drawn to methods of purifying proteins expressed in a non-native limited solubility form in a non-mammalian expression system comprising expressing the protein in a non-mammalian cell, lysing the cell, solubilizing the protein in a solubilization solution,

forming a refold solution, applying the refold solution to a separation matrix, washing the matrix, and eluting the protein.

Oliner *et al.* disclose a method of producing Fc-fusion proteins by expressing them in *E. coli* cells, lysing the cells (which contain the proteins in inclusion bodies), solubilizing the proteins with a solution containing guanidine and DTT, followed by forming a refolding solution by diluting the solubilization solution into a solution containing urea, arginine and cysteine (an alternate solution also contained glycerol and cystamine), which was then filtered and loaded onto an ion exchange chromatography column, followed by elution to remove the protein (see column 76, lines 31-65).

Oliner *et al.* differs from the instant invention in that they do not disclose the use of sarcosyl or sodium dodecylsulfate in the solubilization solution.

Fischer *et al.* state that detergents such as sodium dodecylsulfate have been used to solubilize inclusion bodies (see page 5, paragraph 2).

It would have been obvious to one of ordinary skill in the art, at the time of invention, to use sodium dodecylsulfate in the solubilization solution because it has been used by others in this capacity. According to the Supreme Court *KSR International Co. v. Teleflex Inc.*, No. 04-1350 (U.S. Apr. 30, 2007), simple substitution of one known element for another to obtain predictable results is obvious. In the instant case, sodium dodecylsulfate had been used to solubilize proteins from inclusion bodies. Using it instead of urea or another agent would have been substituting one known element for another. As solubilization of inclusion bodies was well-studied, one would have expected to achieve predictable results.

Conclusion

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Brian J. Gangle whose telephone number is (571)272-1181. The examiner can normally be reached on M-F 9-6.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Nickol can be reached on 571-272-0835. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Brian J Gangle/
Primary Examiner, Art Unit 1645

Electronically Filed: March 1, 2016

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Application of: Joseph Shultz et al.	Docket No.: A-1441-US-DIV
Application No.: 14/599,336	Examiner: Brian J. Gangle
Filed: January 16, 2015	Art Unit: 1645
Confirmation No.: 3003	

For: CAPTURE PURIFICATION PROCESSES
FOR PROTEINS EXPRESSED IN A NON-MAMMALIAN SYSTEM

RESPONSE TO OCTOBER 2, 2015 NON-FINAL OFFICE ACTION

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Madam:

This responds to the October 2, 2015 Non-Final Office Action in the above application. Applicant has petitioned herewith for a two (2) month extension of time to extend the deadline for responding to March 2, 2016. 37 CFR 1.17(a)(2). Accordingly, this Response and accompanying papers are timely filed.

Amendments to the Claims begin on page 2 of this paper.

Remarks begin on page 6 of this paper.

CERTIFICATE OF EFS-WEB TRANSMISSION

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted electronically through EFS-WEB to the Commissioner for Patents, P.O. Box 1450 Alexandria, VA 22313-1450, on the date appearing below.

March 1, 2016	Beverly A. Dynes
Date	Signature

AMENDMENTS TO THE CLAIMS

This Listing of Claims will replace all prior versions and listings in this application.

Listing of Claims

Claim 1. (Currently Amended) A method of purifying a protein expressed in a non-native soluble form in a non-mammalian expression system comprising:

(a) lysing a non-mammalian cell in which the protein is expressed in a nonnative soluble form to generate a cell lysate;

(b) contacting the cell lysate with [[an]] a separation matrix under conditions suitable for the protein to associate with the separation matrix;

(c) washing the separation matrix; and

(d) eluting the protein from the separation matrix.

Claim 2. (Original) The method of claim 1, wherein the protein is a complex protein.

Claim 3. (Original) The method of claim 2, wherein the complex protein is selected from the group consisting of a multimeric protein, an antibody and an Fc fusion protein.

Claim 4. (Original) The method of claim 1, wherein the non-mammalian expression system comprises bacteria or yeast cells.

Claim 5. (Currently Amended) The method of claim 1, wherein the separation matrix is an affinity resin ~~selected from the group consisting of Protein A, Protein G and a synthetic mimetic affinity resin~~.

Claim 6. (Original) The method of claim 1, wherein the separation matrix is a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin.

Claim 7. (Original) The method of claim 1, wherein the cell lysate is filtered before it is contacted with the separation matrix.

Claim 8. (Original) The method of claim 1, further comprising refolding the protein to its native form after it is eluted.

Claim 9. (Currently Amended) A method of purifying a protein expressed in a non-native limited solubility form in a non-mammalian expression system comprising:

~~(a) expressing a protein in a non-native limited solubility form in a nonmammalian cell;~~

~~(b) lysing a non-mammalian cell;~~

~~(c)~~ (a) solubilizing the expressed protein in a solubilization solution comprising one or more of the following:

(i) a denaturant;

(ii) a reductant; and

(iii) a surfactant;

~~(d)~~ (b) forming a refold solution comprising the solubilization solution and a refold buffer, the refold buffer comprising one or more of the following:

(i) a denaturant;

(ii) an aggregation suppressor;

(iii) a protein stabilizer; and

(iv) a redox component;

~~(e)~~ (c) applying the refold solution to a separation matrix under conditions suitable for the protein to associate with the matrix;

~~(f)~~ (d) washing the separation matrix; and

~~(g)~~ (e) eluting the protein from the separation matrix.

Claim 10. (Original) The method of claim 9, wherein the non-native limited solubility form is a component of an inclusion body.

Claim 11. (Original) The method of claim 9, wherein the protein is a complex protein.

Claim 12. (Original) The method of claim 9, wherein the complex protein is selected from the group consisting of a multimeric protein, an antibody, a peptibody, and an Fc fusion protein.

Claim 13. (Original) The method of claim 9, wherein the non-mammalian expression system is bacteria or yeast cells.

Claim 14. (Currently Amended) The method of claim 9, wherein the denaturant of the solubilization solution or the refold buffer comprises one or more of urea, guanidinium salts, dimethyl urea, methylurea and ethylurea.

Claim 15. (Currently Amended) The method of claim 9, wherein the reductant comprises one or more of cysteine, dithiothreitol (DTT), beta-mercaptoethanol and glutathione.

Claim 16. (Original) The method of claim 9, wherein the surfactant comprises one or more of sarcosyl and sodium dodecylsulfate.

Claim 17. (Original) The method of claim 9, wherein the aggregation suppressor is selected from the group consisting of arginine, proline, polyethylene glycols, nonionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes.

Claim 18. (Original) The method of claim 9, wherein the protein stabilizer comprises one or more of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, tris, sodium sulfate, potassium sulfate and osmolytes.

Claim 19. (Original) The method of claim 9, wherein the redox component comprises one or more of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol.

Claim 20. (Previously Presented) The method of claim 9, wherein the separation matrix is:

(i) an affinity resin, selected from the group consisting of Protein A, Protein G, and synthetic mimetic affinity resin; or

(ii) a non-affinity resin selected from the group consisting of ion exchange, mixed mode, and a hydrophobic interaction resin.

Claims 21-29. (Cancelled)

Claim 30. (New) The method of claim 1 or 9, wherein the protein is isolated after elution from the separation matrix.

Claim 31. (New) The method of claim 8, wherein the protein is isolated after refolding.

REMARKS

Summary of Related Cases

Applicant brings to the attention of the Examiner the following related patents, applications, and their respective prosecution histories. Applicant stands ready to provide the Examiner with any specific documents upon request.

Application/Patent No.	Filing/Grant Date	Status
USP 8,940,878	1-27-2015	Issued
USP 8,952,138	2-10-2015	Issued
USPA 14/611,037	1-30-2015	Pending
USPA 14/793,590	7-7-2015	Allowed

The Claim Amendments

Applicant has amended claim 1 to correct an inadvertent typographical error.

Applicant has amended claim 5 to delete the recitation of "selected from the group consisting of Protein A, Protein G and a synthetic mimetic affinity resin." Support for this amendment can be found, for example, on page 2, second paragraph of the Summary of the Invention.

Applicant has amended claim 9 to delete the recitation of (a) and (b). Support for this amendment can be found, for example, on pages 17-25 of the specification.

Applicant has amended claim 14 to more clearly point out what applicant regards as the invention. Specifically, applicant has amended claim 14 to recite that "the denaturant of the solubilization solution or the refold buffer comprises…"

Applicant has amended claim 15 to recite "dithiothreitol (DTT)" as requested by the Examiner.

Applicant has added new claim 30 which depends from claims 1 and 9 and recites that the protein is isolated after elution from the separation matrix. Applicant has added new claim 31 which depends from claim 8 and recites that the protein is isolated after refolding. Support for these claims can be found throughout the application as filed.

Applicant reserves the right to pursue any cancelled subject matter either in this application or an application which claims priority hereto.

None of the new claims or amendments introduces new matter.

After entry of these amendments, claims 1-20 and 30-31 will be pending.

The Information Disclosure Statement

The Examiner states that although the September 2, 2015 Information Disclosure Statement has been considered, the lined through documents were not considered because copies of those references were not provided.

Applicant submits herewith copies of the lined through documents and a Supplementary Information Disclosure Statement (SIDS) referencing those documents. Consideration of applicant's SIDS is respectfully requested.

The Objection

Claim 15 stands objected to for reciting the acronym DTT without including the full recitation followed by the acronym in parentheses in the first instance.

Applicant has amended claim 15 to recite "Dithiothreitol (DTT)," thus rendering the Examiner's rejection moot.

The Rejections

Statutory Double Patenting

Claim 5 stands rejected under 35 U.S.C. § 101 as claiming the same invention as that of claim 1 of U.S. Patent No. 8,940,878 ("the '878 patent").

Applicant has amended claim 5 to delete the recitation of "selected from the group consisting of Protein A, Protein G and a synthetic mimetic affinity resin." Accordingly, claim 5 does not claim the same invention as claim 1 of the '878 patent.

Applicant respectfully requests that the Examiner withdraw the rejection.

Obviousness-type Double Patenting

U.S. Patent No. 8,952,138

Claims 1-4 and 6-20 stand rejected on the ground of nonstatutory double patenting as allegedly unpatentable over claims 1-24 of U.S. Patent No. 8,952,138 ("the '138 patent"). In addition, claims 1-7 stand separately rejected on the ground of nonstatutory double patenting as allegedly unpatentable over claims 1-24 of the '138 patent. Finally, claims 1-7 and 9-20 stand rejected on the ground of nonstatutory double patenting as allegedly unpatentable over claims 1-24 of the '138 patent in view of Fischer et al., Biotechnol. Bioengin., 41:3-13 (1993) ("Fischer"). The Examiner states that although the conflicting claims are not identical, they are not patentably distinct from each other.

Applicant traverses. Without acquiescing to any of the Examiner's contentions, applicant respectfully requests that the double patenting rejections be held in abeyance until the pending claims are indicated to be otherwise allowable.

Copending U.S. Patent Application No. 14/611,037

Claims 1-7 stand provisionally rejected on the ground of nonstatutory obviousness-type double patenting as allegedly unpatentable over claim 1 of copending U.S. Application No. 14/611,037 ("the '037 application"). The Examiner states that "[a]lthough the conflicting claims are not identical, they are not patentably distinct from each other because both claim sets are drawn to purifying a protein expressed in a non-native soluble form."

Applicant traverses. Without acquiescing to any of the Examiner's contentions, applicant respectfully requests that this double patenting rejection be held in abeyance until the pending claims are indicated to be otherwise allowable.

Copending U.S. Patent Application No. 14/793,590

Claims 1-7 stand provisionally rejected on the ground of nonstatutory obviousness-type double patenting as allegedly unpatentable over claims 25-54 of copending Application No. 14/793,590 ("the '590 application"). The Examiner states that "[a]lthough the conflicting claims are not identical, they are not patentably distinct from each other because both claim sets are drawn to purifying a protein expressed in a nonnative soluble form."

Applicant traverses. Without acquiescing to any of the Examiner's contentions,

applicant respectfully requests that this double patenting rejection be held in abeyance until the pending claims are indicated to be otherwise allowable.

35 U.S.C. §112, Second Paragraph: Indefiniteness

The Examiner has rejected claim 14 under 35 U.S.C. §112, second paragraph, as allegedly indefinite. Specifically, the Examiner states that although claim 14 recites the term "the denaturant" in line 1, there is insufficient antecedent basis for the term because the parent claim recites two denaturants and it is allegedly unclear which denaturant is referred to.

Applicant has amended claim 14 to refer to the denaturant of the solubilization solution or the refold buffer of claim 9. Accordingly, applicant respectfully requests that the Examiner withdraw this rejection.

35 U.S.C. §102(b): Anticipation

Pavlinkova et al., Nuclear Med. Biol., 26:27-34 (1999)

Claims 1-4 and 6-7 stand rejected under 35 U.S.C. §102(b) as allegedly anticipated by Pavlinkova et al. ("Pavlinkova"). The Examiner states that "[t]he instant claims are drawn to methods of purifying proteins expressed in a non-native soluble form in a non-mammalian expression system comprising lysing the cells, contacting the lysate with a separation matrix, washing the matrix, and eluting the protein from the matrix." According to the Examiner, Pavlinkova discloses a method where an scFv antibody was expressed in *E. coli*, the cells were lysed, filtered, and applied to an ion exchange resin before being eluted. The Examiner states that scFv's are non-native proteins in that they lack disulfide bonds found in native antibodies.

Applicant traverses. In discussing scFv purification, Pavlinkova recites that "[t]he CC49 scFv protein was purified from the periplasmic fractions using ion-exchange chromatography as described in the Materials and Methods section." *See*, Pavlinkova, page 29, right column. In the referenced Materials and Methods section, Pavlinkova recites that the scFv protein was purified from the periplasmic fraction; pelleted cells were resuspended, incubated, centrifuged, again resuspended, incubated, and recentrifuged; the supernatant containing the E. *coli* periplasmic fraction was filtered; the filtrate containing scFv was

dialyzed and applied to a Mono-Q column. *See*, Pavlinkova, page 28, Section "CC49 *scFv Protein Purification*."

Accordingly, Pavlinkova does not teach or suggest the method of claim 1 which comprises, *inter alia*, (a) lysing a non-mammalian cell in which the protein is expressed in a nonnative soluble form to generate a cell lysate; and (b) contacting the cell lysate with an separation matrix under conditions suitable for the protein to associate with the separation matrix. Specifically, nothing in Pavlinkova teaches or suggests lysing a cell and contacting the cell lysate with a separation matrix. Rather, Pavlinkova, *inter alia*, purifies the scFv protein from the periplasmic fraction and dialyzes the scFv before it is applied to a Mono-Q column.

Because Pavlinkova fails to teach contacting a cell lysate with a separation matrix, Pavlinkova fails to each and every limitation of claim 1. Accordingly, Pavlinkova does not anticipate claim 1 or claims 2-4 and 6-7 (which depend therefrom). For at least these reasons, applicant respectfully requests that the Examiner withdraw this rejection.

Ronnmark et al. (J. Immunologic. Meth., 261:199-211 (2002)

Claims 1-5 and 8 stand rejected under 35 U.S.C. §102(b) as allegedly anticipated by Ronnmark et al. ("Ronnmark"). According to the Examiner, [1] Ronnmark discloses a method of producing affibody-Fc chimera proteins; and [2] the soluble proteins were expressed in *E. coli* cells, which were then lysed by osmotic shock, followed by purification by protein A chromatography, and then refolded under reducing conditions.

Applicant traverses. Ronnmark employs an *E. coli* expression vector that encodes a secreted form of human IgG1 Fc. *See*, Ronnmark, Section 2.1. The secreted protein is targeted to the periplasm. Ronnmark recites that "[a]t harvest, cells were subject to osmotic chock [*sic*]…and the periplasmic fraction was collected." *See*, Ronnmark, Section 2.4. Thus, the method of Ronnmark liberates the periplasmic Fc protein. The periplasmic space is an oxidative environment conducive to formation of disulfide bonds required for native protein formation. Ronnmark notes in the abstract that "affibody-Fc fusion proteins showing spontaneous Fc fragment-mediated homodimerization via disulfide bridges were produced…." Thus, the Fc produced in Ronnmark is a native Fc protein.

In contrast, the claimed invention is to a protein expressed in a "non-native" soluble

form. Because Ronnmark fails to teach each and every element of claim 1, Ronnmark does not anticipate claim 1 or claims 2-5 and 8 (which depend therefrom). For at least these reasons, applicant respectfully requests that the Examiner withdraw this rejection.

Oliner et al., U.S. Patent No. 7,138,370

Claims 9-15 and 17-20 stand rejected under 35 U.S.C. §102(b) as being anticipated by Oliner *et al.*, U.S. Patent No. 7,138,370 ("the '370 patent"). The Examiner states that the instant claims are drawn to methods of purifying proteins expressed in a non-native limited solubility form in a non-mammalian expression system comprising expressing the protein in a non-mammalian cell, lysing the cell, solubilizing the protein in a solubilization solution, forming a refold solution, applying the refold solution to a separation matrix, washing the matrix, and eluting the protein. According to the Examiner, the '370 patent recites a method of producing Fc-fusion proteins by expressing them in *E. coli* cells, lysing the cells (which contain the proteins in inclusion bodies), solubilizing the proteins with a solution containing guanidine and DTT, followed by forming a refolding solution by diluting the solubilization solution into a solution containing urea, arginine and cysteine (an alternate solution also contained glycerol and cystamine), which was then filtered and loaded onto an ion exchange chromatography column, followed by elution to remove the protein. The Examiner also states that the '370 patent recites that protein A affinity chromatography can be used to purify the proteins.

Applicant traverses. Claim 9 recites, *inter alia*, (b) forming a refold solution; and (c) applying the refold solution to a separation matrix under conditions suitable for the protein to associate with the matrix. In contrast, the '370 patent recites that the refolded protein is subject to dialysis, precipitation, and centrifugation. *See*, the '370 patent, col 76, lns 51-59. The supernatant of the '370 patent is then pH adjusted and loaded onto a column. Because the '370 patent does not recite forming a refold solution and applying the refold solution to a separation matrix, the '370 patent fails to teach each and every element of claim 1.

Accordingly, the '370 patent does not anticipate claim 9 or claims 10-15 and 17-20 (which depend therefrom). For at least these reasons, applicant respectfully requests that the Examiner withdraw this rejection.

35 U.S.C. §103: Obviousness

The '370 Patent in view of Fischer

Claims 9-19 stand rejected under 35 U.S.C. §103(a) as allegedly unpatentable over the '370 patent in view of Fischer. The Examiner concedes that the '370 patent differs from the instant invention in that it does not recite the use of sarcosyl or sodium dodecylsulfate in the solubilization solution. The Examiner states, however, that Fischer recites that detergents such as sodium dodecylsulfate have been used to solubilize inclusion bodies.

Thus, the Examiner concludes that "[i]t would have been obvious to one of ordinary skill in the art, at the time of invention, to use sodium dodecylsulfate in the solubilization solution because it has been used by others in this capacity… In the instant case, sodium dodecylsulfate had been used to solubilize proteins from inclusion bodies. Using it instead of urea or another agent would have been substituting one known element for another. As solubilization of inclusion bodies was well-studied, one would have expected to achieve predictable results."

Applicant traverses. As discussed in the context of the 35 U.S.C. §102(b) rejection, above, claim 9 recites, *inter alia*, forming a refold solution and applying the refold solution to a separation matrix. In contrast, the '370 patent recites that the refolded protein is subject to dialysis, precipitation, and centrifugation. The supernatant of the '370 patent is then pH adjusted and loaded onto a column. Thus, the '370 patent does not recite forming a refold solution and applying the refold solution to a separation matrix. Nothing in Fischer cures this deficiency.

For at least these reasons, applicant respectfully requests that the Examiner withdraw this rejection.

CONCLUSION

Applicant submits that the presented claims are in condition for allowance. A favorable action is earnestly requested. Applicant's representative invites the Examiner to call the number below if any issue remains outstanding.

The Commissioner is hereby authorized to charge any additional fees which may be required or credit any overpayment to Deposit Account No. 01-0519.

Please send all future correspondence to:

CUSTOMER NO: 30174
Amgen Inc.
Law Department
1120 Veterans Blvd.
South San Francisco, CA 94080

Respectfully submitted,

/Raymond M. Doss/

Raymond M. Doss
Agent for Applicant(s)
Registration No.: 61,000
Phone: 650-244-2355
Date: March 1, 2016

Electronically Filed: March 1, 2016

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Application of: Joseph Shultz et al. Docket No.: A-1441-US-DIV

Application No.: 14/599,336 Examiner: Brian J. Gangle

Filed: January 16, 2015 Art Unit: 1645

Confirmation No.: 3003

For: CAPTURE PURIFICATION PROCESSES
FOR PROTEINS EXPRESSED IN A NON-MAMMALIAN SYSTEM

INFORMATION DISCLOSURE STATEMENT TRANSMITTAL

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Examiner:

As a means of complying with the duty of disclosure under 37 C.F.R. § 1.56, applicant(s) submit(s) a "List of References Cited by Applicant" or an "Information Disclosure Statement by Applicant" and provide(s) a copy of each listed item of information that is not a U.S. Patent or published U.S. Patent Application for consideration by the Examiner.

Applicant(s) make(s) no determination of relevancy with respect to the item(s) of information submitted herewith and request(s) that the Examiner make an independent determination of relevance and/or materiality of the item(s) of information.

Identification of the listed item(s) of information is not to be construed as an admission by applicant(s) or attorney for applicant(s) that such item(s) of information are available or qualify as "prior art" against the subject application.

SUBMISSION UNDER 37 C.F.R § 1.97(c)

☒ In accordance with 37 C.F.R. § 1.97(c), the information disclosure statement is being filed after the period specified in 37 C.F.R. § 1.97(b), but is being filed before the mailing date of any of a final action under § 1.113, a notice of allowance under § 1.311, or an action that otherwise closes prosecution in the application.

CERTIFICATE OF EFS-WEB TRANSMISSION

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted electronically through EFS-WEB to the Commissioner for Patents, P.O. Box 1450 Alexandria, VA 22313-1450, on the date appearing below.

March 1, 2016 /Beverly A. Dynes/
Date Signature

In accordance with the requirements of 37 C.F.R. § 1.97(c), the information disclosure statement is accompanied by one of:
(1) One of the two statements specified in 37 C.F.R. § 1.97(e); or
(2) The fee set forth in § 1.17(p).

FEE UNDER 37 C.F.R § 1.17(p)

☒ Please charge the fee of $180.00 as set forth in 37 C.F.R. § 1.17(p) to Deposit Account No. 01-0519 in the name of Amgen Inc.

The Commissioner is hereby authorized to charge any additional fees which may be required or credit any overpayment to Deposit Account No. 01-0519.

Please send all future correspondence to:

CUSTOMER NO: 30174
Amgen Inc.
Law Department
1120 Veterans Blvd.
South San Francisco, CA 94080

Respectfully submitted,

/Raymond M. Doss/

Raymond M. Doss
Agent for Applicant(s)
Registration No.: 61,000
Phone: 650-244-2355
Date: March 1, 2016

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/599,336	01/16/2015	Joseph Edward SHULTZ	A-1441-US-DIV	3003

30174 7590 05/23/2016

AMGEN INC.
Law - Patent Operations, M/S 28-2-C
One Amgen Center Drive
Thousand Oaks, CA 91320-1799

EXAMINER	
GANGLE, BRIAN J	

ART UNIT	PAPER NUMBER
1645	

NOTIFICATION DATE	DELIVERY MODE
05/23/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

pair_amgen@firsttofile.com
EFRHelp@amgen.com

UNITED STATES DEPARTMENT OF COMMERCE
U.S. Patent and Trademark Office
Address : COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450

APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR / PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.
14/599,336	16 January, 2015	SHULTZ ET AL.	A-1441-US-DIV

AMGEN INC.
Law - Patent Operations, M/S 28-2-C
One Amgen Center Drive
Thousand Oaks, CA 91320-1799

EXAMINER	
Brian J. Gangle	
ART UNIT	PAPER
1645	20160516

DATE MAILED:

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner for Patents

The reply filed on 3/1/2016 is not fully responsive to the prior Office action because of the following omission(s) or matter(s):
37 CFR 1.111(b) states: In order to be entitled to reconsideration or further examination, the applicant or patent owner must reply to the Office action. The reply by the applicant or patent owner must be reduced to a writing which distinctly and specifically points out the supposed errors in the examiner's action and must reply to every ground of objection and rejection in the prior Office action. If the reply is with respect to an application, a request may be made that objections or requirements as to form not necessary to further consideration of the claims be held in abeyance until allowable subject matter is indicated. The applicant's or patent owner's reply must appear throughout to be a bona fide attempt to advance the application or the reexamination proceeding to final action.
It is noted that only objections or requirements as to form not necessary to further consideration of the claims can be held in abeyance. Applicant has not specifically pointed out the supposed errors in the examiner's action with regard to the Obviousness-type Double Patenting rejections. Though two of these are provisional, the rejection over US Patent 8,952,138 is not provisional and applicant must respond appropriately to the rejection in order for the reply to be fully responsive. Requests to hold the rejection in abeyance do not conform to 37 CFR 1.111.
See 37 CFR 1.111. Since the above-mentioned reply appears to be bona fide, applicant is given a TIME PERIOD of TWO (2) MONTHS or THIRTY (30) DAYS from the mailing date of this notice within which to supply the omission or correction in order to avoid abandonment. EXTENSIONS OF THIS TIME PERIOD MAY BE

/Brian J Gangle/
Primary Examiner, Art Unit 1645

PTO-90C (Rev.04-03)

Electronically Filed: June 8, 2016

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Application of: Joseph Shultz et al.	Docket No.: A-1441-US-DIV
Application No.: 14/599,336	Examiner: Brian J. Gangle
Filed: January 16, 2015	Art Unit: 1645
Confirmation No.: 3003	

For: CAPTURE PURIFICATION PROCESSES
FOR PROTEINS EXPRESSED IN A NON-MAMMALIAN SYSTEM

RESPONSE TO MAY 23, 2016 NOTICE REGARDING NON-RESPONSIVE AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Madam:

This responds to the May 23, 2016 Notice Regarding Non-Responsive Amendment ("Notice") in the above application. A Response is due within 30 days of the mailing of the Notice – i.e., by June 23, 2016. Accordingly, this Response and accompanying papers are timely filed.

Remarks begin on page 2 of this paper.

CERTIFICATE OF EFS-WEB TRANSMISSION

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted electronically through EFS-WEB to the Commissioner for Patents, P.O. Box 1450 Alexandria, VA 22313-1450, on the date appearing below.

June 8, 2016	/Beverly A. Dynes/
Date	Signature

REMARKS

Summary of Related Cases

The Examiner states that the Reply filed on March 1, 2016 is not fully responsive to the prior Office Action because "Applicant has not specifically pointed out the supposed errors in the examiner's action with regard to the Obviousness-type Double Patenting rejections." Specifically, the Examiner states that, although two of the Obviousness-type Double Patenting rejections are provisional rejections, the rejection over US Patent 8,952,138 ("the '138 patent") is not provisional and "applicant must respond appropriately to the rejection in order for the reply to be fully responsive."

For completeness of the record, applicant notes that, in the October 2, 2015 Non-Final Office Action, the Examiner rejected pending claims 1-4 and 6-20 on the ground of nonstatutory double patenting as allegedly unpatentable over claims 1-24 of the '138 patent. In addition, the Examiner separately rejected pending claims 1-7 on the ground of nonstatutory double patenting as allegedly unpatentable over claims 1-24 of the '138 patent. Finally, the Examiner separately rejected pending claims 1-7 and 9-20 on the ground of nonstatutory double patenting as allegedly unpatentable over claims 1-24 of the '138 patent in view of Fischer et al., Biotechnol. Bioengin., 41:3-13 (1993). The Examiner stated that although the conflicting claims are not identical, they are not patentably distinct from each other.

Without acquiescing to any of the Examiner's contentions and solely to expedite prosecution, applicant submits herewith a Terminal Disclaimer over the '138 patent, thus obviating the Examiner's rejection.

With respect to the provisional nonstatutory obviousness-type double patenting rejections over copending U.S. Application No. 14/611,037 and copending U.S. Application No. 14/793,590, applicant understands that, in the Notice, the Examiner considered applicant's previous request to hold the provisional double patenting rejections in abeyance to be responsive.

CONCLUSION

Applicant's representative invites the Examiner to call the number below if any issue remains outstanding.

Please send all future correspondence to:

CUSTOMER NO: 30174
Amgen Inc.
Law Department
1120 Veterans Blvd.
South San Francisco, CA 94080

Respectfully submitted,

/Raymond M. Doss/

Raymond M. Doss
Agent for Applicant(s)
Registration No.: 61,000
Phone: 650-244-2355
Date: June 8, 2016

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Application of: Joseph Shultz et al.	Docket No.: A-1441-US-DIV
Application No.: 14/599,336	Examiner: Brian J. Gangle
Filed: January 16, 2015	Art Unit: 1645
Confirmation No.: 3003	

For: CAPTURE PURIFICATION PROCESSES
FOR PROTEINS EXPRESSED IN A NON-MAMMALIAN SYSTEM

TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A PRIOR PATENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Examiner:

The owner, Amgen Inc., of 100% interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of **prior patent** No. 8,952,138 as the term of said prior patent is defined in 35 U.S.C. 154 and 173, and as the term of said **prior patent** is presently shortened by any terminal disclaimer. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the **prior patent** are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successor or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term as defined in 35 U.S.C. 154 and 173 of the **prior patent**, "as the term of said **prior patent** is presently shortened by any terminal disclaimer," in the event that said **prior patent** later:

expires for failure to pay a maintenance fee;
is held unenforceable;
is found invalid by a court of competent jurisdiction;
is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
has all claims canceled by a reexamination certificate;
is reissued; or
is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

CERTIFICATE OF EFS-WEB TRANSMISSION

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted electronically through EFS-WEB to the Commissioner for Patents, P.O. Box 1450 Alexandria, VA 22313-1450, on the date appearing below.

June 8, 2016 — Date
/Beverly A. Dynes/ — Signature

Application No.: 14/599,336
Date of Terminal Disclaimer: June 6, 2016

Further, the owner does not waive owner's right to obtain, to the full extent provided by law, any patent term extension, restoration and/or pediatric exclusivity.
Check either box 1 ***or*** 2 below, if appropriate.

1. ☐ For submissions on behalf of a business/organization (e.g., corporation, partnership, university, government agency, etc.), the undersigned is empowered to act on behalf of the business/organization.*

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature	Date
Typed or printed name	
Title	Telephone Number

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).

2. ☒ The undersigned is an attorney or agent of record. Reg. No. 61,000

/Raymond M. Doss/	June 8, 2016
Signature	Date
Raymond M. Doss	
Typed or printed name	
650-244-2355	
Telephone Number	

☒ Terminal disclaimer fee under 37 CFR 1.20(d) included. Fees are being paid via EFS-Web.
Please send all future correspondence to:

CUSTOMER NO: 30174
US Patent Operations [RMD]
Amgen Inc.
1120 Veterans Blvd.
Mail Stop ASF3-2
South San Francisco, California 94080

Electronically Filed: June 8, 2016

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Application of: Joseph Shultz et al.	Docket No.: A-1441-US-DIV
Application No.: 14/599,336	Examiner: Brian J. Gangle
Filed: January 16, 2015	Art Unit: 1645
Confirmation No.: 3003	

For: CAPTURE PURIFICATION PROCESSES
FOR PROTEINS EXPRESSED IN A NON-MAMMALIAN SYSTEM

INFORMATION DISCLOSURE STATEMENT TRANSMITTAL

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Examiner:

As a means of complying with the duty of disclosure under 37 C.F.R. § 1.56, applicant(s) submit(s) a "List of References Cited by Applicant" or an "Information Disclosure Statement by Applicant" and provide(s) a copy of each listed item of information that is not a U.S. Patent or published U.S. Patent Application for consideration by the Examiner.

Applicant(s) make(s) no determination of relevancy with respect to the item(s) of information submitted herewith and request(s) that the Examiner make an independent determination of relevance and/or materiality of the item(s) of information.

Identification of the listed item(s) of information is not to be construed as an admission by applicant(s) or attorney for applicant(s) that such item(s) of information are available or qualify as "prior art" against the subject application.

SUBMISSION UNDER 37 C.F.R § 1.97(c)

☒ In accordance with 37 C.F.R. § 1.97(c), the information disclosure statement is being filed after the period specified in 37 C.F.R. § 1.97(b), but is being filed before the mailing date of any of a final action under § 1.113, a notice of allowance under § 1.311, or an action that otherwise closes prosecution in the application.

CERTIFICATE OF EFS-WEB TRANSMISSION

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted electronically through EFS-WEB to the Commissioner for Patents, P.O. Box 1450 Alexandria, VA 22313-1450, on the date appearing below.

June 8, 2016	/Beverly A. Dynes/
Date	Signature

In accordance with the requirements of 37 C.F.R. § 1.97(c), the information disclosure statement is accompanied by one of:
(1) One of the two statements specified in 37 C.F.R. § 1.97(e); or
(2) The fee set forth in § 1.17(p).

FEE UNDER 37 C.F.R § 1.17(p)

☒ Please charge the fee of $180.00 as set forth in 37 C.F.R. § 1.17(p) to Deposit Account No. 01-0519 in the name of Amgen Inc.

The Commissioner is hereby authorized to charge any additional fees which may be required or credit any overpayment to Deposit Account No. 01-0519.

Please send all future correspondence to:

CUSTOMER NO: 30174
Amgen Inc.
Law Department
1120 Veterans Blvd.
South San Francisco, CA 94080

Respectfully submitted,

/Raymond M. Doss/

Raymond M. Doss
Agent for Applicant(s)
Registration No.: 61,000
Phone: 650-244-2355
Date: June 8, 2016

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/599,336	01/16/2015	Joseph Edward SHULTZ	A-1441-US-DIV	3003

30174 7590 09/01/2016

AMGEN INC.
Law - Patent Operations, M/S 28-2-C
One Amgen Center Drive
Thousand Oaks, CA 91320-1799

EXAMINER	
GANGLE, BRIAN J	

ART UNIT	PAPER NUMBER
1645	

NOTIFICATION DATE	DELIVERY MODE
09/01/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

pair_amgen@firsttofile.com
EFRHelp@amgen.com

Office Action Summary	Application No. 14/599,336	Applicant(s) SHULTZ ET AL.	
	Examiner Brian J. Gangle	Art Unit 1645	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *6/8/2016*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-20,30 and 31* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-20,30 and 31* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Applicant's amendment and remarks filed on 6/8/2016 are acknowledged. Claims 1, 9, and 14-15 are amended. New claims 30-31 are added. Claims 1-20 and 30-31 are pending and are currently under examination.

Information Disclosure Statement

The information disclosure statements filed on 3/1/2016 and 6/8/2016 have been considered. Signed copies are enclosed.

Terminal Disclaimer

The terminal disclaimer filed on 6/8/2016 disclaiming the terminal portion of any patent granted on this application which would extend beyond the expiration date of US Patent 8,952,138 has been reviewed and is accepted. The terminal disclaimer has been recorded.

Claim Objections Withdrawn

The objection to claim 15 because the claim contains the acronym DTT is withdrawn in light of applicant's amendment thereto.

Claim Rejections Withdrawn

The rejection of claim 5 under 35 U.S.C. 101 as claiming the same invention as that of claim 1 of prior U.S. Patent No. 8,940,878 is withdrawn in light of applicant's amendment thereto.

The rejection of claims 1-4 and 6-20 on the ground of nonstatutory double patenting as being unpatentable over claims 1-24 of U.S. Patent No. 8,952,138 is withdrawn in light of the terminal disclaimer filed on 6/8/2016.

The rejection of claims 1-7 on the ground of nonstatutory double patenting as being unpatentable over claims 1-24 of U.S. Patent No. 8,952,138 is withdrawn in light of the terminal disclaimer filed on 6/8/2016.

The rejection of claims 1-7 and 9-20 on the ground of nonstatutory double patenting as being unpatentable over claims 1-24 of U.S. Patent No. 8,952,138 in view of Fischer *et al.* (Biotechnol. Bioengin., 41:3-13, 1993; IDS filed 5/13/2011) is withdrawn in light of the terminal disclaimer filed on 6/8/2016.

The rejection of claim 14 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention is withdrawn in light of applicant's amendment thereto.

The rejection of claims 1-4 and 6-7 under 35 U.S.C. 102(b) as being anticipated by Pavlinkova *et al.* (Nuclear Med. Biol., 26:27-34, 1999; IDS filed 9/2/2015) is withdrawn in light of applicant’s arguments thereto.

The rejection of claims 1-5 and 8 under 35 U.S.C. 102(b) as being anticipated by Ronnmark *et al.* (J. Immunologic. Meth., 261:199-211, 2002; IDS filed 9/2/2015) is withdrawn in light of applicant’s arguments thereto.

The rejection of claims 9-15 and 17-20 under 35 U.S.C. 102(b) as being anticipated by Oliner *et al.* (US Patent 7,138,370, 2006; IDS filed 9/2/2015) is withdrawn in light of applicant’s arguments thereto.

The rejection of claims 9-19 under 35 U.S.C. 103(a) as being unpatentable over Oliner *et al.* (US Patent 7,138,370, 2006; IDS filed 9/2/2015) in view of Fischer *et al.* (Biotechnol. Bioengin., 41:3-13, 1993; IDS filed 9/2/2015) is withdrawn in light of applicant’s arguments thereto.

Claim Rejections Maintained

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 1-7 and 30 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claim 1 of copending Application No. 14/611,037 for the reasons set forth in the previous office action in the rejection of claims 1-7. Although the conflicting claims are not identical, they are not patentably distinct from each other because both claim sets are drawn to purifying a protein expressed in a non-native soluble form. Though the copending claims do not address lysing of the cells, one would necessarily lyse the cells in order to accomplish the method. In addition, while the copending claims do recite a soluble protein, they do not specifically state that it is in a non-native form. However, as the point of the method is to refold the protein, the protein must necessarily not have been folded previously and would thus be in a non-native form. In addition, those portions of the specification which provide support for the patent claims may also be examined and considered when addressing the issue of whether a claim in the application defines an obvious variation of an invention claimed in the patent. *In re Vogel*, 422 F.2d 438, 441-42, 164 USPQ 619, 622

(CCPA 1970). The court in *Vogel* recognized "that it is most difficult, if not meaningless, to try to say what is or is not an obvious variation of a claim," but that one can judge whether or not the invention claimed in an application is an obvious variation of an embodiment disclosed in the patent which provides support for the patent claim. According to the court, one must first "determine how much of the patent disclosure pertains to the invention claimed in the patent" because only "[t]his portion of the specification supports the patent claims and may be considered." The court pointed out that "this use of the disclosure is not in contravention of the cases forbidding its use as prior art, nor is it applying the patent as a reference under 35 U.S.C. 103, since only the disclosure of the invention claimed in the patent may be examined." In the instant case, the specification states that the mixture can be contacted with an affinity separation matrix such as Protein A or Protein G resin or with an ion exchange, or mixed mode separation matrix.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Applicant has not provided any traversal of the rejection.

Claims 1-7 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 25-54 of copending Application No. 14/793,590. Although the conflicting claims are not identical, they are not patentably distinct from each other because both claim sets are drawn to purifying a protein expressed in a non-native soluble form. Though the copending claims do not address lysing of the cells, one would necessarily lyse the cells in order to accomplish the method. In addition, while the copending claims do recite a soluble protein, they do not specifically state that it is in a non-native form. However, as the point of the method is to refold the protein, the protein must necessarily not have been folded previously and would thus be in a non-native form. In addition, those portions of the specification which provide support for the patent claims may also be examined and considered when addressing the issue of whether a claim in the application defines an obvious variation of an invention claimed in the patent. *In re Vogel*, 422 F.2d 438, 441-42, 164 USPQ 619, 622 (CCPA 1970). The court in *Vogel* recognized "that it is most difficult, if not meaningless, to try to say what is or is not an obvious variation of a claim," but that one can

judge whether or not the invention claimed in an application is an obvious variation of an embodiment disclosed in the patent which provides support for the patent claim. According to the court, one must first "determine how much of the patent disclosure pertains to the invention claimed in the patent" because only "[t]his portion of the specification supports the patent claims and may be considered." The court pointed out that "this use of the disclosure is not in contravention of the cases forbidding its use as prior art, nor is it applying the patent as a reference under 35 U.S.C. 103, since only the disclosure of the invention claimed in the patent may be examined." In the instant case, the specification states that the mixture can be contacted with an affinity separation matrix such as Protein A or Protein G resin or with an ion exchange, or mixed mode separation matrix.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Applicant has not provided any traversal of the rejection.

New Claim Rejections

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 1-20 and 30-31 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-25 of U.S. Patent No. 8,940,878. Although the claims at issue are not identical, they are not patentably distinct from each other because the limitations of the instant claims (with the exception of claims 30-31) are recited in the patented claims. Therefore the instant claims are anticipated by the patented claims. With regard to claims 30-31, as the methods are methods of purifying the protein, it would have been obvious to actually isolate the protein as part of the method.

Conclusion

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Brian J. Gangle whose telephone number is (571)272-1181. The examiner can normally be reached on M-F 9-6.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Nickol can be reached on 571-272-0835. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Brian J Gangle/
Primary Examiner, Art Unit 1645

Exhibit 6

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/422,327	02/01/2017	Joseph Edward SHULTZ	A-1500-US-CNT2	4838

21069 7590 04/17/2017

AMGEN INC.
Law - Patent Operations, M/S 28-2-C
One Amgen Center Drive
Thousand Oaks, CA 91320-1799

EXAMINER
KIM, YUNSOO

ART UNIT	PAPER NUMBER
1644	

NOTIFICATION DATE	DELIVERY MODE
04/17/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

pair_amgen@firsttofile.com
EFRHelp@amgen.com

Office Action Summary	Application No. 15/422,327	Applicant(s) SHULTZ ET AL.	
	Examiner YUNSOO KIM	Art Unit 1644	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *3/8/17*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *25-54* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *25-54* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *2/1/17* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date *2/1/17,2/27/17*.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

DETAILED ACTION

1. The present application is being examined under the pre-AIA first to invent provisions.

2. Claims 25-54 are pending and are under consideration.

Upon further consideration, the restriction requirement mailed on 3/6/17 has been withdrawn.

3. Applicant's IDS filed on 2/1/17 and 2/27/17 have been acknowledged.

4. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

5. Claims 25-54 are rejected under 35 U.S.C. 102(b) as being anticipated by U. S. Pat. No. 7,138,370 (IDS reference) as is evidenced by specification p. 20-26 of the instant application.

The '370 patent teaches purification methods of proteins in buffer containing 4M urea (denaturant), 160mM arginine (protein stabilizer), 20% of glycerol (aggregation suppressor) 4mM cysteine and 1mM cystamine (redox components) (col. 76, example 5).
As is evidenced by specification in p. 20-26 of the instant application and the redox components cysteine and cystamine and thiol-pair ratio was met in typically 2-20mM (p. 22), claims 51-54 are included in this rejection

Given that the proteins or binding molecules include antibody, Fc fusion constructs, multimeric conjugates of Fc (col. 25-26), claims 30-31, 44-45 are included in this rejection.

Further, the '370 patent teaches that the proteins are expressed in E.coli (non-mammalian), the expression vector is prepared recombinantly and the inclusion bodies are observed (col. 76), claims 39-50 are included in this rejection. Moreover, the '370 patent teaches that the bacterial culture was incubated at 37°C with CO_2 (col. 76), claim 36 is included in this rejection.

In addition, the '370 patent teaches that the additional chromatographical means including protein A, ion exchange resins and/or various filtration methods (col. 53-54), claims 26 and 41 reciting isolating steps are included in this rejection. Therefore, the reference teaching anticipates the claimed invention.

6. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office Action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

7. Claims 25-54 are rejected under 35 U.S.C. 103(a) as being unpatentable over U.S. Pub 2007/02348860 (IDS reference) in view of Hevehan et al (Biotechnology and Bioengineering, 1996, 54(3):221-230, IDS reference).

The '860 publication discloses methods for protein refolding of proteins from non-mammalian expression system including bacterial and yeast using refolding buffer ([0004, 0036, 0041, 0060, 0075]). The '860 publication further teaches that the protein is present at 16.5mg/ml (16.5g/L)

before dilution ([0075]) and examples of the '860 publication discloses refold buffer of redox system (GSH/GSSG) and thiol-ratio pair of 2 ([0075]).

In addition, the '860 publication teaches L-arginine, Tris-HCl and GdmHCl in the presence of detergent ([0036, 0074, 0060]) and claims 51-54 are included in this rejection.

The disclosure of the ‘860 publication differs from the instant claimed invention in that it does not teach the thiol pair buffer strength of 2mM or greater as in (ii) of claim 25 of the instant application.

Hevehan et al. teach dilution of protein with refold buffer and the experimental matrix to change parameters of the refold buffer. The concentrations of reducing agent, dithiothreitol (DTT) and oxidizing agent (GSSG) in the redox buffer was varied and Hevehan et al. concluded the yields were strongly depended on thiol concentrations of the renaturation buffer. The high yield was obtained using 4mM DTT and 7mM GSSG (Fig. 4, thiol pair ratio is 0.3-9).

It would have been obvious to one of ordinary skill in the art at the time the invention was made to use the thiol pair ratio of the Hevehan reference into the methods of refolding taught by the '860 publication.

One of ordinary skill in the art at the time the invention was made would have been motivated to do so because the particular thiol-pair ratio taught by the Hevehan reference reduces aggregation while improving yield of protein production.

From the teachings of references, it would have been obvious to one of ordinary skill in art to combine the teachings of the references and there would have been a reasonable expectation of success in producing the claimed invention. Therefore, the invention as a whole was prima facie obvious to one of the ordinary in the art at the time of invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

8. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

9. Claims 25-54 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-24 of U.S.Pat. 8,952,138.

Although the conflicting claims are not identical, they are not patentably distinct from each other because both recite methods of purifying proteins in the presence of refold buffer, denaturant, aggregation suppressor and protein stabilizer and isolate the proteins of interest.

10. Claims 25-54 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-25 of U.S.Pat. 8,940,878.

Although the conflicting claims are not identical, they are not patentably distinct from each other because both recite methods of purifying proteins in the presence of refold buffer, denaturant, aggregation suppressor and protein stabilizer and isolate the proteins of interest.

11. No claims are allowable.

12. Any inquiry concerning this communication or earlier communications from the examiner should be directed to YUNSOO KIM whose telephone number is (571)272-3176. The examiner can normally be reached on M-F, 9-5. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Daniel Kolker can be reached on 571-272-3181. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Yunsoo Kim
Patent Examiner
Technology Center 1600
April 12, 2017

/Yunsoo Kim/
Primary Examiner, Art Unit 1644

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Amgen Inc.
Serial No.:	15/422,327
Title:	REFOLDING PROTEINS USING A CHEMICALLY CONTROLLED REDOX STATE
Filed:	February 1, 2017
Inventor:	Joseph Edward SHULTZ et al.
Examiner:	Yunsoo Kim
Art Unit:	1644
Confirmation No.:	4838

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT IN RESPONSE TO APRIL 17, 2017 NON-FINAL OFFICE ACTION

Dear Sir:

This Amendment is submitted in response to the non-final Office Action mailed on April 17, 2017.

Amendments to the Claims begin on page **2**.

Remarks begin on page **9**.

Amendments to the Claims:

This listing of claims will replace all prior versions and listings of claims in the application.

Claims 1-24 (Cancelled)

25. (Currently amended) A method of refolding proteins expressed in a non-mammalian expression system to consistently yield at least about 25% properly refolded proteins, the method comprising:

~~(a)~~ contacting the proteins with a preparation that supports the renaturation of at least one of the proteins to a biologically active form, to form a refold mixture, the preparation comprising:

~~(i) a solution that comprises~~ at least one ingredient selected from the group consisting of a denaturant, an aggregation suppressor and a protein stabilizer;

an amount of oxidant;

an amount of reductant,

wherein the amounts of the oxidant and the reductant are related through a thiol-pair ratio and a thiol-pair buffer strength,

~~(A)~~ wherein the ~~a~~ thiol-pair ratio is in the range of 0.001-100; and

~~(B)~~ wherein the ~~a~~ thiol-pair buffer strength maintains the solubility of the preparation and is selected based on a desired~~;~~

~~wherein the thiol-pair ratio and the thiol-pair buffer strength~~ yield ~~at least about 25%~~ of properly refolded protein~~,~~; and

~~(ii) at least one ingredient selected from the group consisting of a denaturant, an aggregation suppressor and a protein stabilizer; and(b)~~ wherein the thiol-pair ratio and the thiol-pair buffer strength are such that incubating the refold mixture achieves consistent yields of at least about 25% properly refolded proteins.

26. (Previously presented) The method of claim 25, wherein the refold mixture has a protein concentration in a range of 1-40 g/L.

27. (Previously presented) The method of claim 25, wherein the refold mixture has a protein concentration of 2.0 g/L or greater.

28. (Previously presented) The method of claim 25, wherein the thiol-pair ratio is selected from the group consisting of 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50, and 40 to 50.

29. (Previously presented) The method of claim 25, wherein the thiol-pair buffer strength is 2 mM or greater.

30. (Previously presented) The method of claim 25, wherein the thiol-pair buffer strength is increased proportionally to an increase in a total protein concentration in the refold mixture.

31. (Previously presented) The method of claim 25, wherein the thiol-pair buffer strength is decreased proportionally to a decrease in a total protein concentration in the refold mixture.

32. (Previously presented) The method of claim 25, wherein the thiol-pair ratio and the thiol-pair buffer strength yield at least about 30% properly refolded protein.

33. (Previously presented) The method of claim 25, wherein the at least one of the proteins is a complex protein.

34. (Currently amended) The method of claim 25, wherein the thiol-pair ratio is calculated, and thus derived, according to the following equation:

$$\frac{\text{~~[reductant]~~}^2\ [\text{the } reductant]^2}{\text{~~[oxidant]~~}\quad [\text{the } oxidant]}$$

35. (Currently amended) The method of claim 25, wherein the thiol-pair buffer strength is calculated, and thus derived, according to the following equation:

$$2[\text{the oxidant}] + [\text{the reductant}].$$

36. (Currently amended) A method of refolding proteins expressed in a non-mammalian expression system to consistently yield at least about 25% properly refolded proteins, the method comprising:

(a) selecting at least one ingredient selected from the group consisting of a denaturant, an aggregation suppressor and a protein stabilizer;

(b)~~(a)~~ generating a solution that comprises:

an amount of oxidant;

an amount of reductant,

wherein the amounts of oxidant and the reductant are related through a thiol-pair ratio and a thiol pair buffer strength,

wherein the ~~(i) a~~ thiol-pair ratio is in the range of 0.001-100, ~~; and~~

wherein the thiol-pair ratio and the thiol-pair buffer strength are correlated, and

wherein the ~~(ii) a~~ thiol-pair buffer strength maintains the solubility of the preparation and is based on a desired ~~, wherein the thio pair ratio~~ yield ~~at least about 25%~~ of properly refolded proteins;

~~(b) selecting at least one ingredient selected from the group consisting of a denaturant, an aggregation suppressor and a protein stabilizer;~~

(c) combining the at least one ingredient with the solution of (b~~a~~) to form a preparation that supports the refolding of the proteins; and

(d) contacting the proteins with the preparation to achieve a chemically-controlled refold mixture;

(e) incubating the refold mixture that results in consistent yields of at least about 25% properly refolded proteins.

37. (Previously presented) The method of claim 36, wherein the refold mixture has a protein concentration in a range of 1-40 g/L.

38. (Previously presented) The method of claim 36, wherein the refold mixture has a protein concentration of 2.0 g/L or greater.

39. (Previously presented) The method of claim 36, wherein the thiol-pair ratio is selected from the group consisting of 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50, and 40 to 50.

40. (Previously presented) The method of claim 36, wherein the thiol-pair buffer strength is 2 mM or greater.

41. (Previously presented) The method of claim 36, wherein the thiol-pair buffer strength is increased proportionally to an increase in a total protein concentration in the refold mixture.

42. (Previously presented) The method of claim 36, wherein the thiol-pair buffer strength is decreased proportionally to a decrease in a total protein concentration in the refold mixture.

43. (Previously presented) The method of claim 36, wherein the thiol-pair ratio and the thiol-pair buffer strength yield at least about 30% properly refolded protein.

44. (Previously presented) The method of claim 36, wherein the at least one of the proteins is a complex protein.

45. (Currently amended) The method of claim 36, wherein the thiol-pair ratio is calculated, and thus derived, according to the following equation:

$$\frac{\text{~~[reductant]~~}^{\text{~~2~~}}\,[reductant]^{2}}{\text{~~[oxidant]~~}\quad[oxidant]}$$

46. (Currently amended) The method of claim 36, wherein the thiol-pair buffer strength is calculated, and thus derived, according to the following equation:

2[the oxidant] + [the reductant].

47. (Currently amended) A method of refolding proteins expressed in a non-mammalian expression system to consistently yield at least about 30-80% properly refolded proteins, the method comprising:

~~(a)~~ contacting the proteins with a preparation that supports the renaturation of at least one of the proteins to a biologically active form, to form a refold mixture, the preparation comprising:

~~(i) a solution that comprises~~ at least one ingredient selected from the group consisting of a denaturant, an aggregation suppressor and a protein stabilizer;

an amount of oxidant;

an amount of reductant,

wherein the amounts of the oxidant and the reductant are related through a thiol-pair ratio and a thiol-pair buffer strength,

wherein the ~~(A) a~~ thiol-pair ratio is in the range of 0.001-100; and

wherein the ~~(B) a~~ thiol-pair buffer strength maintains the solubility of the preparation and is effected based on a desired

~~, wherein the thiol-pair ratio and the thiol-pair buffer strength~~ yield of ~~about 30-80%~~ properly refolded proteins; and

wherein the thiol-pair ratio and the thiol pair buffer strength are such that ~~(ii) at least one ingredient selected from the group consisting of a denaturant, an aggregation suppressor and a protein stabilizer, and~~

~~(b)~~ incubating the refold mixture achieves consistent yields of at least about 30-80% properly refolded proteins.

48. (Previously presented) The method of claim 47, wherein the refold mixture has a protein concentration in a range of 1-40 g/L.

49. (Previously presented) The method of claim 47, wherein the thiol-pair ratio is selected from the group consisting of 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50, and 40 to 50.

50. (Previously presented) The method of claim 47, wherein the thiol-pair buffer strength is 2 mM or greater.

51. (Currently amended) A method of refolding proteins expressed in a non-mammalian expression system so that at least about 30-80% properly refolded proteins are consistently refolded, the method comprising:

(a) selecting at least one ingredient selected from the group consisting of a denaturant, an aggregation suppressor and a protein stabilizer;

~~(a)~~(b) generating a solution that comprises:

an amount of oxidant;

an amount of reductant,

wherein the amounts of oxidant and the reductant are related through a thiol-pair ratio and a thiol pair buffer strength,

wherein the ~~(i)~~a thiol-pair ratio is in the range of 0.001-100, ~~and~~

wherein the thiol-pair ratio and the thiol-pair buffer strength are correlated, and

wherein the ~~(ii) a~~ thiol-pair buffer strength maintains the solubility of the preparation and is based on a desired yield of ~~about 30-80%~~ properly refolded proteins;

~~(b) selecting at least one ingredient selected from the group consisting of a denaturant, an aggregation suppressor and a protein stabilizer;~~

(c) combining the at least one ingredient with the solution of (b~~a~~) to form a preparation that supports the refolding of the proteins;

(d) contacting the proteins with the preparation to achieve a chemically-controlled refold mixture; and

(e) incubating the refold mixture results in consistent yields of at least about 30-80% properly refolded proteins.

52. (Previously presented) The method of claim 51, wherein the refold mixture has a protein concentration in a range of 1-40 g/L.

53. (Previously presented) The method of claim 51, wherein the thiol-pair ratio is selected from the group consisting of 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50, and 40 to 50.

54. (Previously presented) The method of claim 51, wherein the thiol-pair buffer strength is 2 mM or greater.

REMARKS

Applicant brings to the attention of the Examiner the following patents, applications, and their respective prosecution histories. Applicant stands ready to provide the Examiner with any specific documents relating to such patents, applications, and prosecution histories upon request.

Application/Patent No.	Filing/Grant Date	Status
USP 8,940,878	1-27-2015	Issued
USP 8,952,138	2-10-2015	Issued
USP 9,643,997	5-9-2017	Issued
USPA 14/611,037	1-30-2015	Pending
USPA 14/793,590	7-7-2015	Pending
USPA 15/476,691	3-31-2017	Pending

The Claim Amendments

Claims 25-54 are pending in this application. Claims 25, 36, 47 and 51 are independent. The claim amendments are supported by the application, as filed. None of the claim amendments adds new matter.

35 U.S.C. §102(b): Anticipation

Claims 25-54 have been rejected under 35 U.S.C. §102(b) as being anticipated by U.S. Patent No. 7,138,370 to Oliner et al. ("Oliner"). Applicant traverses.

This application is a Track 1 application in which Applicant has three months to address all rejections raised in the Office Action. As set forth in the MPEP §707.07d, the burden is on the Office to establish any prima facie case of unpatentability, thus the reasoning behind any rejection must be clearly articulated. With this context, the Office Action ("Action") fails to mention the first independent, claim 25, as well as the third

independent claim, claim 47, in this §102 rejection. In fact, the seven sentences that consist of the entire rejection fails to mention claims 25, 27-29, 32-35, 37-40, 42-43 and 46-50 that are more than one-half of the pending claims. Respectfully, for this reason alone, should this Amendment not result in a Notice of Allowance, the next Action must be a new, non-final Action.

With respect to the §102(b) rejection, independent claim 25 recites, in relevant part, a method of refolding proteins expressed in a non-mammalian expression system to consistently achieve at least about 25% properly refolded proteins. The method comprises contacting the proteins with a preparation that supports the renaturation of at least one of the proteins to a biologically active form, to form a refold mixture. The preparation comprises at least one ingredient selected from the group consisting of: a denaturant, an aggregation suppressor and a protein stabilizer; an amount of oxidant; and an amount of reductant. The amounts of the oxidant and the reductant are related through a thiol-pair ratio and a thiol-pair buffer strength. The thiol-pair ratio is in the range of 0.001-100. The thiol-pair buffer strength maintains the solubility of the preparation and is effected based on a desired yield of properly refolded protein. Also, the thiol-pair ratio and the thiol-pair buffer strength are such that incubating the refold mixture achieves consistent yields of at least about 25% properly refolded proteins.

The Action relies on Example 5 of the Oliner reference, which discloses the purification of protein in a solution containing 4 M urea, 20% glycerol, 50 mM Tris, 160 mM arginine, 4 mM cysteine and 1 mM cystamine.

First, Oliner is silent regarding the yield of properly refolded protein that would result from the steps described in Example 5.

Second, Oliner fails to disclose that the thiol-pair buffer strength maintains the solubility of the preparation and is effected based on a desired amount yield of properly refolded protein.

Moreover, Oliner fails to disclose or suggest that the thiol-pair ratio and the thiol-pair buffer strength are such that incubating the refold mixture results in a consistent yield of the properly refolded proteins. Thus, as distinguished from Oliner, the presently claimed method advantageously controls parameters, via the claimed thiol-pair ratio range and the thiol-pair buffer strength, to consistently yield at least about 25% properly refolding protein.

Accordingly, Oliner fails to disclose or suggest independent claim 25.

Claims 26-35 depend from claim 25, and for this reason alone, are patentable over Oliner.

Dependent claims 26, 27, 30, 31, 34 and 35 are also patentable for the additional reasons discussed briefly below.

Dependent claim 26 recites that the refold mixture has a protein concentration in a range of 1-40 g/L. Dependent claim 27 recites that the refold mixture has a protein concentration of 2.0 g/L or greater.

To the contrary, Oliner discloses a 250-fold dilution (a factor of 1/10 from the solubilization step and a factor of 1/25 from the oxidation step) that does not lend itself to the creation of a high concentration refold mixture, as recited in claims 26 and 27. In fact, the cited example of Oliner fails to disclose any protein concentrations in the refold mixture. Thus, Oliner clearly fails to anticipate claims 26 and 27.

Dependent claim 30 recites that the thiol-pair buffer strength is increased proportionally to an increase in a total protein concentration in the refold mixture. Dependent claim 31 recites that the thiol-pair buffer strength is decreased proportionally to a decrease in a total protein concentration in the refold mixture.

Example 5 of Oliner provides different refolding conditions for linear peptides and cyclic peptides. However, Oliner does not disclose or suggest that the thiol-pair buffer strength is increased or decreased in proportion to any increase or decrease in total protein concentration in the refold mixture. Thus, Oliner also does not anticipate dependent claims 30 and 31.

Dependent claim 32 recites that the thiol-pair ratio and the thiol-pair buffer strength yield at least about 30% properly refolded protein. As discussed above, Oliner fails to disclose any yield of properly refolded proteins. Thus, Oliner also fails to disclose or suggest the yield of properly refolded proteins recited in dependent claim 32.

Dependent claim 34 recites the thiol-pair ratio is calculated, and thus derived, according to the following equation:

$$\frac{[\text{the } reductant]^2}{[\text{the } oxidant]}.$$

Dependent claim 35 recites that the thiol-pair buffer strength is calculated, and thus derived, according to the following equation:

$$2[\text{the oxidant}] + [\text{the reductant}].$$

Oliner does not disclose either of the above equations. Oliner does not even suggest that either equation is used to calculate the thiol-pair ratio value or the thiol-pair buffer strength. It appears that the Office Action simply used hindsight gleaned from the claimed present invention to select data from a single example in Oliner, and insert that data into the claimed equations in an attempt to show the claimed thiol-pair ratio range. Clearly, Oliner did not use the equations to derive the claimed thiol-pair ratio range, or the thiol-pair buffer strength.

Independent claims 36, 47 and 51 are each similar to independent claim 25. However, independent claims 47 and 51 further limit the consistent yield amount of the properly refolded proteins.

Thus, each independent claim is allowable over Oliner for at least the same reasons presented above for claim 25. Since dependent claims 37-46, 48-50 and 52-54 depend from one of independent claims 36, 47 and 51, these dependent claims are likewise allowable.

Lastly, the Action refers to isolating steps of Oliner, and expressly states that "claims 26 and 41 reciting isolating steps are included in this rejection." Applicant respectfully points out that claims 26 and 41, and in fact no previously pending claim, recited an isolating step. This is noted as still another reason why if this application is not in condition for immediate allowance, the next Action must be a non-final Action.

In view of the above, reconsideration and withdrawal of the 35 U.S.C. § 102(b) rejection of claims 25-54 over Oliner are respectfully requested.

35 U.S.C. §103(a): Obviousness

Claims 25-54 have been also rejected under 35 U.S.C. §103(a) over U.S. Patent Application Publication No. 2007/02348860 to Schlegl in view of Hevehan et al. (Biotechnology and Bioengineering, 1996, 54(3):221-230) ("Hevehan"). Applicant traverses.

As noted above, independent claim 25 recites a method of refolding proteins expressed in a non-mammalian expression system to consistently achieve at least about 25% properly refolded proteins. Claim 25 recites that the amounts of the oxidant and the reductant are related through a thiol-pair ratio and a thiol-pair buffer strength. Claim 25 further recites that the thiol-pair buffer strength maintains the solubility of the preparation and is effected based on a desired yield of properly refolded protein. Claims 25 still further

recites that the thiol-pair ratio and the thiol-pair buffer strength are such that incubating the refold mixture achieves consistent yields of at least about 25% properly refolded proteins.

The cited specific example of Schlegl discloses refolding of the purified model protein bovine α-LA (paragraph [0075]). Significantly, Schlegl discloses that redox chemicals are optional for refolding of α-LA. This is because α-LA is capable of refolding without any redox chemicals.

Accordingly, Schlegl fails to disclose that the amounts of the oxidant and the reductant are related through a thiol-pair ratio and a thiol-pair buffer strength. Also, Schlegl fails to disclose that the thiol-pair buffer strength maintains the solubility of the preparation and is effected based on a desired amount yield of properly refolded protein. Further, Schlegl fails to disclose that the thiol-pair ratio and the thiol-pair buffer strength are such that incubating the refold mixture achieves consistent yields of at least about 25% properly refolded proteins. Significantly, the failure of Schlegl to disclose or suggest that the thiol-pair ratio and the thiol-pair buffer strength in conjunction are looked at to achieve consistent yields of properly refolded proteins, clearly distinguishes Schlegl from claim 25 and the remaining pending claims. Moreover, Schlegl further fails to disclose that the thiol-pair ratio and the thiol-pair buffer strength are such that incubating the refold mixture achieves consistent yields of at least about 25% properly refolded proteins.

The Action cites to Hevehan to overcome the acknowledged deficiencies in Schlegl. Hevehan discloses a method of refolding another purified model protein, namely hen egg white lysozyme.

Hevehan does not disclose that the thiol-pair buffer strength maintains the solubility of the preparation and is effected based on a desired amount yield of properly refolded protein. Moreover, Hevehan does not disclose or suggest that the thiol-pair ratio and the thiol-pair buffer strength are such that the incubated refold mixture achieves consistent yields of at least about 25% properly refolded proteins.

Thus, both Schlegl and Hevehan, whether taken alone or in combination, fail to disclose several features of claim 25. Thus, independent claim 25 is allowable over the cited combination of Schlegl and Hevehan. Since claims 26-39 depend from claim 25, for this reason alone, they are likewise allowable over this cited combination of art.

Dependent claims 26, 27, 33, 34 and 35 are further patentable for the additional reasons below.

Dependent claim 26 recites that the refold mixture has a protein concentration in a range of 1-40 g/L. Dependent claim 27 recites that the refold mixture has a protein concentration of 2.0 g/L or greater.

The Action relies on paragraph [0075] of Schlegl, which discloses one example in which the renaturation buffer has, among other components, 2mM cystine and 2 mM cysteine. However, as noted above, this example of Schlegl provides a final protein concentration of 0.516 g/L, which is below the presently claimed protein concentrations in the refold mixture of 1-40 g/L (claim 26) and 2.0 g/L or greater (claim 27). Further, the Action does not explain why one of skill in the art would use the same concentrations of cystine and cysteine from paragraph [0075] of Schlegl for higher protein concentrations, i.e., for a refold mixture having a protein concentration of 2.0 g/L or greater, or in a range of 1-40 g/L. Even Hevehan, which was cited as a secondary reference, discloses that thiol concentrations that are optimized for low protein concentrations might not be appropriate when folding a protein at 1 mg/mL or higher concentrations. Accordingly, one of skill in the art would expect that the concentrations of cystine and cysteine in the refold buffer of Schlegl would need to be adjusted to account for a higher protein concentration, as clearly stated in Hevehan. Thus, even if one were to modify the refolding conditions of Schlegl to obtain higher protein concentrations, there is no disclosure or suggestion in the cited combination how to adjust the redox chemicals for the specific protein in Schlegl.

Dependent claim 33 recites that at least one of the proteins is a complex protein.

The present application defines a complex protein as being a protein that is (a) larger than 20,000 MW, or comprises greater than 250 amino acid residues, and (b) comprises two or more disulfide bonds in its native form. However, the α-LA disclosed in Schlegl has only 123 amino acid residues and a molecular weight of 14176. Thus, the α-LA of Schlegl does not meet the above-noted requirement (a) for a complex protein.

Hevehan discloses refolding of hen egg white lysozyme, a protein that has 129 amino acids and a molecular weight of 14389.68. Accordingly, the hen egg white lysozyme of Hevehan also does not meet the above requirement (a), and is similarly not a complex protein as defined by the present application. Thus, the cited combination of Schlegl and Hevehan fails to render obvious claim 33.

Dependent claim 34 recites that the thiol-pair ratio is calculated, and thus derived, by the equation: $\frac{[\text{the } reductant]^2}{[\text{the } oxidant]}$.

Dependent claim 35 recites that the thiol-pair buffer strength is calculated, and thus derived, by the equation: 2[the oxidant] + [the reductant].

Schlegl does not disclose or suggest either equation. Thus, Schlegl does not even suggest that either equation is used in deriving the thiol-pair ratio range or the thiol-pair buffer strength.

Further, the Action relies on portions of both Schlegl and Hevehan as disclosing the claimed thiol-pair ratio range of 0.001 to 100 calculated according to the equation $\frac{[\text{the } reductant]^2}{[\text{the } oxidant]}$. However, Hevehan, like Schlegl, also fails to disclose any equation. Further, Hevehan, like Schlegl fails to teach that efficient refolding of proteins can be achieved by the thiol-pair ratio and thiol-pair buffer strength.

Significantly, both references instead admittedly rely on trial-and-error to determine redox conditions that can be used for refolding the specifically disclosed proteins. This trial and error contrasts to the methods of claims 34 and 35 (and the other claimed methods herein).

Independent claims 36, 47 and 51 have been amended somewhat similarly as amended claim 25. For at least the same reasons stated above with respect to claim 25, independent claims 36, 47 and 51, and claims 37-46, 48-50 and 52-54 that depend therefrom, are likewise allowable over the cited combination of Schlegl in view of Hevehan.

Accordingly, reconsideration and withdrawal of the 35 U.S.C. §103(a) rejection of claims 25-54 over Schlegl in view of Hevehan are respectfully requested.

Nonstatutory Obviousness-type Double Patenting

Claims 25-54 have been rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-24 of U.S. Patent No. 8,952,138, as well as over claims 1-25 of U.S. Patent No. 8,940,878. Applicant believes that the amended claims patentably distinguish over both cited references. Thus, reconsideration and withdrawal of these rejections are requested.

Information Disclosure Statement by Applicant

Applicant submits herewith an "INFORMATION DISCLOSURE STATEMENT BY APPLICANT" listing documents for consideration by the Examiner. The documents include, *inter alia*, those relating to a Federal Court litigation concerning USP 8,952,138 (the parent to this application); an IPR of USP 8,952,138; and a Federal Court litigation concerning USP 8,940,878. The Examiner is respectfully requested to do her own independent careful consideration of these documents.

CONCLUSION

In view of the above, claims 25-54 are now in condition for allowance. Such action is solicited.

Accordingly, it is respectfully submitted that the above application is in condition for allowance. Should the application not be immediately allowed, Applicant invites the Examiner to contact the undersigned attorney prior to another action on the merits to arrange a formal interview.

Respectfully submitted,

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Date: July 17, 2017

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/422,327	02/01/2017	Joseph Edward SHULTZ	A-1500-US-CNT2	4838

21069 7590 08/22/2017

AMGEN INC.
Law - Patent Operations, M/S 28-2-C
One Amgen Center Drive
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EXAMINER
KIM, YUNSOO

ART UNIT	PAPER NUMBER
1644	

NOTIFICATION DATE	DELIVERY MODE
08/22/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

pair_amgen@firsttofile.com
EFRHelp@amgen.com

Office Action Summary	Application No. 15/422,327	Applicant(s) SHULTZ ET AL.	
	Examiner YUNSOO KIM	Art Unit 1644	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *7/17/17*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *25-54* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *25-54* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date *7/17/17(3)*.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

DETAILED ACTION

1. The present application is being examined under the pre-AIA first to invent provisions.

2. Claims 25-54 are pending and are under consideration.

Upon further consideration, the restriction requirement mailed on 3/6/17 has been withdrawn.

3. Applicant's IDS filed on 7/17/17 has been acknowledged.

4. In light of Applicant's amendment to the claims filed on 7/17/17, the rejection under 35 U.S.C. 102, 35 U.S.C. 103(a) and the nonstatutory obviousness-type double patenting rejection over U.S. Pat 8,940,878 (see sections 4-7 and 10 of the office action mailed on 4/17/17) have been withdrawn.

Currently amended claims require the claimed method consistently yield at least about 25% properly folded proteins using the preparation comprising a (a)denaturant, aggregation suppressor or a protein stabilizer, (b) oxidant and (c) reductant and the oxidant and reductant are related through a thiol-pair ratio between 0.001-100and thiol-pair buffer strength maintains the solubility.

5. The following rejection remains.

6. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re*

Vogel, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

7. Claims 25-54 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-24 of U.S.Pat. 8,952,138.

Although the conflicting claims are not identical, they are not patentably distinct from each other because both recite methods of purifying proteins in the presence of refold buffer, denaturant, aggregation suppressor and protein stabilizer and isolate the proteins of interest.

Applicant's response filed on 7/17/17 have been fully considered but they were not persuasive.

Applicant has asserted that the currently amended claims no longer read on the patented claims.

However, the patented claims and the claims of the instant application are essentially identical in that they both teach contacting the proteins from non-mammalian system with "refold buffer" for patented claims and preparation comprising a (a)denaturant, aggregation suppressor or a protein stabilizer, (b) oxidant and (c) reductant. Even though the refold buffer is named as “preparation”, the components comprising both refold buffer and the preparation used in the claimed invention are identical. Especially, both refold buffer and the oxidant and reductant are related through a thiol-pair ratio between 0.001-100and thiol-pair buffer strength of 2.25mM-15mM. As both claims comprise identical method steps, the patented claims will inherently yield at least about 25% properly folded proteins. Therefore, the rejection is maintained.

8. The following new ground of rejection is set forth upon further consideration.

9. The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out this invention.

10. Claims 25-54 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This is a New Matter rejection.

The specification or the original claims as filed does not provide a written description the phrase " to consistently yield at least about 25% properly refolded protein…refold mixture achieves consistent yield of at least 25% properly refolded proteins" in claim 25. The recitation of "about at least 25% properly refolded protein" has been made in the amendment filed on 2/2/17. The claims are not entered as a preliminary amendment. The preliminary amendment was made on 2/1/17. The specification or original claims do not support such phrases and the claims are new matter. Further, claim 47 recites "consistently yield at least about 30-80% properly folded proteins".

The specification mentions 25% or 30-80% at once in mentioning "isolation" or "purify" in conjunction to reduce heterogenous elements in [0032] but no quantification has been made with yield of properly refolded protein as is amended.

The instant claims now recite a limitation which was not clearly disclosed in the specification as filed, and now changes the scope of instant disclosure as filed.

Such limitations recited in the present claims, which did not appear in the specification as filed, introduce new concepts and violate the description requirement of the first paragraph of 35 U.S.C.112.

11. No claims are allowable.

12. Any inquiry concerning this communication or earlier communications from the examiner should be directed to YUNSOO KIM whose telephone number is (571)272-3176. The examiner can normally be reached on M-F, 9-5. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Daniel Kolker can be reached on 571-272-3181. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Yunsoo Kim
Patent Examiner
Technology Center 1600
August 17, 2017

/Yunsoo Kim/
Primary Examiner, Art Unit 1644

Exhibit 7

US007138370B2

(12) **United States Patent**
Oliner et al.

(10) **Patent No.: US 7,138,370 B2**
(45) **Date of Patent: Nov. 21, 2006**

(54) **SPECIFIC BINDING AGENTS OF HUMAN ANGIOPOIETIN-2**

(75) Inventors: **Jonathan Daniel Oliner**, Newbury Park, CA (US); **Hosung Min**, Newbury Park, CA (US)

(73) Assignee: **Amgen Inc.**, Thousand Oaks, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 619 days.

(21) Appl. No.: **10/269,695**

(22) Filed: **Oct. 10, 2002**

(65) **Prior Publication Data**

US 2003/0229023 A1 Dec. 11, 2003

Related U.S. Application Data

(60) Provisional application No. 60/414,155, filed on Sep. 27, 2002, provisional application No. 60/328,624, filed on Oct. 11, 2001.

(51) **Int. Cl.**
A61K 38/08 (2006.01)
A61K 38/10 (2006.01)
A61K 38/16 (2006.01)

(52) **U.S. Cl.** **514/2**; 514/12; 514/13; 514/15; 514/16; 514/17; 530/324; 530/325; 530/326; 530/327; 530/328; 530/329; 530/330

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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(Continued)

OTHER PUBLICATIONS

Putaporntip et al. Diversity in the Thrombospondin-Related Adhesive Protein Gene (Trap_of Plasmodium Vivax, Gene 268, pp. 97-104 (2001).*

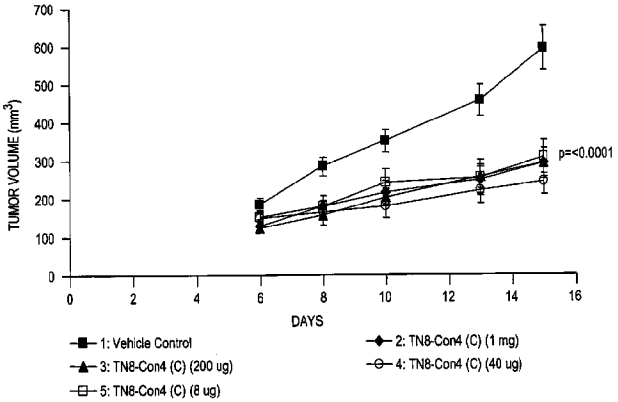
(Continued)

Primary Examiner—Anish Gupta
(74) *Attorney, Agent, or Firm*—Raz E. Fleshner; Scott N. Bernstein

(57) **ABSTRACT**

Disclosed are peptides that bind to Ang-2. Also disclosed are peptibodies comprising the peptides, methods of making such peptides and peptibodies, and methods of treatment using such peptides and peptibodies.

25 Claims, 19 Drawing Sheets



with a polylinker containing ApaLI and XhoI sites. Using strain 3788 as a template, PCR was performed with Expand Long Polymerase, using the oligonucleotide of SEQ ID NO: 8, below, as the 5' primer and a universal 3' primer, SEQ ID NO: 9, below. The resulting PCR product was gel purified and digested with restriction enzymes NdeI and BsrGI. Both the plasmid and the polynucleotide encoding the peptide of interest together with its linker were gel purified using Qiagen (Chatsworth, Calif.) gel purification spin columns. The plasmid and insert were then ligated using standard ligation procedures, and the resulting ligation mixture was transformed into *E. coli* cells (strain 2596). Single clones were selected and DNA sequencing was performed. A correct clone was identified and this was used as a vector source for the modified peptides described herein.

5'Primer:

ACAAACAAACATATGGGTGCACA-GAAAGCGGCCGCAAAAAAA CTCGAGGGTG-GAGGCGGTGGGGACA (SEQ ID NO: 8)

3' Primer:

GGTCATTACTGGACCGGATC (SEQ ID NO: 9)

In addition to making these modified peptides as N-terminal fusions to Fc (N-terminal peptibodies), some of them were also made as C-terminal fusion products (C-terminal peptibodies). The vector used for making the C-terminal fusions is described below.

Construction of Fc C-Terminal Vector

The Fc C-terminal vector for modified peptides was created using *E. coli* strain 3728, pAMG21 Fc_Gly5_Tpo monomer, as a template. Information on the cloning of this strain can be found in WO 00/24782 (See Example 2 and FIG. **7** therein). A 3' PCR primer (SEQ ID NO: 10) was designed to remove the Tpo peptide sequence and to replace it with a polylinker containing ApaLI and XhoI sites. Using strain 3728 as a template, PCR was performed with Expand Long Polymerase using a universal 5' primer (SEQ ID NO: 11) and the aforementioned 3' primer. The resulting PCR product was gel purified and digested with restriction enzymes BsrGI and BamHI. Both the plasmid and the polynucleotide encoding each peptides of interest with its linker were gel purified via Qiagen gel purification spin columns. The plasmid and insert were then ligated using standard ligation procedures, and the resulting ligation mixture was transformed into *E. coli* (strain 2596) cells. Single clones were selected and DNA sequencing was performed. A correct clone was identified and used as a source of vector for modified peptides described herein.

5' Primer:

CGTACAGGTTTACGCAAGAAAATGG (SEQ ID NO: 10)

3' Primer:

TTTGTTGGATCCATTACTC-GAGTTTTTTTGCGGCCGCTTTCTGTG CACCAC-CACCTCCACCTTTAC (SEQ ID NO: 11)

GM221 (#2596). Host strain #2596, used for expressing Fc-peptide fusion proteins, is an *E. coli* K-12 strain modified to contain the lux promoter, and both the temperature sensitive lambda repressor cI857s7 in the early ebg region and the $lacI^{Q}$ repressor in the late ebg region. The presence of these two repressor genes allows the use of this host with a variety of expression systems The ATCC designation for this strain is 202174.

EXAMPLE 5

Production of Peptibodies

Expression in *E. coli*. Cultures of each of the pAMG21-Fc fusion constructs in *E. coli* GM221 were grown at 37° C. in Terrific Broth medium (See Tartof and Hobbs, "Improved media for growing plasmid and cosmid clones", Bethesda Research Labs Focus, Volume 9, page 12, 1987, cited in aforementioned Sambrook et al. reference). Induction of gene product expression from the luxPR promoter was achieved following the addition of the synthetic autoinducer, N-(3-oxohexanoyl)-DL-homoserine lactone, to the culture medium to a final concentration of 20 nanograms per milliliter (ng/ml). Cultures were incubated at 37° C. for an additional six hours. The bacterial cultures were then examined by microscopy for the presence of inclusion bodies and collected by centrifugation. Refractile inclusion bodies were observed in induced cultures, indicating that the Fc-fusions were most likely produced in the insoluble fraction in *E. coli*. Cell pellets were lysed directly by resuspension in Laemmli sample buffer containing 10% β-mercaptoethanol and then analyzed by SDS-PAGE. In most cases, an intense coomassie-stained band of the appropriate molecular weight was observed on an SDS-PAGE gel.

Purification. Cells were broken in water (1/10) using high pressure homogenization (two passes at 14,000 PSI), and inclusion bodies were harvested by centrifugation (4000 RPM in a J-6B centrifuge, for one hour). Inclusion bodies were solubilized in 6 M guanidine, 50 mM Tris, 10 mM DTT, pH 8.5, for one hour at a 1/10 ratio. For linear peptides fused to Fc, the solubilized mixture was diluted twenty-five times into 2 M urea, 50 mM Tris, 160 mM arginine, 2 mM cysteine, pH 8.5. The oxidation was allowed to proceed for two days at 4° C., allowing formation of the disulfide-linked compound (i.e., Fc-peptide homdimer). For cyclic peptides fused to Fc, this same protocol was followed with the addition of the following three folding conditions: (1) 2 M urea, 50 mM Tris, 160 mM arginine, 4 mM cysteine, 1 mM cystamine, pH 8.5; (2) 4 M urea, 20% glycerol, 50 mM Tris, 160 mM arginine, 2 mM cysteine, pH 8.5; and (3) 4 M urea, 20% glycerol, 50 mM Tris, 160 mM arginine, 4 mM cysteine, 1 mM cystamine, pH 8.5. The refolded protein was dialyzed against 1.5 M urea, 50 mM NaCl, 50 mM Tris, pH 9.0. The pH of this mixture was lowered to pH 5 with acetic acid. The precipitate was removed by centrifugation, and the supernatant was adjusted to a pH of from 5 to 6.5, depending on the isoelectric point of each fusion product. The protein was filtered and loaded at 4° C. onto an SP-Sepharose HP column equilibrated in 20 mM NaAc, 50 mM NaCl at the pH determined for each construct. The protein was eluted using a 20-column volume linear gradient in the same buffer ranging from 50 mM NaCl to 500 mM NaCl. The peak was pooled and filtered.

The peptibodies generated using the procedures above are set forth in Table 4 below.

TABLE 4

Peptibody	Peptibody Sequence	
L1 (N)	MGAQKFNPLDELEETLYEQFTFQQLEGGGGG-Fc	(SEQ ID NO:12)
L1 (N) WT	MKFNPLDELEETLYEQFTFQQLEGGGGG-Fc	(SEQ ID NO:13)
L1 (N) 1K WT	MKFNPLDELEETLYEQFTFQQGSGSATGGSGSTASSGS GSATHLEGGGGG-Fc	(SEQ ID NO:14)
2xL1 (N)	MGAQKFNPLDELEETLYEQFTFQQGGGGGGGGGKFNPL DELEETLYEQFTFQQLEGGGGG-Fc	(SEQ ID NO:15)
2xL1 (N) WT	MKFNPLDELEETLYEQFTFQQGGGGGGGKFNPLDELEE TLYEQFTFQQLEGGGGG-Fc	(SEQ ID NO:16)
Con4 (N)	MGAQQEECEWDPWTCEHMLEGGGGG-Fc	(SEQ ID NO:17)
Con4 (N) 1K-WT	MQEECEWDPWTCEHMGSGSATGGSGSTASSGSGSATH LEGGGGG-Fc	(SEQ ID NO:18)
2xCon4 (N) 1K	MGAQQEECEWDPWTCEHMGSGSATGGSGSTASSGSGS ATHQEECEWDPWTCEHMLEGGGGGFC	(SEQ ID NO:19)
L1 (C)	M-Fc-GGGGGAQKFNPLDELEETLYEQFTFQQLE	(SEQ ID NO:20)
L1 (C) 1K	M-Fc- GGGGGAQGSGSATGGSGSTASSGSGSATHKFNPLDELE ETLYEQFTFQQLE	(SEQ ID NO:21)
2xL1 (C)	M-Fc- GGGGGAQKFNPLDELEETLYEQFTFQQGGGGGGGGGKF NPLDELEETLYEQFTFQQLE	(SEQ ID NO:22)
Con4 (C)	M-Fc-GGGGGAQQEECEWDPWTCEHMLE	(SEQ ID NO:23)
Con4 (C) 1K	M-Fc- GGGGGAQGSGSATGGSGSTASSGSGSATHQEECEWDP WTCEHMLE	(SEQ ID NO:24)
2xCon4 (C) 1K	M-Fc- GGGGGAQQEECEWDPWTCEHMGSGSATGGSGSTASS GSGSATHQEECEWDPWTCEHMLE	(SEQ ID NO:25)
Con4-L1 (N)	MGAQEECEWDPWTCEHMGGGGGGGGKFNPLDELEET LYEQFTFQQGSGSATGGSGSTASSGSGSATHLEGGGGG Fc	(SEQ ID NO:26)
Con4-L1 (C)	M-Fc- GGGGGAQGSGSATGGSGSTASSGSGSATHKFNPLDELE ETLYEQFTFQQGGGGGQEECEWDPWTCEHMLE	(SEQ ID NO:27)
TN-12-9 (N)	MGAQ-FDYCEGVEDPFTFGCDNHLE-GGGGG-Fc	(SEQ ID NO:28)
C17 (N)	MGAQ-QYGCDGFLYGCMINLE-GGGGG-Fc	(SEQ ID NO:29)
TN8-8 (N)	MGAQ-KRPCEEMWGGCNYDLEGGGGG-Fc	(SEQ ID NO:30)
TN8-14 (N)	MGAQ-HQICKWDPWTCKHWLEGGGGG-Fc	(SEQ ID NO:31)
Con1 (N)	MGAQ-KRPCEEIFGGCTYQLEGGGGG-Fc	(SEQ ID NO:32)

In Table 4, "Fc" refers to the human Fc IgG1 sequence. Column two sets forth the amino acid sequence of the peptibody. The Fc portion thereof is labeled "Fc", and is as set forth in SEQ ID NO: 60 below. It will be appreciated that where a label is used, for example, "Con4" or "Con-4", this refers to the Con-4 peptide, whereas use of the suffix "C", "(C)", or "—C"; or "N", "(N)", or "—N" thereon indicates that the molecule is a peptibody as described herein. The suffixes "N", "(N)", or "—N" in a peptibody name indicate that the Ang-2-binding peptide (or peptides) is/are N-terminal to the Fc domain, and the suffixes "C", "(C)" or "—C" indicate that the Ang-2-binding peptide (or peptides) is/are C-terminal to the Fc domain. Furthermore, 2xCon4 (C) 1K, as defined in SEQ ID NO: 25, may also be referred to without the "1K" suffix herein.

The amino acid sequence of the Fc portion of each peptibody is as follows (from amino terminus to carboxyl terminus):

(SEQ ID NO: 60)

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV

SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL

HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS

Exhibit 8

1UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ADELLO BIOLOGICS. LLC, APOTEX INC. and APOTEX CORP.
Petitioners

v.

AMGEN INC. and AMGEN MANUFACTURING LIMITED
Patent Owner

Post-Grant Review No.: PGR2019-00001

PETITION FOR POST-GRANT REVIEW
OF U.S. PATENT NO. 9,856,287

Mail Stop **Patent Board**
Patent Trial and Appeal Board
U.S. Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

protein stabilizer and aggregation suppressor) and "6 M GdmHCl" (a denaturant). Ex. 1007 at [0074]; Ex. 1002 at ¶116.

Schlegl further indicates that the refold buffer should be customized for the protein of interest and may contain "a redox system (e.g., reduced glutathione GSH/oxidized glutathione GSSG)." Ex. 1005 at 19; Ex. 1007 at [0036]; Ex. 1002 at ¶117.

Therefore, Schlegl discloses contacting the protein with a preparation that supports the renaturation of a protein to a biologically active form, to form a refold mixture as described in claims 1 and 10. And Schlegl discloses preparing a solution comprising the proteins and the other components described in claims 16 and 26. Ex. 1002 at ¶118.

c. Components of the mixture

Schlegl discloses a refold buffer containing guadinium chloride, DTT and optionally a redox system (*e.g.*, GSH/GSSG), EDTA, detergents, salts, and refolding additives like L-arginine. Ex. 1007 at [0036]. These are "typical buffer components." Ex. 1007 at [0036]. Schlegl also discloses that compounds may be added to the refolding buffer to "suppress or completely prevent unfolding/aggregation" that were "known in the art," including "L-arginine, Tris, [and] detergents." Ex. 1005 at 40; Ex. 1007 at [0041]. Schlegl further discloses a

refold buffer containing "0.1 M Tris-HCl" (a protein stabilizer and aggregation suppressor) and "6 M GdmHCl" (a denaturant). Ex. 1005 at 38; Ex. 1007 at [0074]; Ex. 1002 at ¶119.

Therefore, Schlegl teaches a refold buffer containing an aggregation suppressor, protein stabilizer, and a denaturant. Ex. 1002 at ¶120.

d. Redox Components

Schlegl teaches optimizing the refold buffer for the particular protein to be refolded. Ex. 1007 at [0036]. This optimized refold buffer will include a redox component when refolding a protein containing disulfide bonds. Ex. 1007 at [0073]-[0082], and Ex. 1002 at ¶121; Ex. 1005 at 18. The optimized refold buffer containing a redox component will contain an amount of a reductant and an amount of an oxidant that allow for the disulfide bonds to reshuffle. Ex. 1007 at [0073]-[0082], and Ex. 1002 at ¶121.

The Example in Schlegl discloses contacting the bovine α-lactalbumin with a refold buffer comprising a redox component to form a refold mixture. Ex. 1005 at 26; Ex. 1007 at [0075]. As indicated in Schlegl, the refolding buffer may contain "a redox system (e.g., reduced glutathione GSH/oxidized glutathione GSSG)," Ex. 1007 at [0036], and a POSA would understand that the addition of cysteine and cystine here serve as the redox system or redox component for bovine

α-lactalbumin. Ex. 1005 at 26; Ex. 1002 at ¶122. That redox component has a thiol-pair ratio of 2. Ex. 1005 at 13 ("Schlegl futher describes a refolding buffer with a redox system having a defined thiol-pair ratio and redox buffer strength."), also at 26-27; Ex. 1007 at [0036], [0075]; Ex. 1002 at ¶122. Therefore, Schlegl discloses a thiol-pair ratio within the range of 0.001-100. Ex. 1005 at 27.

Finally, Schlegl teaches that the thiol-pair buffer strength maintains the solubility of the preparation (claims 1 and 10) and the solution (claims 16 and 26). Schlegl teaches that its method results in properly refolded proteins. Ex. 1007 at [0082]. This result would not be possible unless the redox components maintained the solubility of the protein while the protein refolded. Ex. 1002 at ¶123.

e. Incubating the refold mixture

Schlegl discloses: "[c]omplete refolding, including formation of disulfide bonds, proline isomerization and domain pairing may take hours and up to several days." Ex. 1007 at [0016]. Schlegl also discloses further incubation in the refolding tank to allow complete refolding of the protein. *Id*. at [0060]. Schlegl further teaches that its method yields "refolded protein at equilibrium [of] 63% for the batch system and 81% for the fed-batch system." *Id.* at [0082]. Schlegl confirmed that its results represented properly refolded protein:

> Native conformation of refolded protein is also confirmed by circular dichroism (see FIG. 6). The spectra

clear from the specification how the thiol-pair buffer strength maintains such solubility. Ex. 1002 at ¶¶192-193.

X. CONCLUSION

For the foregoing reasons, challenged claims 1-30 of the '287 Patent recite subject matter that is unpatentable. Therefore, Petitioners respectfully request institution of this post-grant review to cancel these claims.

Respectfully submitted,

October 1, 2018

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75, at 16-18. (December 30, 2016), referencing *In Re Packard*, 751 F.3d 1307, 1310 (Fed. Cir. 2014).

Exhibit 9

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

APOTEX INC. and ADELLO BIOLOGICS, LLC
Petitioners

v.

AMGEN INC. and AMGEN MANUFACTURING LIMITED
Patent Owner

Post Grant Review No.: PGR2019-00001

DECLARATION OF ANNE S. ROBINSON, PH.D

that were "known in the art," including "L-arginine, Tris, [and] detergents." *Id.* at [0041]. Schlegl further discloses a refold buffer containing "0.1 M Tris-HCl" (a protein stabilizer and aggregation suppressor) and "6 M GdmHCl" (a denaturant). *Id.* at [0074].

120. Therefore, Schlegl teaches a refold buffer containing an aggregation suppressor, protein stabilizer, and a denaturant.

(d) Redox Components

Claims 1 and 10	Claims 16 and 26
"an amount of oxidant; and an amount of reductant, *wherein the amounts of the oxidant and the reductant are related through a thiol-pair ratio and a thiol-pair buffer strength,* *wherein the thiol-pair ratio is in the range of 0.001-100,* *wherein the thiol-pair buffer strength maintains the solubility of the* ***preparation****"*	*"an amount of oxidant; and an amount of reductant,* *wherein the amounts of the oxidant and the reductant are related through a thiol-pair ratio and a thiol-pair buffer strength,* *wherein the thiol-pair ratio is in the range of 0.001-100,* *wherein the thiol-pair buffer strength maintains the solubility of the* ***solution****"*

121. Schlegl teaches optimizing the refold buffer for the particular protein to be refolded. *Id.* at [0036]. In my view, this optimized refold buffer will include a redox component when refolding a protein containing disulfide bonds, as proteins with disulfide bonds would not properly fold without the use of a redox

system. *Id.* at [0073]-[0082]. The optimized refold buffer containing a redox component will contain an amount of a reductant and an amount of an oxidant that allow for the disulfide bonds to reshuffle. *Id.* at [0073]-[0082].

122. The Example in Schlegl discloses contacting the bovine α-lactalbumin with a refold buffer comprising a redox component as part of the dilution refold method of Schlegl to form a refold mixture. *Id.* at [0075]. As indicated in Schlegl, the refolding buffer may contain "a redox system (e.g., reduced glutathione GSH/oxidized glutathione GSSG)," Ex. 1007 at [0036], and in my opinion, a POSA would understand that the addition of cysteine and cystine here serve as the redox system or redox component for bovine α-lactalbumin. That redox component has a thiol-pair ratio of 2. Ex. 1007 at [0036], [0075], [0079]-[0080] also includes the section entitled, "Quenching of Oxidative Refolding," which teaches both the use of redox chemistry in protein refolding, and in fact suggests customizing the refold buffer. *Id.* at [0036]. The TPR and RBS calculated in the refold mixture are 1.94 and 5.8, respectively.[7] Therefore, Schlegl discloses a thiol-pair ratio within the range of 0.001-100.

---

[7] Applying Equation 1 of the '287 patent ([reductant]^2/[oxidant]), the thiol-pair ratio of the refold mixture in the Example of Schlegl is 2. However, as one must also include the dilution by protein (1/32 of total volume), resulting in a value of 1.94:

$$[\text{Cysteine}]^2/[\text{Cystine}] = [2*31/32]^2/[2*31/32] = 1.94$$

123. Finally, Schlegl teaches that its method results in properly refolded proteins. *Id.*at [0082]. This result would not be possible unless the redox components maintained the solubility of the protein while the protein refolded. Even the loss of one disulfide bond (Cys6-Cys120) results in formation of a partially unfolded (molten globule) form that is not the native structure, as it lacks the tertiary structure of the native protein, and appears more similar to the unfolded protein. In fact, a variant lacking these cysteines spontaneously rearranges its disulfide bonds, forming some native and some non-native disulfide bonds. Ex. 1041 at 104.

(e) Incubating the refold mixture

Claims 1 and 16	Claims 10 and 26
"incubating the refold mixture so that at least about 25% of the proteins are properly refolded."	*"incubating the refold mixture so that about 30-80% of the proteins are properly refolded."*

124. Schlegl discloses: "[c]omplete refolding, including formation of disulfide bonds, proline isomerization and domain pairing may take hours and up

---

Applying Equation 2 of the '287 patent (2[oxidant] + [reductant]), the redox buffer strength of the refold mixture in the Example of Schlegl is 6 mM. Applying the dilution, the value is 5.8 mM:

2[Cystine] + [Cysteine] = 2[2*31/32] + [2*31/32] = 5.8 mM.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Respectfully submitted,

Dr. Anne S. Robinson

Date: 10/1/2018

Exhibit 10

US 20070238860A1

(19) **United States**

(12) **Patent Application Publication**

SCHLEGL

(10) **Pub. No.: US 2007/0238860 A1**

(43) **Pub. Date: Oct. 11, 2007**

(54) **METHOD FOR REFOLDING A PROTEIN**

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(21) Appl. No.: **11/695,950**

(22) Filed: **Apr. 3, 2007**

(30) **Foreign Application Priority Data**

Apr. 10, 2006 (EP) ..... EP 06112 443

Publication Classification

(51) **Int. Cl.**
C07K 14/47 (2006.01)

(52) **U.S. Cl.** **530/350**

(57) **ABSTRACT**

A method for refolding a protein by mixing a protein solution with a refolding buffer at mixing conditions that approximate ideal mixing. The method can be carried out batch wise, in a fed-batch mode or continuously with on-line solubilization of inclusion bodies.

Figure 1

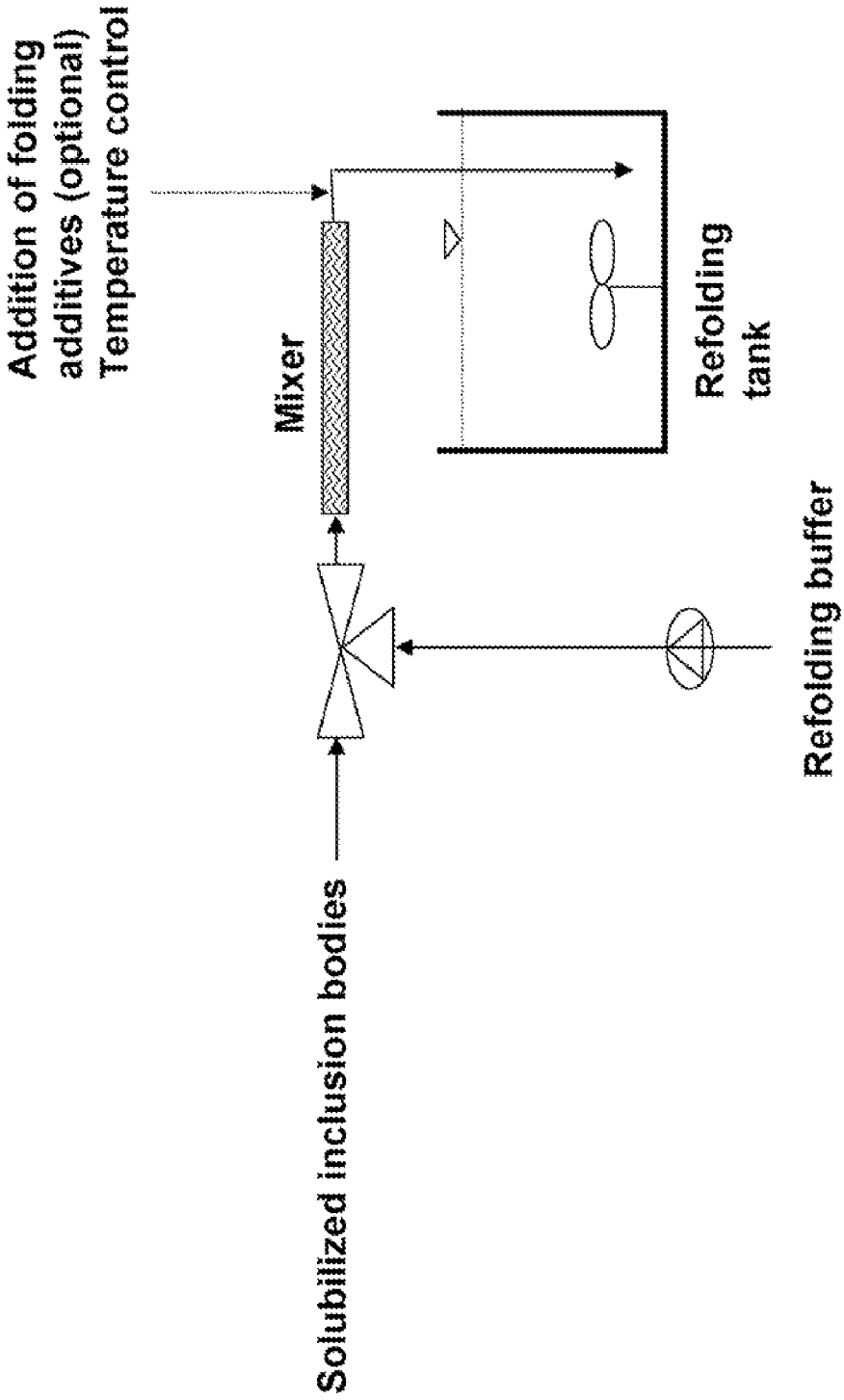

Addition of folding
additives (optional)
Temperature control
Mixer
Refolding
tank
Refolding buffer
Solubilized inclusion bodies

Figure 2

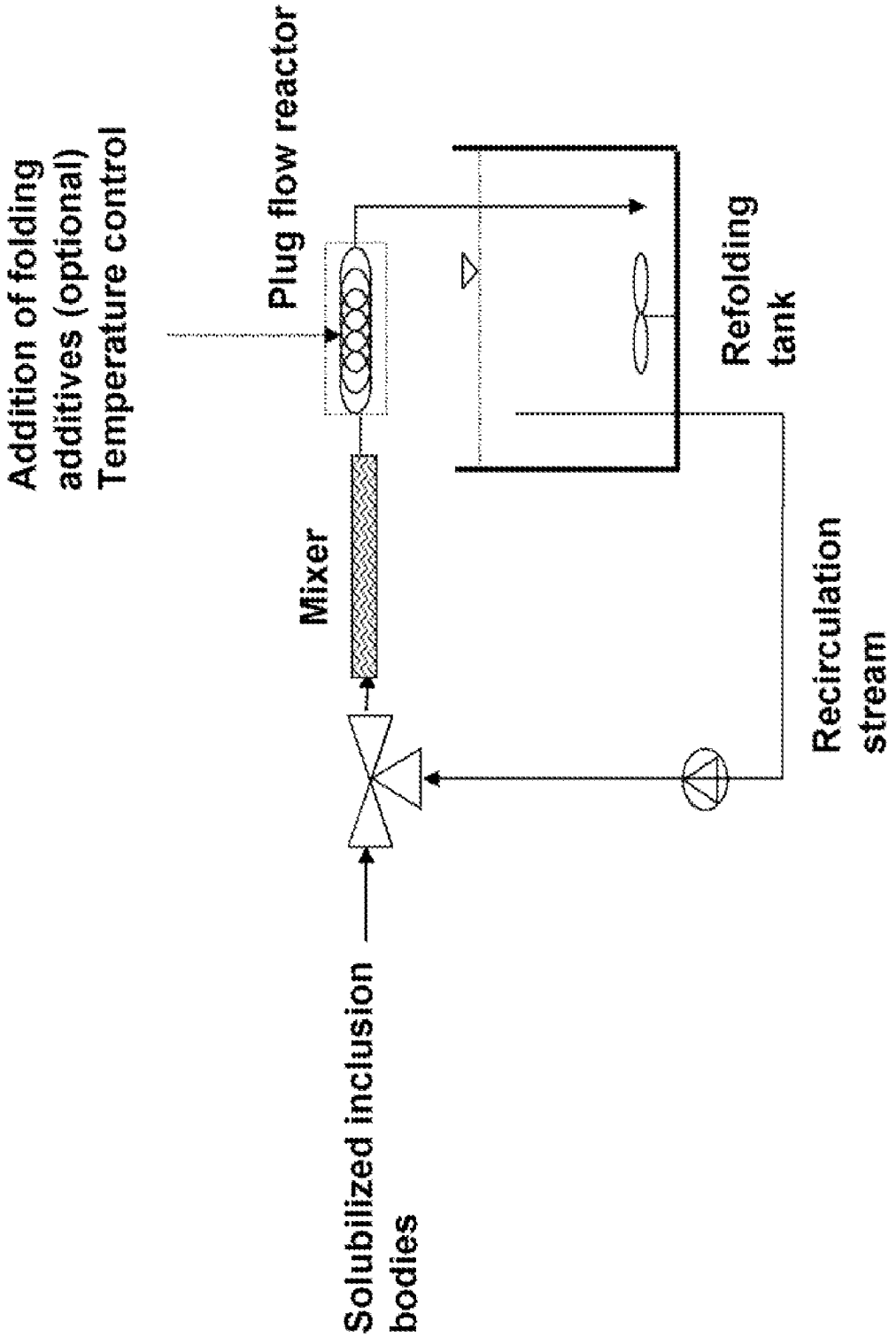

Addition of folding
additives (optional)
Temperature control
Plug flow reactor
Refolding
tank
Mixer
Recirculation
stream
Solubilized inclusion
bodies

Figure 3

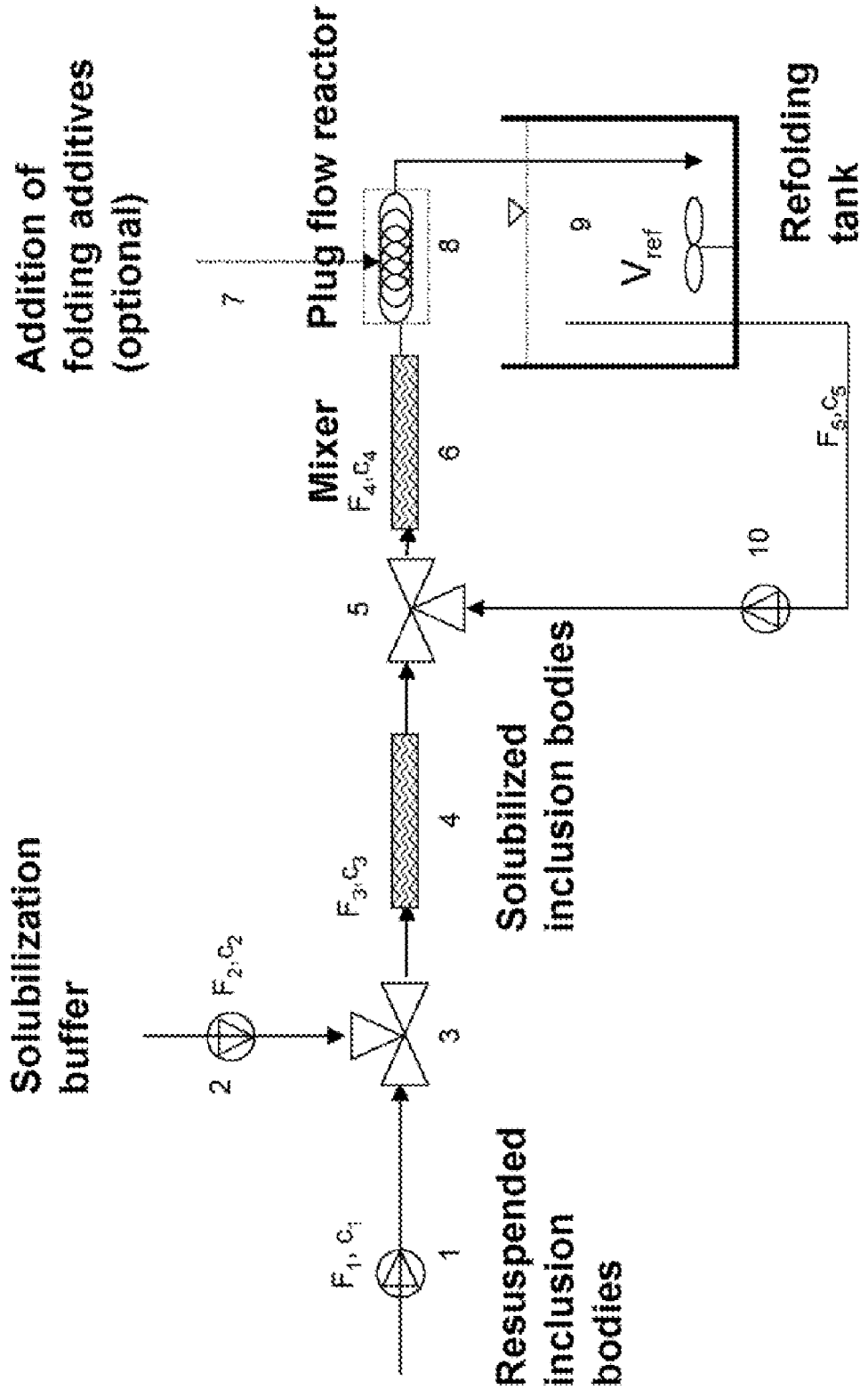

Addition of folding additives (optional)
Plug flow reactor
Mixer
Refolding tank
Solubilization buffer
Solubilized inclusion bodies
Resuspended inclusion bodies
F_1, c_1
F_2, c_2
F_3, c_3
F_4, c_4
F_5, c_5
V_{ref}
1
2
3
4
5
6
7
8
9
10

Figure 4

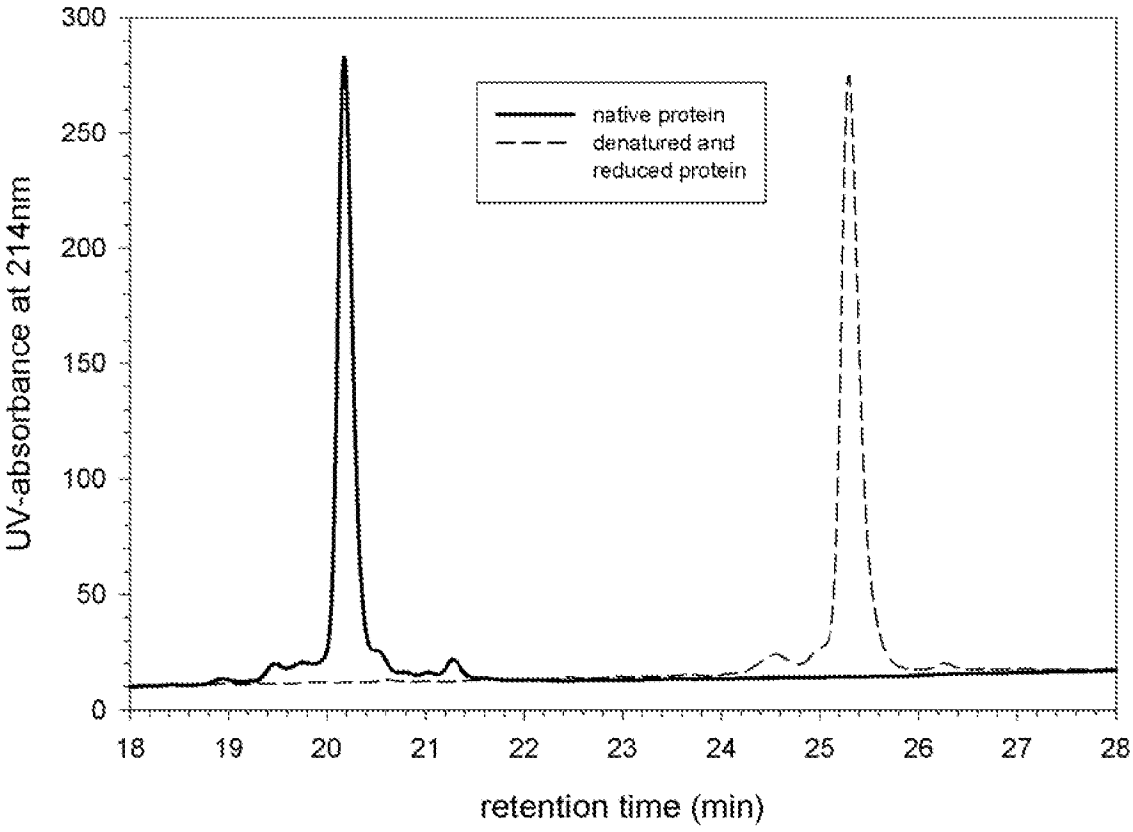

300
250
200
150
100
50
0
UV-absorbance at 214nm
native protein
denatured and
reduced protein
18
19
20
21
22
23
24
25
26
27
28
retention time (min)

Figure 5

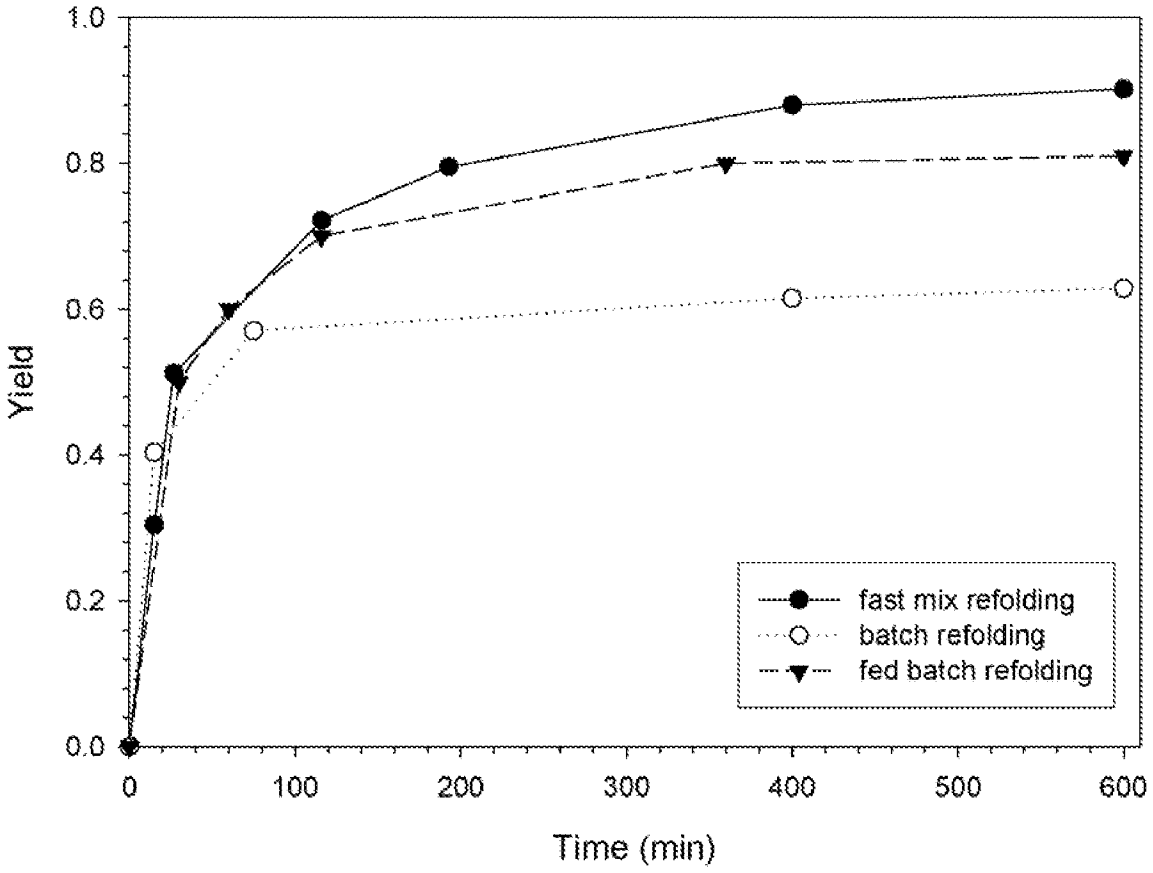
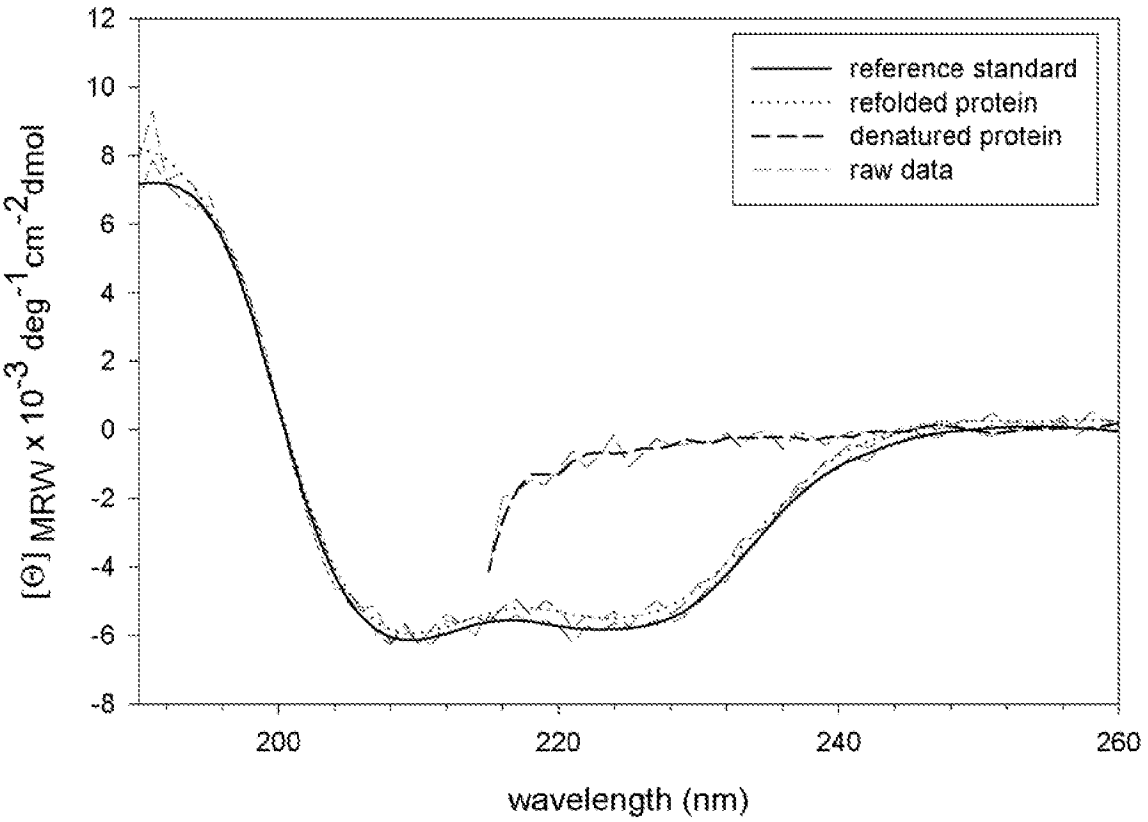

1.0
0.8
0.6
0.4
0.2
0.0
Yield
0
100
200
300
400
500
600
Time (min)
fast mix refolding
batch refolding
fed batch refolding

Figure 6


reference standard
refolded protein
denatured protein
raw data
[Θ] MRW x 10^-3 deg^-1 cm^-2 dmol
wavelength (nm)
12
10
8
6
4
2
0
-2
-4
-6
-8
200
220
240
260

METHOD FOR REFOLDING A PROTEIN

[0001] This application claims priority benefit to EP 06 112 443, dated Apr. 10, 2006, the entirety of which is incorporated herein.

[0002] The invention relates to the field of recombinant protein production

[0003] Proteins for industrial applications, e.g. for use as biopharmaceuticals or fine chemicals, are either obtained by extraction and purification from a natural source, such as a plant or animal tissue or microorganisms, or by means of recombinant DNA technology.

[0004] To produce a recombinant protein, the cDNA encoding the protein of interest is inserted into an expression vector and the recombinant vector is transformed into host cells, which are grown to express the protein. The host cells may be selected from microorganisms such as bacteria, yeast or fungi, or from animal or plant cells.

[0005] Expression of a recombinant protein is a complex event. To obtain the correct conformation, the protein is associated with so-called "folding helper proteins" and enzymes. The folding helper proteins, also termed "chaperones" or "minichaperones", interact in a complex way so that the protein regains its native conformation after passing through various intermediate states. Some of the intermediate states may be quite stable. Enzymes involved in protein maturation either catalyze the rapid formation of disulfide bridges (1; 2), the isomerization of prolyl-peptide linkages (3-6) or more complex modifications, such as the truncation of the protein, side chain modifications or modifications of the N- and C-terminus. When a protein is efficiently overexpressed, the production of the nascent peptide chain occurs faster than the folding of the protein. For some proteins, an intermediate state may also form aggregates (in the following, the term "intermediate" forms also encompasses aggregate forms). Overall, aggregate formation occurs much faster than the complete folding of a protein (7; 8).

[0006] In expression systems, in which such conditions are present, the protein is deposited in the cells in a paracrystalline form, so-called "inclusion bodies", also termed "refractile bodies".

[0007] Since the protein, when present in the form of insoluble inclusion bodies, is shielded from enzymatic attack like proteolysis, it cannot interfere with the physiology of the cells. Therefore, recombinant DNA technology has taken advantage of this aberrant way of protein secretion, e.g. for the production of the proteins that are toxic for the cells (9).

[0008] Various steps have to be taken to obtain a protein from host cells, in which it is accumulated in a denatured form, i.e. a conformational state without biological activity, in its correctly refolded form. For example, bacterial cells carrying inclusion bodies are disintegrated, the inclusion bodies harvested by centrifugation and then dissolved in a buffer containing a chaotropic agent. The denatured protein is then transferred into an environment that favors the recovery of its native conformation. Before adopting its native state, the protein undergoes a transition through various semi-stable intermediates. Since intermediates in the early stages of the folding pathway have highly exposed hydrophobic domains, which are prone to associate, they tend to form aggregates. Obviously, intramolecular interactions are concentration-independent, whereas intermolecular interactions are concentration-dependent. The higher the protein concentration, the higher the risk of intermolecular misfolding, and vice versa. In principle, refolding, also termed "renaturation", may be considered as a race against aggregate formation, which usually follows second or higher order reaction kinetics, while refolding of the protein follows first order reaction kinetics (10).

[0009] A protein can be refolded from its denatured conformation to the correctly folded conformation by transferring it into an environment that favors the change to the native conformation. During this rearrangement, the protein passes through several intermediate conformational states, which are prone to form aggregates. Depending on the individual protein and on the environmental conditions, the aggregates may precipitate. Independent of whether the aggregates remain soluble or whether they precipitate, this process leads to dramatic losses in the yield of correctly folded protein.

[0010] During a folding reaction, several characteristic conformations are formed. Although the transition from one conformation to another is smooth and a characterization of the distinct conformations is not available yet, similar states have been reported for different proteins. Immediately after initiation of the folding reaction, the unfolded protein collapses and a partly structured intermediate state is formed. This change in conformation is called burst phase and appears in the sub millisecond time scale. Rapid changes in spectroscopic properties, such as fluorescence and far UV-CD are due to the molecular collapse of the protein. For lysozyme, molecular compaction and formation of globular shape was detected with small angle X-ray scattering and tryptophan fluorescence (11). Other examples of proteins where a burst phase was detected are ovalbumin (12), barstar (13), cytochrom C (14), dihydrofolat reductase (15) and α-lactalbumin (16). After the burst phase, a more compact structure is formed, the 'molten globule' intermediate. The molten globule is defined as state with native-like secondary structure but fluctuating tertiary structure (17). It was proposed as a common intermediate in folding pathways and a number of proteins pass through a molten globule structure during folding. Intermediates in early folding steps cannot be detected, either due to very rapid or very little structural changes. In later folding events, reorganization of tertiary contacts takes place. These reactions are slow compared to formation of secondary and tertiary structure. They comprise generation and reshuffling of disulfide bonds, proline isomerization and domain pairing. Disulfide bond intermediates can be detected for example with reversed phase chromatography. Association of native monomers to biologically active oligomers is the final step in the case of larger proteins.

[0011] With some currently available methods, refolding of proteins is achieved either by diluting the protein in a refolding buffer in a batch or continuous mode (18-20). In these methods, batch wise dilution results in highly diluted protein solutions and therefore large process volume, which often is the bottleneck in industrial processes.

[0012] In another approach the naturally occurring folding pathway is simulated by adding chaperons and/or minichaperons, and/or enzymes that catalyze certain steps in the in vivo folding pathway (2; 21-25). Complex refolding reactor systems comprising series of tanks have been designed to improve the refolding reaction (26).

[0013] In another approach, the helper proteins and enzymes are immobilized to a solid phase. Then the protein solution is passed over a so-called "Packed Bed" that contains the immobilized helper proteins and/or helper enzymes, whereby the protein is folded into its native conformation (27-30). Since the folding helper proteins and enzymes must be present in a stoichiometric ratio, this process requires almost the same amount of helper proteins, which in turn have to be produced by recombinant DNA technology, as the finally obtained protein of interest. In addition, to improve folding, the helper proteins are usually fused to the protein of interest, which requires further processing of the fusion protein. For these reasons, this strategy is very cost intensive and not applicable on an industrial scale.

[0014] WO 02/057296 discloses an on-line method for refolding a protein by dilution and subsequent separation. The solution containing unfolded protein is diluted with refolding buffer by mixing in a mixing chamber and the output of this dilution step is directly loaded onto the separation device, e.g. a chromatographic column. By optionally varying the length of the tubing between the mixing chamber and the column, the time for refolding the protein in solution—before it is bound to the column—can be adjusted. This system is limited to proteins with fast refolding kinetics and to proteins with low requirements as regards adjusting the conditions of the separation step to those of the antecedent refolding step.

[0015] Dilution of the unfolded protein with the refolding buffer using a flow-type reactor was described by Terashima et al (31): Denatured lysozyme is continuously diluted in a small mixing unit and directed to a packed column with a flow that closely approaches a plug flow. The achieved refolding efficiencies in the flow type reactor are hardly superior to those of a batch system.

[0016] Among the known refolding strategies, dilution is still the simplest methodology. In industrial scale applications, dilution is commonly used for refolding of recombinant proteins, expressed as inclusion bodies. Typically, dilution is carried out in one step by mixing/diluting the solution containing solubilized protein with a diluent containing a solubilizing agent in an amount necessary to reach the optimal level of dilution. When the concentration of solubilizing agent is below a certain threshold level, the protein start to regain its biologically active three-dimensional conformation. Depending on the specific protein and the chosen folding conditions, refolding begins within milliseconds to seconds. In this initial burst phase, the protein is highly susceptible to aggregation. To minimize aggregation, the protein concentration has to be kept low. After this initial refolding phase, the protein forms into a more compact structure. This intermediate structure, which is sometimes termed 'molten globule', is defined as a state with a secondary structure that resembles that of the native protein and that is less susceptible to aggregation. Complete refolding, including formation of disulfide bonds, proline isomerization and domain pairing may take hours and up to several days.

[0017] Usually, such dilution is carried out as a so-called "batch" dilution, in which the diluent is added in a defined volume, the "batch", to the unfolded protein solution. Batch dilution has many disadvantages when carried out at large scale. In commercial protein purification methods, depending on the dilution rate, the total volumes being handled at the same time can be very large, usually between several hundreds or thousand liters. In such processes, variations in refolding efficiency are caused by ill-defined operating variables with regard to feed rate and mixing, which result in non-robust processes during scale-up with (32).

[0018] During batch refolding, all of the protein in the reactor is transiently present in the form of reactive intermediates, resulting in a brief period of aggregation. Therefore, optimum operation occurs at extremely low overall protein concentration. Additionally, refolding a protein in large volumes by batch dilution may cause some re-aggregation of the protein, probably because the solution, at least as initially present in batch dilutions, is not homogeneous. This may result in a lower net yield of refolded protein. The non-homogeneity of the solution in batch dilutions results from the difficulty in timely achieving "ideal" mixing conditions, which are required for obtaining homogeneity, in large volumes.

[0019] Ideal mixing conditions in a refolding mixture are given when the composition of the mixture with respect to its physical-chemical properties is identical at each time interval for each infinite small volume element in the refolding tank. In theory, "ideal" mixing conditions result in a homogenous solution without concentration gradients of unfolded or partially refolded protein during dilution. Ideal mixing conditions are a function of a solution's "mixing time". Mixing time is the time needed for the molecules in a droplet between addition of the droplet to the solution and their even dispersion in the total volume of the solution. Variables affecting mixing time include the total volume of the solution, the size of the added volume, the size and configuration of the mixing chamber (vessel, tank), and other characteristics of the mixing device, e.g. whether stirring occurs and which type of stirrer is used, and the location of the inlets in the mixing chamber. The larger the volume of the solution and the larger the size of the reaction vessel, the longer is the mixing time and thus the longer it takes until the mixture, e.g. the solubilized protein solution and the diluent; will not be homogenous. As reported by Ram et al. (33), mixing time in process vessels used in biopharmaceutical manufacturing can last up to several minutes.

[0020] Due to the concentration gradient present in a non-homogenous solution, there are variations of the pH value and ionic strength, which results in variations of the charges of the unfolded or partially folded protein causing the protein to refold incorrectly or interact improperly with nearby protein molecules. A high local concentration of unfolded protein in the regions of the mixing chamber where the unfolded protein is fed into the reactor, may lead to higher aggregation compared to an "ideal" mixing chamber.

[0021] In so-called "fed-batch" processes, the unfolded protein is added to the refolding tank in a semi-continuous or pulse wise manner, which results in a lower actual concentration of folding intermediates and therefore less aggregation (34). Such methods have the advantage that the actual concentration of unfolded protein is kept low, while the final concentration of refolded protein can be increased. The composition in terms of the protein's state in the refolding mixture changes from the first molecule (virtual isolation, best chance of successful folding into native conformation) to the last molecule, which is added to a volume containing the correctly folded or misfolded proteins (worst chance of successful refolding). Like in batch meth-

ods, renaturation that is conducted pulse-wise (fed-batch mode) can be only operated in a discontinuous way. In a fed-batch reactor, the amount of denaturing and reducing agents from the feed solution start to accumulate during addition of the unfolded protein until they reach a critical level at which the protein starts to unfold. Design equations for fed-batch refolding with regard to the folding and aggregation kinetics have been described by Dong et al. (35) and Kotlarsky et al. (36).

[0022] It was an object of the invention to provide an improved method for obtained a protein in its refolded, biologically active form.

[0023] The solution of the problem underlying the invention is based on refolding the protein under defined mixing conditions.

[0024] The present invention relates to a method for obtaining a biologically active recombinant protein by reconstituting the protein from a denatured state to its active form, said method containing a steps of mixing a feed solution containing the protein in its denatured form and/or its biologically inactive intermediate forms with a refolding buffer under conditions that approximate ideal mixing, wherein

[0025] i. the mixing time is ca. 1 msec to ca. 10 sec; and

[0026] ii. the dilution rate F_P:F_B is ca. 1:1 to ca. 1:100000, wherein

[0027] F_P is the flow rate of said protein feed solution and

[0028] F_B is the flow rate of said refolding buffer.

[0029] "Denatured form", in the meaning of the present invention, designates the biologically inactive, unfolded or predominantly misfolded form of the expressed protein of interest, as obtained as a product of the recombinant production process, e.g. as obtained after dissolving the inclusion bodies.

[0030] "Intermediate forms" or "intermediates" in the meaning of the present invention, designates the forms that the protein passes through between its denatured form and its reconstituted (refolded) native and biologically active state. The intermediates, which are biologically inactive or have a lower biological activity than the native protein, may form aggregates.

[0031] A "protein" in the meaning of the present invention is any protein, protein fragment or peptide that requires refolding upon recombinant expression in order to obtain such protein in its biologically active form.

[0032] Preferred mixing times range from ca. 10 msec and ca. 5 sec, preferably from ca. 100 msec to ca. 1 sec.

[0033] By maintaining a very high flow rate of the refolding buffer and a low flow rate of the feed stream containing the unfolded protein, the method of the invention provides very high local dilution rates; preferred dilution rates range from 1:5 to 1:50000 and from 1:10 to 1:10000.

[0034] Depending on the dimensions of the system, the flow rates may vary within a wide range, e.g. from μL/min in the case of laboratory scale to Liters/min in the case of industrial scale manufacturing.

[0035] The concentration of the protein after dilution with refolding buffer is in the range of ca. 1 ng/ml to 10 mg/ml, for example ca. 100 ng/ml to ca. 5 mg/ml or ca. 1 μg/ml to ca. 1 mg/ml.

[0036] The refolding buffer used for a given protein of interest is customized to the refolding requirements/kinetics of that protein. Refolding buffers are known in the art and commercially available; typical buffer components are guadinium chloride, dithiothreitol (DTT) and optionally a redox system (e.g. reduced glutathione GSH/oxidized glutathione GSSG), EDTA, detergents, salts, and refolding additives like L-arginine.

[0037] As mentioned above, "ideal mixing" refers to conditions that result in a homogenous solution without substantial concentration gradients in solution. By ideal mixing, infinitive short mixing times are achieved.

[0038] Since the mixing conditions according to the method of the invention are close to ideal mixing, mixing of the protein feed stream with the refolding buffer occurs with similar or faster kinetics than the unfolding/aggregation kinetics of the protein, thereby reducing or completely preventing aggregation of the protein

[0039] In the process of the invention, the actual protein concentration immediately after mixing is much lower as compared to conventional refolding methods.

[0040] In its simplest embodiment, the method of the invention is a batch process that comprises, as its essential step, the above-defined mixing operation, in which a feed stream having a high concentration of unfolded protein and a low flow rate is combined with a refolding buffer solution having a high flow rate.

[0041] This embodiment of the invention, which is schematically shown in FIG. **1**, is particularly useful for proteins that have very fast refolding kinetics, e.g. peptides and smaller protein fragments. The refolding buffer and the protein feed solution are independently fed from reservoirs to the mixing device. Having passed the mixing device, the highly diluted solution containing the refolded protein is collected in a tank. Optionally, before entering the tank, refolding additives may be added in the case of proteins that have not yet completely refolded during mixing to suppress or completely prevent unfolding/aggregation. Compounds useful as refolding additives are known in the art, examples are L-arginine, Tris, detergents, redox systems like GSH/GSSG, ionic liquids like N'-alkyl and N'-(omega-hydroxy-alkyl)-N-methylimidazolium chlorides etc. The end of the process is reached when the reservoir of refolding buffer and/or protein solution is exhausted. At this point, the feed of unfolded protein (or the feed of buffer, respectively) is interrupted and the solution containing the protein in its refolded, biologically active form is withdrawn from the tank. In this embodiment of the invention, it is advantageous to have the mixing device equipped with means that control the temperature to exclude any, even minimal aggregation, e.g. cooling means.

[0042] Mixing devices suitable for use in the method of the invention are any mixers that ensure fast mixing and short mixing times, e.g. tubular jet mixers or static mixers, e.g. commercially available mixers from Fluitec, CH, or Sulzer Chemtech, CH. In the simplest form of the method of the invention, the two streams can be combined into one stream by a branch connection without any additional specific mixing devices. Such a simple device can be used to achieve the desired mixing efficiency, albeit without precise control of mixing efficiency. In the case that the mixer is a high-throughput continuous flow device, accurate control of the flows is of particular importance. With such mixers, mixing times as low as a few milliseconds on the small scale or a few seconds on the large scale can be achieved. The mixing characteristics of such mixers most closely approximate "ideal mixing". The mixing ratio of the two streams is

adjusted such that a low protein concentration is maintained to minimize aggregation. After mixing the two streams, the protein starts to refold.

[0043] The method of the invention is also referred to as "fast mix refolding".

[0044] Except for proteins with fast refolding kinetics, which may already be completely refolded during mixing, the initial refolding steps take place in the mixing device and refolding is completed in the refolding tank or in the optionally present adjustment zone, e.g. in the plug flow reactor (PFR), as described below.

[0045] In a further embodiment, the method of the invention comprises in addition, subsequent to the mixing step defined above and before the solution enters the refolding tank, a step in which the highly diluted mixture is transferred to a zone in which the protein is allowed to form more stable folding intermediates under precisely controlled conditions such that unfolding and formation of aggregates is suppressed or completely prevented. This step is also referred to as "adjustment step", and the zone or the reactor in which adjustment occurs is referred to as "adjustment zone" or "adjustment reactor", respectively.

[0046] In a preferred embodiment, the adjustment reactor is a plug flow reactor, i.e. a chemical reactor where the fluid passes through in a coherent manner, so that the residence time is the same for all elements. An ideal plug flow reactor has a fixed residence time:

[0047] Any fluid that enters the reactor at time t will exit the reactor at time t+τ, where τ is the residence time of the reactor. In its simplest form, the plug flow reactor is a tube, optionally packed with solid material.

[0048] The adjustment step provides the possibility of generating, for a defined volume and period of time, conditions that favor stabilization of the partially refolded protein. This may be achieved by a short-term change of the pH value (increase or decrease) and/or change of the temperature (heating or cooling) and/or addition of refolding additives, as defined above, in the adjustment zone. The adjustment step provides the advantage that optimal refolding conditions, e.g. heating or cooling or addition of additives, need to applied only to a small volume as compared to the refolding tank, thus saving energy, reagents and costs.

[0049] The mean residence time, i.e. the time that it takes for the solution to pass through the adjustment reactor, i.e. the tube in the case of a plug flow reactor, depends on the flow rate and the tube volume. The residence time should be long enough to allow the protein to fold into a more compact and stable structure, e.g. into a so-called 'molten globule' intermediate.

[0050] By varying the design of the adjustment reactor, e.g. length and/or diameter of the tube, the residence time of a specific protein in the adjustment zone and thus its exposure to the selected adjustment conditions is adapted to the requirements of the protein, i.e. its specific refolding kinetics. In the case of fast refolding kinetics, refolding is usually completed already in the adjustment zone.

[0051] After leaving the adjustment zone, the protein solution containing the refolded protein and/or the partially refolded stabilized intermediates is collected in the refolding tank, where, if still necessary, refolding is completed.

[0052] In the embodiment that provides an adjustment step, the method of the invention may be conducted batch-wise or preferably, by recycling the protein solution from the refolding tank, in the fed-batch mode.

[0053] In the batch mode, the end of the process is reached when the reservoir of refolding buffer and/or protein solution is exhausted. At this point, the feed of unfolded protein, or of the refolding buffer, respectively, is interrupted and the protein solution is withdrawn from the tank. FIG. **2** shows the schematic drawing of the embodiment of the invention that is a fed-batch process comprising an adjustment step in combination with recycling of the protein solution. In this embodiment, the protein solution circulates at high flow rates from the refolding tank back to the feed inlet, where unfolded protein is freshly introduced into the system. In such embodiment, the recycled protein solution forms the refolding buffer solution.

[0054] In a preferred embodiment, the method of the invention is performed on-line and, even more preferred, in a continuous mode. "On-line" means that refolding is connected to one or more other steps, e.g. antecedent steps, of the overall process, e.g. solubilization of inclusion bodies.

[0055] By running refolding continuously and on-line with solubilization of inclusion bodies, as depicted in FIG. **3**, time consumption and costs can be reduced and the yield of refolded protein increased as compared to known methods. The method of the invention ensures, in particular in its continuous on-line embodiment, fast and efficient processing of inclusion body proteins, thereby reducing inadvertent variations, such as variations in refolding efficiency or product homogeneity. On-line solubilization of suspended inclusion bodies is preferred to their batch-wise solubilization in a stirred tank, where the contact time between the molecules and the solubilizing agent has to be minimized or precisely controlled to avoid irreversible modification of the proteins. This is often the case when solubilization of the inclusion bodies is carried out at extreme pH values. Such irreversible modification of amino acid side chains could lead to reduced activity of the molecule.

[0056] Exemplified by the embodiments in which the protein solution is recycled (FIGS. **2** and **3**), very high local dilution rates (1:1000, 1:10000 or more) can be easily achieved depending on the ratio of the flow rate of the solution of unfolded protein F_B (designated F_3 in the Figure) and the flow rate of the circulating refolding buffer F_B (designated F_5 in the figure). The protein concentration C_4 after
dilution can be calculated by a simple mass balance as

$$C_3F_3+C_5F_5=C_4F_4 \text{ and } F_4=F_3+F_5$$

[0057] C_3 is the concentration of the unfolded protein in the feed stream and C_4 is the concentration of unfolded protein immediately after mixing. The flow rate of the circulating stream necessary to achieve the desired concentration of unfolded protein after dilution (C_4) can be simply calculated by neglecting C_5 (refolded protein present in the reaction system, which is less susceptible to aggregation) as

$$F_5=((C_3\times F_3)/C_4)-F_3$$

[0058] The total protein concentration in the reaction system increases between the addition of the unfolded protein and the time point when the desired final concentration is reached, e.g. at 1 μg/ml/min. Addition of the solution of unfolded protein is either stopped when the desired concentration is reached or when the concentrations of denaturing and reducing chemicals of the feed stream exceed a value that is critical for the protein to unfold.

[0059] The volume of refolding buffer solution V_{ref} in the refolding tank prior to starting the addition of the unfolded

protein stream depends on the desired final protein concentration in the reactor (C_5) after complete processing the unfolded protein solution V_{denat} and can be calculated by

$$V_{ref}=((C_3\times V_{denat})/C_5)-V_{denat}$$

[0060] When addition of the desired volume/amount of unfolded protein is completed, the solution can be further incubated in the refolding tank to allow complete refolding of the protein. The time period for such subsequent refolding depends on the refolding kinetics of the protein.

[0061] In the continuous mode, the refolding solution circulates via an additional pump back to the inlet of the feed solution containing unfolded protein. Depending on the flow rate of the feed stream and the flow rate of the refolding solution, high dilution rates can be achieved after mixing of the two streams. This effect and the continuous supply of the unfolded protein (or approximately continuous by fed-batch addition, respectively) result in higher conversion of unfolded protein into the native, biologically active protein as compared to batch or fed-batch refolding without recirculation Addition of the feed solution is stopped when the concentration of denaturing agents from the feed stream, e.g. urea or DTT, has reached a critical threshold value.

[0062] Particularly in the continuous mode, precise control of the dilution step (protein concentration, mixing time) as well as residence time and selected refolding parameters in the adjustment zone allow a more efficient renaturation of the protein as compared to the known batch or fed-batch dilution methods.

[0063] The feed has been obtained from fermentation of bacterial, yeast, fungal, plant or animal cells carrying an expression vector to produce a heterologous protein of interest. The feed is a protein solution, usually obtained from solubilization of the inclusion bodies. In the on-line mode of the method of the invention, the feed stream, when it enters the system, contains the resuspended inclusion bodies, which are solubilized on-line before the protein feed is combined with refolding buffer and enters the mixing zone.

[0064] The protein feed contains, besides buffer substances, components that promote the solubilization of inclusion bodies, e.g. chaotropic agents such as urea, guanidinium chloride (GdmCl), sodium and/or potassium thiocyanate, and reducing agents such as mercaptoethanol, dithiothreitol, monothiogylcerol. Suitable compositions and conditions for solubilization of inclusion bodies are known in the art, they have been extensively described in the literature (11; 12; 37).

[0065] In a final step, the protein is separated and purified according to methods known in the art, including, but not limited to, dialysis, filtration, extraction, precipitation and chromatography techniques.

BRIEF DESCRIPTION OF THE FIGURES

[0066] FIG. **1**: Setup for fast mix refolding in its simplest form

[0067] FIG. **2**: Setup for fast mix refolding, including an adjustment zone and recycling of the refolded protein

[0068] FIG. **3**: Setup for continuous fast mix refolding in combination with on-line solubilization of inclusion bodies

[0069] 1) feed pump delivering the resuspended inclusion bodies; 2) solubilization buffer pump; 3, 5) branch connection; 4, 6) mixing device; 7) addition of folding additives (optional); 8) plug flow reactor; 9) refolding mixture vessel; 10) recirculation pump

[0070] FIG. **4**: Reversed phase HPLC chromatogram of native and denatured and reduced α-lactalbumin

[0071] FIG. **5**: Time course of protein refolding in a batch, fed-batch and fast mix refolding reactor

[0072] FIG. **6**: CD spectroscopic analysis of native, refolded and denatured α-lactalbumin

EXAMPLE

Refolding of α-Lactalbumin

[0073] Bovine α-lactalbumin (α-LA) (38) is used as a model protein. The native protein contains 123 amino acid residues (Mr **14176**) and four disulfide bonds. The oxidative folding pathway is well characterized and the protein has an additional calcium-binding site, which increases the stability of the native protein (39) and is thus well suited as a model protein. The protein goes through a molten globule folding state as determined by stopped-flow X-ray scattering (16). The kinetic folding intermediate of α-LA has a native-like secondary structure in the α-domain and a loose hydrophobic core accessible to solvent, bur lacks most of the specific side-chain packing.

Preparation of Denatured and Reduced Protein

[0074] For refolding experiments, α-LA is denatured and reduced in a refolding buffer containing 0.1 M Tris-HCl, pH 8.0, 6 M GdmHCl, 1 mM EDTA and 20 mM DTT and incubated for 1 h at room temperature. The concentration of denatured protein is analyzed by RP-HPLC as described below.

Refolding by Dilution

[0075] Denatured and reduced aliquots at 16.5 mg/ml are rapidly diluted (batch-dilution) 32 fold into renaturation buffer consisting of 100 mM Tris-HCl, 5 mM $CaCl_2$, 2 mM cystine and 2 mM cysteine, pH 8.5,
to a final protein concentration of 0.516 mg/ml. The refolding volume is 2 ml.

[0076] In fed-batch refolding (pulse renaturation), 6.25 ml of the feed solution are fed over a time period of 90 min (flow rate of 70 μl/min) into refolding buffer. The final refolding volume is 200 ml.

[0077] Refolding according to the method of the present invention (fast mix refolding) is done by feeding 6.25 ml of unfolded protein solution at a flow rate of 0.434 ml/min to the recirculating refolding buffer stream (80 ml/min). The two streams are combined via a branch connection and mixed in a static mixing device (Sulzer Chemtech, Germany). The mixing device had a hold-up volume of 1.25 ml. The mixing time is less than one second as determined with a test solution containing bromphenol blue as tracer substance. The outlet of the mixer is connected to a plug flow reactor in the form of a silicone tubing (5 mm inner diameter×80 cm) resulting in a mean residence time of 12 sec. The refolding mixture is then collected in a stirred glass vessel and continuously re-circulated.

[0078] Samples are drawn after various time intervals and analyzed for native protein by RP-HPLC and circular dichroism (CD).

Quenching of Oxidative Refolding

[0079] The kinetic of the oxidative refolding process is monitored by removing 100 μl samples at specific time

intervals and quenching the formation of disulphide bonds by addition of 15 μl 6.4% HCl, resulting in pH 2.

Analytical Methods

[0080] Prior to analysis, all samples are centrifuged at 12.000 g for 3 min to remove insoluble material. For RP-HPLC a C4 column (Vydac 214TP54) is connected to an Agilent 1100 HPLC system (Agilent Technologies). Fully denatured α-LA is separated from oxidative folding intermediates and native protein by linear gradient elution from 36% to 45% acetonitrile/water containing 0.1% TFA in 30 min at 1 ml/min and 30° C. The system is calibrated with solutions of native and denatured α-LA. No difference in absorbance at 214 nm is detected for equivalent amounts of injected samples of native and fully reduced protein. For determination of total protein samples are denatured and reduced by 1:6 dilution in 0.1 M Tris-HCl, 6 M Gdn HCl, 50 mM DTT and 1 mM EDTA, pH 8.0, and analyzed by RP-HPLC. When the refolded protein eluted at the same position as the native protein standard, we assumed that the protein is in its native conformation. The completely unfolded and reduced protein exhibits a higher retention time compared to the native protein.

[0081] Far UV CD spectra of native, refolded and denatured α-LA are recorded on a Jasco J-600 spectropolarimeter. The CD spectra of native and refolded protein are measured in 2 mM phosphate buffer pH 7.7 containing 1 mM $CaCl_2$ at 25° C. Protein concentrations are 90 μg/ml. Far-UV wavelength scans are performed in a 1 mm quartz cuvette using five repeats with an averaging time of 4 s at each wavelength and a spectrometer bandwidth of 1.0 nm. All spectra are averaged and smoothed taking the mean of the five data points. Due to the high amount of Gdn-HCl in the denatured protein sample, significant data could not be recorded at wavelengths below 215 nm.

Results

[0082] Refolding experiments with the model protein α-LA at a final protein concentration of approx. 0.5 mg/ml are carried out. Samples are quenched after various time intervals and analyzed by RP-HPLC (see FIG. **4**) for refolded protein. The time course of protein refolding during conventional batch and fed-batch methods on the one hand and fast mix refolding on the other hand is shown in FIG. **5**. The final yield of refolded protein at equilibrium is 63% for the batch system and 81% for the fed-batch system. Using the fast mix refolding method of the present invention, the yield of refolded protein is 90%. In all experiments, the final dilution rate of the unfolded protein and refolding buffer is 32 fold. The temporary dilution rate as calculated as the ratio of the circulating folding buffer and feed stream is 184. The unfolded protein concentration after mixing of the two streams is therefore approx. 3 μg/ml followed by a 13 sec hold up time in a plug flow reactor. Within this period of time, the unfolded protein collapses and forms folding intermediates, which are less susceptible to aggregation.

[0083] Native conformation of refolded protein is also confirmed by circular dichroism (see FIG. **6**). The spectra of the refolded and native protein are identical, whereas the unfolded protein shows a completely different spectrum. The CD spectra of the refolded and native protein resemble those published by Wu et al. (39).

1) A method for obtaining a biologically active recombinant protein by reconstituting the protein from a denatured state to its active form, said method comprising the step of mixing a feed solution containing the protein in its denatured form and/or its biologically inactive intermediate forms with a refolding buffer under conditions that approximate ideal mixing, wherein

i. the mixing time is ca. 1 msec to ca. 10 sec; and

ii. the dilution rate F_P:F_B is ca. 1:1 to ca. 1:100000, wherein F_P is the flow rate of said protein solution and F_B is the flow rate of said refolding buffer.

2) The method of claim **1**, wherein said mixing time is ca. 10 msec to ca. 5 sec.

3) The method of claim **2**, and wherein the mixing time is ca. 100 msec to ca. 1 sec.

4) The method of claim **1**, wherein the dilution rate obtained in step a) is ca. 1:5 to ca. 1:50000.

5) The method of claim **4**, wherein the dilution rate ca. 1:10 to ca. 1:10000.

6) The method of claim **1**, wherein the concentration of the protein after mixing with the refolding buffer is between ca. 1 ng/ml and ca. 10 mg/ml.

7) The method of claim **6**, wherein the concentration of the protein is between ca. 100 ng/ml and ca. 5 mg/ml.

8) The method of claim **7**, wherein the concentration of the protein is between ca. 1 μg/ml and ca. 1 mg/ml.

9) The method of claim **1**, wherein the protein solution obtained after mixing is collected in a tank and incubated until the protein is completely present in its biologically active form.

10) The method of claim **9**, comprising, subsequent to said mixing step, a step wherein the protein solution is adjusted to conditions that favor refolding of the protein.

11) The method of claim **10**, wherein said adjusting comprises increasing or decreasing the temperature and/or increasing or decreasing the pH value and/or adding refolding additives.

12) The method of claim **10**, wherein said adjustment step is carried out in a plug flow reactor.

13) The method of claim **10**, wherein the protein solution is recycled from the tank and combined with said protein feed solution.

14) The method of claim **13**, wherein said protein feed solution has been obtained by solubilization of inclusion bodies.

15) The method of claim **14**, wherein said solubilization is on-line.

16) The method of claim **15**, which is run continuously.

* * * * *

Exhibit 11

Trials@uspto.gov
571-272-7822

Paper 60
Entered: February 15, 2018

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

APOTEX INC. and APOTEX CORP.,
Petitioner

v.

AMGEN INC. and AMGEN MANUFACTURING LIMITED,
Patent Owner.

Case IPR2016-01542
Patent 8,952,138 B2

Before JAMES T. MOORE, MICHAEL J. FITZPATRICK, and CHRISTOPHER G. PAULRAJ, *Administrative Patent Judges.*

MOORE, *Administrative Patent Judge.*

FINAL WRITTEN DECISION
35 U.S.C. § 318(a)

INTRODUCTION

Petitioner, Apotex, Inc. and Apotex Corp.[1] (hereinafter jointly "Petitioner") filed a Petition requesting institution of an *inter partes* review of claims 1–24 of U.S. Patent No. 8,952,138 B2 ("the '138 patent"). Paper 2 ("Pet."). Patent Owner, Amgen Inc. and Amgen Manufacturing Corp. (herein collectively "Patent Owner") filed a Preliminary Response. Paper 9 ("Prelim. Resp."). We instituted trial to determine whether the challenged claims were patentable. Paper 10. Patent Owner filed a response. Papers 14/15.[2] ("Resp."). Petitioner filed a reply. Papers 25/26. ("Reply"). Oral Argument was heard on December 13, 2017, and a transcript of the record has been made of record. Paper 59. Multiple unopposed motions to seal and multiple opposed motions to exclude and submit supplemental information are pending in this proceeding. See, *e.g.* Papers 16, 27, 33, 31, 37, 40, 43/44, 47, and 50.

For the reasons that follow, and based upon the totality of evidence in the record, we determine that Petitioner has carried its burden of persuasion that claims 1–17 and 19–24 of this patent are unpatentable. We also

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[1] Apotex Pharmaceuticals Holdings, Inc., Apotex Holdings, Inc., and ApoPharma USA, Inc., and Intas Pharmaceuticals Limited are said to be additional real parties in interest. Pet. 2.

[2] As we grant certain of the motions to seal, we use this designation to indicate the paper numbers of the unredacted and redacted (public) versions of the same document where applicable.

determine that Petitioner has not carried its burden of persuasion that claim 18 of this patent is unpatentable

A. Related Matters

Petitioner asserts that the '138 patent is the subject matter of district court litigation in the United States District Court for the Southern District of Florida.[3] Pet. 2. Petitioner further cites to related administrative matters, including nonprovisional patent applications, as related.[4] Pet. 2. Patent Owner points out that the district court litigation concerning Petitioner's invalidity defenses was resolved in its favor. Prelim. Resp. 4, Ex. 2004, 4–5. ("The Court finds that Apotex failed to meet its burden of establishing by clear and convincing evidence that the '138 patent is invalid for obviousness. The Court thus finds that each of the asserted claims 1-3, 6, 7, 13, 15-17, 22-23 of the '138 Patent is not invalid for obviousness under 35 U.S.C. § 103.") *Id*. at 5. While informative, the standards are different between the two proceedings, and the district court's decision is not binding upon this board.

B. The '138 Patent (Ex. 1001)

The '138 patent is entitled "Refolding Proteins Using a Chemically Controlled Redox State." Ex. 1001, (54). The '138 patent issued on

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[3] *Amgen Inc. et al. v. Apotex Inc. et al.*, No. 0:15-CV-61631-JIC/BSS (S.D. Fla.).

[4] U.S. Patent Application Serial Numbers 14/611,037 and 14/793,590.

February 10, 2015, from an application that was filed June 21, 2010. *Id.*, (22), (45). The '138 patent describes that the expression of recombinant proteins in the prior art prokaryotic systems is problematic in that the expressed proteins have limited solubility precipitates called inclusion bodies, which are improperly folded proteins. *Id.* at 1:18–33. According to the specification of the '138 patent:

> [V]arious methods have been developed for obtaining correctly folded proteins from bacterial inclusion bodies. These methods generally follow the procedure of expressing the protein, which typically precipitates in inclusion bodies, lysing the cells, collecting the inclusion bodies and then solubilizing the inclusion bodies in a solubilization buffer comprising a denaturant or surfactant and optionally a reductant, which unfolds the proteins and disassembles the inclusion bodies into individual protein chains with little to no structure. Subsequently, the protein chains are diluted into or washed with a refolding buffer that supports renaturation to a biologically active form.

Id. at 1:34–47.

According to the Specification, a problem existing until the present invention is said to be that "[m]ore complex molecules, such as antibodies, peptibodies and other large proteins, are generally not amenable to detergent refold conditions and are typically refolded" in so-called chaotropic refold solutions. *Id.* at 2:10–13. "These more complex molecules often have greater than two disulfide bonds, often between 8 and 24 disulfide bonds, and can be multi-chain proteins that form homo- or hetero-dimers." *Id.* at 13–16.Until the present invention, the specification states that "these types of complex molecules could not be refolded at high concentrations, i.e., concentrations

of 2.0 g/L and higher, with any meaningful degree of efficiency on a small scale, and notably not on an industrial scale." *Id.* at 2:17–21.

Thus, the invention of the '138 patent is said to be a method of refolding a protein expressed in a non-mammalian expression system (e.g. bacterial or viral) and present in a volume at a concentration of 2.0 g/L or greater comprising:

> (a) contacting the protein with a refold buffer comprising a redox component comprising a final thiol-pair ratio having a range of 0.001 to 100 and a redox buffer strength of 2 mM or greater and one or more of: (i) a denaturant; (ii) an aggregation suppressor; and (iii) a protein stabilizer; to form a refold mixture; (b) incubating the refold mixture; and (c) isolating the protein from the refold mixture.

Id. 2:52–61.

C. Illustrative Claim

All of the patent claims are challenged. In particular, they are claims 1–24. Pet. 3. Of these challenged claims, claim 1 is independent. Claims 2–24 depend, either directly or indirectly, from claim 1.

Claim 1 is illustrative, and reproduced below:

> 1. A method of refolding a protein expressed in a non-mammalian expression system and present in a volume at a concentration of 2.0 g/L or greater comprising:
>
> (a) contacting the protein with a refold buffer comprising a redox component comprising a final thiol-pair ratio having a range of 0.001 to 100 and a redox buffer strength of 2 mM or greater and one or more of:
>
> (i) a denaturant;
>
> (ii) an aggregation suppressor; and

(iii) a protein stabilizer;

to form a refold mixture;

(b) incubating the refold mixture; and

(c) isolating the protein from the refold mixture.

Ex. 1001, 17:47–59.

D. Prior Art Relied Upon

This proceeding utilizes the following prior art references:

Reference		Date	Exhibit
Schlegl	US 2007/0238860 A1	Oct. 11, 2007	Ex. 1003
Hevehan	"Oxidative Renaturation of Lysozyme at High Concentrations," *Biotechnology and Bioengineering*, 1996, 54(3):221-230	1996	Ex. 1004
Hakim[5]	"Inclonals" *mAbs*, 1:3, 281-287	June 2009	Ex. 1006

Petitioner also relies on the Declarations of Anne S. Robinson, Ph. D. ("Dr. Robinson"). Exs. 1002; 1056. Dr. Robinson's *curriculum vitae* is Exhibit 1049.

[5] Referred to throughout the Petition as "Inclonals." We use the first author's name, for consistency.

E. Instituted Grounds of Unpatentability

We instituted trial as to claims 1–24 of the '138 patent based on the following two grounds (Pet. 37–38):

Challenged Claim(s)	Basis	Reference(s)
1–11 and 13–24	§ 103(a)	Schlegl and Hevehan
12	§ 103(a)	Schlegl, Hevehan, and Hakim

II. ANALYSIS

The question of obviousness is resolved on the basis of underlying factual determinations including: (1) the scope and content of the prior art; (2) any differences between the claimed subject matter and the prior art; (3) the level of ordinary skill in the art; and (4) objective evidence of nonobviousness. *Graham v. John Deere Co.*, 383 U.S. 1, 17–18 (1966). One seeking to establish obviousness based reference combination of teachings also must articulate sufficient reasoning with rational underpinnings to combine teachings. *See KSR Int'l Co. v. Teleflex, Inc.*, 550 U.S. 398, 418 (2007).

A. The Person of Ordinary Skill In the Art at the Time of Invention

Petitioner proposes that the person of ordinary skill in the art to which the '138 Patent is directed "would have had at least a Bachelor's degree (or the equivalent) in Biochemistry or Chemical Engineering with several years' experience in biochemical manufacturing, protein purification, and protein refolding, or alternatively, an advanced degree (Masters or Ph.D.) in Biochemistry or Chemical Engineering with emphasis in these same areas."

Pet. 18. "This person may also work in collaboration with other scientists and/or clinicians who have experience in protein refolding or related disciplines." Pet. 18–19 Finally, Petitioner asserts that this person "would have easily understood the prior art references referred to herein and would have had the capacity to draw inferences from them." *Id.*

Patent Owner asserts that a person of ordinary skill in the relevant art, (the art of protein refolding in June of 2009, the priority date of the '138 Patent) "would have had a Ph.D. degree in biochemistry, biochemical engineering, molecular biology, or a related biological/chemical/engineering discipline, or a master's degree in such disciplines and several years of industrial experience producing proteins in non-mammalian expression systems." Prelim. Resp. 18; Resp. 14.

These two descriptions are mostly consistent, but we adopt the slightly higher level recited by Patent Owner, requiring a graduate level of education and experience. Ex. 2001, ¶ 17. This is due to the sophistication and complexity in the area of protein refolding. Ex. 2001, ¶ 16. A person of ordinary skill in the art would have an advanced degree in biochemistry with an engineering component and significant experience in protein production, including refolding. *Id.* ¶ 17. This is also the level of ordinary skill in the art reflected by the prior art of record. *See Okajima v. Bourdeau*, 261 F.3d 1350, 1355 (Fed. Cir. 2001); *In re GPAC Inc.*, 57 F.3d 1573, 1579 (Fed. Cir. 1995); *In re Oelrich*, 579 F.2d 86, 91 (CCPA 1978).

B. Claim Construction

In an *inter partes* review, claim terms in an unexpired patent are interpreted according to their broadest reasonable construction in light of the

specification of the patent in which they appear. 37 C.F.R. § 42.100(b); *Cuozzo Speed Techs., LLC v. Lee*, 136 S. Ct. 2131, 2142–46 (2016). Consistent with that standard, claim terms also are given their ordinary and customary meaning, as would be understood by one of ordinary skill in the art in the context of the entire disclosure. *See In re Translogic Tech., Inc.*, 504 F.3d 1249, 1257 (Fed. Cir. 2007).

There are, however, two exceptions to that rule: "1) when a patentee sets out a definition and acts as his own lexicographer," and "2) when the patentee disavows the full scope of a claim term either in the specification or during prosecution." *See Thorner v. Sony Computer Entm't Am. LLC*, 669 F.3d 1362, 1365 (Fed. Cir. 2012).

If an inventor acts as his or her own lexicographer, the definition must be set forth in the specification with reasonable clarity, deliberateness, and precision. *Renishaw PLC v. Marposs Societa' per Azioni*, 158 F.3d 1243, 1249 (Fed. Cir. 1998). Although it is improper to read a limitation from the specification into the claims, (*In re Van Geuns*, 988 F.2d 1181, 1184 (Fed. Cir. 1993)) claims still must be read in view of the specification of which they are a part. *Microsoft Corp. v. Multi-Tech Sys., Inc.*, 357 F.3d 1340, 1347 (Fed. Cir. 2004).

Only terms which are in controversy need to be construed, and only to the extent necessary to resolve the controversy. *See Wellman, Inc. v. Eastman Chem. Co.*, 642 F.3d 1355, 1361 (Fed. Cir. 2011); *Vivid Techs., Inc. v. Am. Sci. & Eng'g, Inc.*, 200 F.3d 795, 803 (Fed. Cir. 1999). In view of the arguments made in the Response and Reply, we have altered some of the constructions adopted in the institution decision, as discussed below.

protein

Petitioner argues that "protein" should not be construed as a "complex protein." Pet. 20.

The following passage of the Specification, which defines "protein" gives us a clear definition:

> As used herein, the terms "protein" and "polypeptide" are used interchangeably and mean any chain of at least five naturally or non-naturally occurring amino acids linked by peptide bonds.

Ex. 1001, 5:47-50.

Accordingly, guided by the express definition in the Specification, we adopt the above-described minimum of five amino acids as the construction of "protein." Prelim. Resp. 12–13. This construction has not changed from the institution decision.

Final thiol-pair ratio "TPR"

The term "final thiol-pair ratio" is interpreted to mean the relationship of the reduced and oxidized redox species used in the redox component of the refold buffer as defined by the equation

$$\frac{[\text{reductant}]^2}{[\text{oxidant}]}$$

Ex. 1001, 6:20-27, Resp. 18, fn. 4. This construction has changed from that in the institution decision to reflect the claim language more accurately. *See* Response 18, n. 4.

Redox buffer strength "RBS"

The term "Redox buffer strength" is interpreted to mean the following:

2[oxidant] + [reductant].

Ex. 1001, 6:29-38. Resp. 20–21, fn. 5. This construction also has changed from that in the institution decision to reflect the claim language more accurately. *See* Response 18, n. 5.

refold mixture

The broadest reasonable interpretation for "refold mixture" is "a mixture formed from contacting (1) the protein with (2) a refold buffer." Ex. 1001, 17:50–57. We find that the protein volume and refold volumes combine to form the refold mixture volume. Resp. 16.

complex protein

Patent Owner argues that the specification defines complex protein. Prelim. Resp. 16.

> The protein can be a complex protein, i.e., a protein that (a) is larger than 20,000 MW, or comprises greater than 250 amino acid residues, and (b) comprises two or more disulfide bonds in its native form

Ex. 1001, 12:58-61. A similar statement is found at 5:64–69 as regards a "complex molecule."

We also observe that the specification also provides a slightly different description in a different location.

> The method can be applied to any type of protein, including simple proteins and complex proteins (e.g., proteins comprising 2-23

> disulfide bonds *or* greater than 250 amino acid residues, *or* having a MW of greater than 20,000 daltons)

Ex. 1001, 4:23–27 (emphases added). There was discussion at the oral argument as to which of these descriptions was the broadest reasonable definition. Paper 59, 13–14 and 31–35. Dr. Robinson testifies that the single use of the broader description is correct. Ex. 1056, ¶¶ 5–7. Patent Owner urges otherwise. Resp. 17, citing Ex. 2020 ¶ 9.

We agree with Patent Owner that the evidence of record in the specification is more persuasive. The specification has set forth a definition multiple times, and that it is the definition is evidenced by the use of "i.e." (*id est*, or "that is"). In contrast, "e.g." (*exempli gratia*, or "for example") does not indicate a definition. We also observe that the use of the "e.g." appears intended to exemplify both the simple protein and complex protein antecedents expansively defining how the method may be applied. Ex. 1001, 4:23–27.

We need not expressly interpret any additional terms.

C. Obviousness Grounds – The Prior Art

Petitioner asserted, and we instituted trial upon, two obviousness grounds of unpatentability that rely on Schlegl, combined with two other discrete references. A short summary of these references, Dr. Robinson's testimony, and our analysis of these grounds follow.

(1) Schlegl (Exhibit 1003)

Schlegl, U.S. Patent Application Publication 2007/0238860 A1, is a publication of application 11/695,950, filed April 3, 2007 and published

October 11, 2007, and entitled "Method for Refolding a Protein." Ex. 1003 (10), (21), (22), (43), (54). Based on its publication date, Schlegl is prior art.

Schlegl describes methods for protein refolding, including the refolding and production of recombinant proteins. Ex.1003 at Abstract, ¶ 4. Schlegl utilizes a dilution method of protein refolding that results in a protein concentration up to 10 mg/ml. *Id*. ¶¶ 4–8, 16.

Schlegl delineates a continuous process that optimizes flow rate by keeping the concentration of unfolded proteins low and adding the protein solution at a flow rate that gives the unfolded protein time to properly fold. *Id.* ¶¶ 33–61. Before mixing, Schlegl starts with a high concentration of unfolded protein. *Id*. at ¶ 40.

Schlegl further describes a refolding buffer with a redox system having a defined thiol-pair ratio and redox buffer strength. *Id.* ¶¶ 36, 41, 75. The refolding buffer also contains a denaturant, an aggregation suppressor, and/or a protein stabilizer. *Id.* ¶¶36, 41, 74-75.

(2) Hevehan (Ex. 1004)

Hevehan is prior art to the '138 Patent. Ex. 1004

Hevehan describes refolding proteins from inclusion bodies at high concentrations. Using multiple dilution profiles, Hevehan created an experimental matrix to investigate different effects and the relationship between variables to optimize yields at higher concentrations, arriving at concentrations higher than 2 g/L. *Id.* at 5–6, Figure 4.

By varying the concentrations of reducing agent dithiothreitol ("DTT") and oxidizing agent oxidized glutathionone ("GSSG") in the redox

mixture, the *Hevehan* authors observed that renaturation yields were "strongly dependent on thiol concentrations in the renaturation buffer." *Id.* at 5.

The refold buffer used in *Hevehan* also included two folding aids, GdmCl (a denaturant) and L-arginine (a protein stabilizer and aggregation suppressor). *Id.* at Abstract. The authors found that such folding aids present in low concentrations during refolding can limit aggregation resulting in reactivation yields as high as 95%. *Id.* Finally, the authors of *Hevehan* incubated the refold mixture. *Id.* at 3.

(3) Hakim (Ex. 1006)

Hakim was published online on May 1, 2009. Ex. 1006, 1. Thus, Hakim is prior art to the '138 Patent under 35 U.S.C. § 102(a). Patent Owner attempts to antedate Hakim, which is discussed in more detail *infra*.

Hakim describes the production of fusion proteins. *Id.* at 4. Specifically, it describes the production of "PE38" fusions of the heavy chain or the light chain. *Id*. The bacterial expression system developed by Hakim allowed the production of antibodies in 8-9 days, instead of the eight weeks required when expressed in mammalian cells. *Id.* Hakim is pertinent to the proposed ground involving claim 12.

(4) Dr. Robinson's Initial Testimony Concerning the Combination

Dr. Robinson testifies that Hevehan explains the viewpoint of one of skill in the art looking to tackle the known problems of refolding proteins in 2009. Ex. 1002, ¶ 112. According to Dr. Robinson, Hevehan shows the systematic approach that those skilled in the art would take to refold a protein of interest. *Id.*, citing Ex. 1004 at 1–2. Specifically, Dr. Robinson

testifies that Hevehan considered conditions known to successfully refold proteins at low concentrations, minimizing aggregation, and applied those techniques to higher concentrations. Ex. 1002, ¶ 112, citing Ex. 1004 at 2.

Dr. Robinson further testifies that Hevehan authors found optimal refolding of proteins expressed in a non-mammalian expression system at higher concentrations is related to the thiol-pair ratio and redox buffer strength. Ex. 1002, ¶ 113, citing Ex. 1004 at 5. Hevehan concluded that yields are "strongly dependent" on thiol concentrations in the renaturation buffer. Ex. 1004 at 5. The optimum thiol-pair ratio was between 0.57 and 2.3 (DTT/GSSG). Ex. 1004 at Fig. 4 and ¶ 67, fn 5.

According to Dr. Robinson, one of ordinary skill would also be motivated to use the teachings of Schlegl and Hevehan to refold a "complex" protein, and would have a reasonable expectation of success in doing so. This is said to be so because both references teach the refolding of "complex" proteins by a dilution refolding method. Ex. 1002, ¶ 117.

(5) Analysis

a. Obviousness of Claims 1-11 and 13–24 in View of Schlegl and Hevehan

(i) Overview – Motivation to Combine

Petitioner asserts that one of ordinary skill in the art would have been motivated to combine Schlegl and Hevehan and would have had a reasonable expectation of success in doing so. Pet. 38. Specifically, Petitioner urges that the authors of Hevehan considered conditions already known to successfully refold proteins at low concentrations, minimizing aggregation. Pet. 39, citing Ex. 1004 at 2; Ex. 1002 at ¶ 112.

This position is supported by the testimony of Dr. Robinson, as noted above. We find Dr. Robinson is qualified to testify to the subject matter of this proceeding. Ex. 1002 ¶¶ 3–11; Ex. 1049. She testifies that one of ordinary skill in the art would look to Hevehan to solve the problem of refolding proteins at higher concentrations, and would have known the methods of Hevehan could apply to the dilution refolding methods of Schlegl. Ex. 1002, ¶ 115.

Petitioner is of the view that a person of ordinary skill in the art would have known that the refolding methods of Hevehan and Schlegl would be just as applicable to the refolding of proteins in inclusion bodies as to the proteins in denatured native proteins. Pet. 40.

Patent Owner, on the other hand, assert that Schlegl and Hevehan are fundamentally different and incompatible approaches to protein refolding. Resp. 2–3. Schlegl's method is said to be a "mechanical approach" to achieve protein refolding at dilute protein concentrations. *Id*.

We are provided with the declaration testimony of Richard C. Willson, Ph. D. ("Dr. Willson") as Exhibits 2001 and 2020. We find Dr. Willson qualified to testify to the subject matter of this proceeding. Ex. 2001, ¶¶ 7–14, Ex. 2002. His testimony is the basis for Patent Owner's contrary assertions.

According to Patent Owner, Hevehan's method is a different approach – a chemical approach (focused on denaturant and oxidant, but not reductant, in the refold buffer) to achieve protein refolding at high protein concentrations. Ex. 2001, ¶111. In Schlegl, protein aggregation is avoided by physically separating the protein molecules by dilution. *Id*. ¶112. In

Hevehan, refolding proteins at high concentrations necessarily reduces or eliminates such physical separation; chemicals are necessary to avoid aggregation and to achieve proper refolding. *Id*.

Dr. Willson further testifies that the equations involving the reactants (thiol pair ratio and redox buffer strength) are significant – reflecting the indiscovery that the refold efficiency is mostly impacted by the redox state of the refold system. Ex. 2001, ¶ 58.

Dr. Robinson responded to these positions in her second declaration. Ex. 1056. According to her testimony, the two approaches of protein refolding in Schlegl and Hevehan's refolding complement each other and Hevehan optimizes the refolding conditions. Ex. 1056, ¶ 18. She testifies that "Hevehan considered conditions known to successfully refold proteins at low concentrations, and applied those conditions to refolding of proteins expressed in a non-mammalian expression system at higher concentrations." *Id.* (citingEx.1004, 2). "Hevehan found that optimal refolding of proteins expressed in a non-mammalian expression system at higher concentrations is related to the thiol-pair ratio and redox buffer strength." *Id.* (citing Ex.1004, 5). "By varying the conditions of a reductant (DTT) and an oxidant (GSSG) and recording the outcomes, Hevehan concluded that yields are "strongly dependent" on thiol concentrations in the renaturation buffer." *Id.* (citing Ex.1004, 2.) *Id.*

Dr. Robinson also testifies that Schlegl has a clear indication of the use of redox chemistry. Ex. 1056, ¶ 22. She points to Schlegl claim 9 in her testimony, which recites "wherein the protein solution obtained after mixing is collected in a tank and incubated until the protein is completely present in

its biologically active form." Ex. 1056 ¶24 (citing Ex. 1003, 13). She testifies that it is her view that "the method of claim 9 of Schlegl cannot be practiced without redox chemistry for proteins with disulfide bonds in the native state. If one is working with a protein with disulfide bonds, it is unlikely that one can obtain a biologically active form without the use of redox components." Ex. 1056, ¶ 24.

Dr. Willson testified in his second declaration that Schlegl and Hevehan, alone or in combination, do not teach elements of claim 1; a person of ordinary skill in the art would not combine the references; and the art does not render the claims obvious. Ex. 2020, *passim*.

Dr. Robinson was cross-examined on May 8, 2017, in Washington, DC. A transcript of that deposition testimony is in the record as Exhibit 2019. Dr. Willson likewise was cross-examined, on August 9, 2017, in New York, NY. A transcript of that deposition testimony is in the record as Exhibit 1055. Subsequent to her second declaration, Dr. Robinson was again cross-examined on September 26, 2017, and a transcript of that cross-examination is in the record as Exhibit 2059. We have carefully reviewed the testimony provided by both witnesses.

We credit the testimony of Dr. Robinson on this point over that of Dr. Willson. We are especially persuaded by the fact that simply diluting the protein concentration will not necessarily result in refolding. Reply, 5. Dr. Robinson also makes a compelling point that using a dilution technique to contact a protein-containing volume with a refold buffer does not exclude the use of redox agents. Ex. 1056, ¶ 15.

She further testifies that Schlegl teaches the use of redox chemistry and a customized refold buffer. *Id.* at ¶ 17, citing Ex. 1003, ¶ 36. Paragraph 36 is reproduced below:

> The refolding buffer used for a given protein of interest is customized to the refolding requirements/kinetics of that protein. Refolding buffers are known in the art and commercially available; typical buffer components are guadinium chloride, dithiothreitol (DTT) and optionally a redox system (e.g. reduced glutathione GSH/oxidized glutathione GSSG), EDTA, detergents, salts, and refolding additives like L-arginine.

Ex. 1003, ¶ 36. It appears to us that glutathione discussed in Schlegl is also listed as an exemplary redox component in the optimized refold buffer of the '138 patent. Ex. 1001, 10:53–54.

Dr. Willson, on the other hand states that Schlegl "does not focus on" the use of redox chemicals. Ex. 2001, ¶ 93. The Response then asserts that because Schlegl's example was a well-known model protein and easy to refold, that "redox chemicals do not play a role in Schlegl's refolding method." Resp. 36. Focusing on the sole example, the Response notes that protein was simple to refold and uses calcium. *Id.*

This testimony of Dr. Willson, while literally true, cannot in our view be reconciled with Schlegl's express teaching of a customizable refolding buffer with a redox buffer option. We further find that the discussion in Schlegl does not support the Patent Owner's assertion that these references are "incompatible." Resp. 23.

Patent Owner also asserts that redox systems used for refolding at low protein concentrations "are inappropriate" when refolding at high protein

concentrations. *Id.* at 26(citing Dr. Willson's second declaration, Ex. 2020, ¶ 22). Dr. Willson makes the statement that "[a]cknowledging that what worked at low protein concentrations 'might not be appropriate when folding a protein at 1 mg/mL or higher concentrations,' *Hevehan* reports the use of a trial-and-error matrix approach to find appropriate conditions." Ex. 2020(citing Ex. 1004, 5).

The problem with this analysis is that, like that with respect to Schlegl above, it strays by incremental degrees from the original evidence of record, and it goes too far. According to Hevehan:

> The above thiol concentrations were optimized for oxidative renaturations at low protein concentrations (0.01–0.1 mg/mL) and might not be appropriate when folding a protein at 1 mg/mL or higher concentrations.

Ex. 1004, 5.

We read this paragraph, contained in a section headed *Thiol Concentration Dependence on Renaturation,* and sandwiched between a discussion of the prior art thiol concentrations in the renaturation buffer and empirical studies of different ranges as suggesting quite the opposite – as teaching that one of ordinary skill in the art could find workable ranges by routine experimentation.

Patent Owner also asserts that host-cell contaminants would lead one of ordinary skill in the art not to have an expectation of success as model proteins are not predictive of or applicable to recombinant proteins expressed in mammalian expression systems. Resp. 2–3 and 27–32. The evidence relied upon for this proposition is a publication originating from

the same laboratory that the authors of Hevehan occupied. *Id.* (citing Ex. 2033).

Again, the weakness in this position is that the authors of the relied upon exhibit(*i.e.* Ex. 2033) came to no such conclusion themselves. Patent Owner selectively relies upon a single example to state: "It decreased by 40% to 50%." Resp. 30. While this again may be literally true, and Patent Owner includes a chart referencing what appears to be the single worst example in the reference, we reproduce the abstract of the reference below to provide additional context:

> The effect of typical contaminants in inclusion body preparations such as DNA, ribosomal RNA, phospholipids, lipopolysaccharides, and other proteins on renaturation rate and yield of hen egg white lysozyme was investigated. Separate experiments were conducted in which known amounts of individual contaminants were added to test their effect on renaturation kinetics. **On the basis of a simplified model for the kinetic competition between folding and aggregation, it was found that none of the above contaminants had an effect on the rate of the folding reaction, but some of them significantly affected the rate of the aggregation reaction and, thus, the overall renaturation yield**. **While ribosomal RNA did not seem to affect the aggregation reaction, plasmid DNA and lipopolysaccharides increased the aggregation rate, resulting in a decrease of about 10% in the overall renaturation yield. Phospholipids were found to improve refolding yields by about 15% by decreasing the overall rate of the aggregation reaction without affecting the rate of the folding reaction.** Proteinaceous contaminants which aggregate upon folding, such as β-galactosidase and bovine serum albumin, were found to significantly decrease renaturation yields by promoting aggregation. The effect was strongly dependent on the concentration of the proteinaceous impurity. **On the other hand, the presence of refolding ribonuclease A, which does**

> **not significantly aggregate upon folding under the conditions tested in this work, did not affect the renaturation kinetics of lysozyme, even at concentrations as high as 0.7 mg/ml.**

Ex. 2033, Abstract. (Emphases added).

Dr. Willson does not address adequately any of the content we have emphasized in the exhibit's Abstract, which observations either cause lesser overall losses or, in one case, increase yield. While we have no doubt contamination can result in some reduction, none of the highlighted portions, which would appear to somewhat undercut the testimony, are sufficiently acknowledged by or appear to be adequately discussed or countered in the testimony. Ex. 2020, ¶ 43.

Dr. Willson cites to additional references Georgiou (Ex. 2034, at 2) and Darby (Ex. 2035, at 1–2) as further support. Ex. 2020, ¶ 42–43. As above, the cited references do not provide support sufficient to establish that for which they are cited. For example, while Georgiou does state that the efficiency of refolding is inversely proportional to the level of contamination (Ex. 2034, 2), Georgiou also states "[n]onetheless, as was shown with β-lactamase, it is often possible to modify the expression conditions to reduce the amount of extraneous material incorporated within the inclusion bodies" (*Id.* at 4). (footnotes omitted). Likewise, Darby (a letter to the Editor of Nature Magazine) mentions losses but then also concludes with "[n]evertheless, awareness of the possible presence of complexes should suggest ways of resolving them as well as the stage in the purification process at which refolding of the protein should be attempted." Ex. 2035, 2.

We also are further persuaded of the appropriateness of the combination by Dr. Robinson's observation that Schlegl describes quenching of oxidative refolding, and her view that claim 9 could not be practiced without redox chemistry. Ex. 1056, ¶¶ 23 and 24.

Patent Owner, on the other hand, through the testimony of Dr. Willson, asserts that one of ordinary skill in the art would not expect success for myriad reasons including Hevehan's kinetic model being inaccurate because: (1) the model incorrectly assumes that each step is irreversible and proceeds in only one direction (Ex. 2020, ¶74 citing Ex. 1004, 8, Figure 7); (2) the assumption in the model that the aggregation pathway follows third-order kinetics does not apply to all protein aggregation pathways(*id.* at ¶ 73 citing Ex. 2043, *passim*); (3) the model incorrectly assumes that only proteins in the intermediate state (between folded and unfolded) aggregate (*id.* at ¶ 74 citing Ex. 2046, 1, Ex. 2047, 1 6, and Fig. 7, and Ex. 2042 ("Buswell") at 1); and (4) the model incorrectly assumes that there is a single pathway for converting one protein state to another (*id.* at ¶75 citing Ex. 1004 at 8, Figure 7).

Relying on the above testimony and evidence, Patent Owner urges that it was known prior to Schlegl that Hevehan does not accurately predict refolding of its own model protein and therefore a person of ordinary skill in the art would not have applied Hevehan's teachings to refolding any other proteins. Resp. 39–42.

Petitioner asserts in reply that Patent Owner has misapplied Buswell, which teaches that Hevehan's model does not work at low-protein concentrations (defined therein as 0.01-0.02 mg/L), which are not the

conditions Hevehan was using for its measurements. Reply, 10. Petitioner also observes that Buswell's theory has been discredited. *Id.* (citing Ex. 1056 ¶¶34-35, Ex. 1057, 91, 95).

We note that Ex. 1057 does expressly negate a principal conclusion of Buswell:

> Buswell and Middelberg (2003) reported that the presence of native lysozyme significantly decreased the effective refolding yield. This was because that native lysozyme was able to polymerize with aggregates (Buswell and Middelberg, 2002). We checked this possibility by adding pure native sGFPmut3.1 in our refolding buffer before refolding.
>
> In contrast to decrease in yields in the presence of native lysozyme (Buswell and Middelberg, 2003), refolding yields remained unaffected in the presence of pure native sGFPmut3.1 (Fig. 3).

Ex. 1057, 5.

Accordingly, while it is a close call with competing evidence, we find that a person of ordinary skill in the art would look to Hevehan to solve the problem of refolding proteins at higher concentrations, and would have known the methods of Hevehan could apply to the dilution refolding methods of Schlegl. We also find that a person of ordinary skill in the art (who would have been highly skilled as discussed above) would have had a reasonable expectation of success in combining Schlegl and Hevehan.

We next turn to comparison of the claimed subject matter against the prior art.

The Claimed Subject Matter vs. the Prior Art

Claim 1

> *1. A method of refolding a protein expressed in a non-mammalian expression system and present in a volume at a concentration of 2.0 g/L or greater comprising:*

Petitioner asserts that Schlegl describes refolding of recombinant proteins expressed using nonmammalian expression systems such as bacterial and yeast expression systems. Pet. 43. (citing Ex. 1003 ¶ 4). We find that Schlegl describes expression vectors including microorganisms such as bacteria. *Id.*

Schlegl is also said to describe protein present at a volume of 16.5 mg/mL (16.5 g/L) before being diluted by the refold buffer. Ex. 1003 at ¶ 75.

We find that Schlegl describes that denatured and reduced protein aliquots of 16.5 mg/ml are batch-diluted into a renaturation buffer. *Id.* While we observe that the end dilution is lower than 2.0 g/L, the protein is contained in a volume at an initial concentration greater than 2.0 g/L.

> *(a) contacting the protein with a refold buffer comprising a redox component comprising a final thiol-pair ratio having a range of 0.001 to 100 and a redox buffer strength of 2 mM or greater and one or more of:*
>
> *(i) a denaturant;*
>
> *(ii) an aggregation suppressor; and*
>
> *(iii) a protein stabilizer;*
>
> *to form a refold mixture;*

Ex. 1001, 17:47–59.

Petitioner asserts that the example in Schlegl discloses contacting bovine α-lactalbumin (a denatured model protein) with a refold buffer comprising a redox component as part of the dilution refold method of Schlegl to form a refold mixture. Pet. 44–45 (citing Ex. 1003 ¶ 75).

We find that, in the example, the protein is denatured and reduced in what Schlegl calls a refold buffer. Ex. 1003 ¶ 74. The Schlegl 'refold buffer' contains 0.1 M Tris-HCL, pH 8.0, 6 M GdmHCl, 1 mM EDTA and 20 mM DTT. *Id.*

We also find that the protein is rapidly diluted into a renaturation buffer containing 100 mM Tris-HCl, 5 mM $CaCl_2$, 2 mM cystine and 2 mM cysteine, pH 8.5. *Id.* ¶ 75.

Petitioner asserts that a person of ordinary skill in the art would understand that the addition of cystine and cysteine here serve as the redox system or redox component for bovine α-lactalbumin. Pet. 45 (citing Ex. 1002 at ¶ 124). We find this testimony to be credible. See also Ex. 2001, ¶ 53. (Dr. Willson testifying that cystine is the oxidant and cysteine is the reductant).

Petitioner asserts that this redox component has a thiol-pair ratio of 2 and a redox buffer strength of 6 mM. Pet. 45 (citing Ex. 1003 at ¶¶ 36, 0075). Dr. Robinson testifies to this fact. Ex. 1002 ¶ 124. She calculates the ratio at footnote 3 of paragraph 59 of her declaration (Ex. 1002). She states:

> Based upon the '138 patent, the thiol pair ratio (TPR) is defined by the equation $TPR = [reductant]^2/[oxidant]$, where the TPR is calculated in the redox component. Since these ratios will be the same in the refolding buffer, in this case, the

$$TPR = \frac{[cysteine]^2}{[cystine]} = \frac{[2\ mM]^2}{[2\ mM]} = \ 2\ mM$$

Ex. 1002, fn3.

Two is within the claimed ratio range of 0.001-100.

Dr. Robinson calculates the redox buffer strength as well:

> Based on the '138 patent, the redox buffer strength (BS) is defined by the equation, [R]BS=*2oxidant* + [*reductant*]. In this case, [R]BS=2[cysteine]+[cysteine]=6.[6]

Ex. 1002, fn4.

Six is within the claimed range of "greater than two."

Petitioner further asserts that Hevehan describes contacting a hen egg white lysozyme with a refold buffer comprising a redox component to form a refold mixture. Pet. 45 (citing Ex. 1004 at 6). Petitioner urges that the redox component "has a thiol-pair ratio of between 0.3 and 9 and a redox buffer strength of 5 mM to 19 mM, the optimum being between 10-16 mM." Pet. 45, citing Ex. 1003 at 5; Ex. 1002 ¶ 124.

Patent Owner urges that the above two assertions are incorrect.

Patent Owner has provided an illuminating diagrammatic representation of the claim to illustrate their point, which is reproduced below. The red box is said to indicate the "contacting" step.

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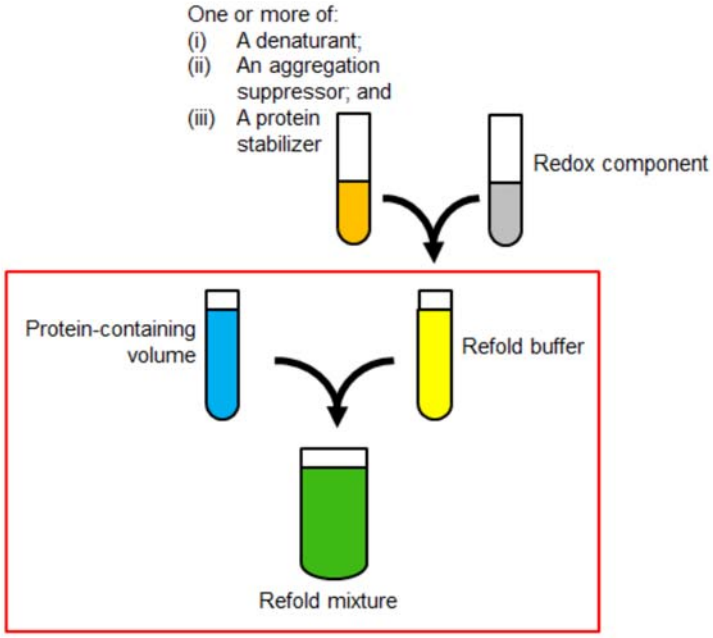
[6] We observe that Dr. Robinson did not show all of her work; however, it is readily apparent to us that RBS=2[2 mM]+2 mM = 4+2 = 6 mM.



Resp. 15.

Patent Owner's first argument is that neither Schlegl or Hevehan describe the TPR and RBS equations. *Id.* at 22. According to Patent Owner, Dr. Robinson utilized the equations from the '138 Patent which is hindsight. *Id.* at 23.

While an interesting argument, we are not persuaded of its legal correctness. The TPR and RBS equations define ratios and concentrations of oxidant and reductant. In order to discern whether the claims are obvious, we of necessity must determine whether the prior art ratios and concentrations render the claimed range obvious. Petitioner is correct in observing that "where the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by

routine experimentation." Reply 3 (citing *In re Aller*, 220 F.2d. 454, 456 (CCPA 1955)).

To hold otherwise would eviscerate long-standing legal precedent and simply allow for the patenting of inventions whose only contribution was to quantify into a previously unwritten equation relationships that were discernible to one of ordinary skill in the art from the prior art. For example, if we were to follow Patent Owner's logic to its conclusion, and if another inventor calculated the TPS using a third order relationship, creating an even broader claim, we might be compelled to conclude that the new, broader claim was unobvious simply because the formula was not known. See., e.g. *Brown & Williamson Tobacco Corp. v. Philip Morris Inc*., 229 F. 3d. 1120 (Fed. Cir. 2000)(affirming judgment that patent claiming a reduced circumference cigarette was invalid as obvious over prior art cigarettes also with a different reduced circumference, despite argument that tobacco utilization efficiency "TUE" recited in the claim, defined by the formula TUE= amount of tobacco consumed/puff, was an unobvious advance and not known in the prior art).

Patent Owner's second argument, that one of ordinary skill would not combine Schlegl and Hevehan, has been addressed above and found to be unpersuasive. Resp. 23–31.

Patent Owner's third argument is that the combination of Schlegl and Hevehan does not teach the claimed TPR limitation. Resp. 32. More specifically, Patent Owner urges that the TPR of the combination of Schlegl and Hevehan is zero, and falls outside the claimed range. *Id.*

This argument was initially raised in passing in the Preliminary Response, page 26, citing to testimony of Dr. Willson that the addition of a reductant was not necessary. Ex. 2001, ¶ 109.

This argument was further developed in the Response, 32–35. More specifically Patent Owner amplifies:

> But *Hevehan* explicitly teaches that there is no reductant in the refold buffer. EX2020 at ¶54. *Hevehan* teaches two volumes: a protein-containing volume and a refold buffer (called the renaturation media). EX2019 at 74:20-75:3; EX2020 at ¶55; EX1004 at 2-3. *Hevehan*'s protein-containing volume contains, in relevant part, HEWL (the protein) and DTT (which Dr. Robinson identifies as the reductant). EX1004 at 2; EX1002 at ¶68, fn. 5; EX2020 at ¶55. *Hevehan*'s refold buffer contains Tris-HCl, EDTA, GSSG (the oxidant), and possibly some GdmCl and L-arginine—none of which are reductants. EX1004 at 2-3; EX2019 at 75:4-25 (GdmCl and L-arginine are not redox chemicals); EX2020 at ¶55 (Tris-HCl and EDTA are not redox chemicals).
>
> Critically, *Hevehan* explicitly teaches that the reductant is not necessary in the refold (renaturation) buffer:
>
> > Addition of GSSG's reducing partner, GSH, to the renaturation system was not necessary due to the DTT carried over from the denatured [protein] solution.
>
> EX1004 at 3; EX2019 at 77:8-16. And Dr. Robinson admitted at deposition that there is no DTT reductant in the refold buffer. EX2019 at 76:1-5; EX2020 at ¶56.

Response 33.

Were there a teaching of no reductant in the refold buffers in Hevehan, then it appears to us that the Patent Owner would prevail. However, Petitioner correctly observes that the very next sentence in Hevehan states:

> In a typical experiment, the refolding solution contained 5 mM GSSG and 2 mM DTT, resulting in a glutathione ratio [GSH]/[GSSG] of 1.33/1.

Reply 8 (citing Ex. 1004, 3).

Petitioner also observes that the TPR in Hevehan cannot be zero, as Hevehan states that protein yields are "strongly dependent" on thiol concentrations in the renaturation buffer. Reply 9 (citing Ex. 1004, 5). Petitioner asserts that this conclusion would not be possible if Hevehan were teaching a TPR of zero. Petitioner observes that Hevehan discloses that the optimum thiol-pair ratio is between 0.57 and 2.3 (DTT/GSSG). Ex. 1004, Fig. 4; Ex. 1002, ¶68; Ex. 1056, ¶¶31–33.

We find Petitioner's evidence more credible and compelling. Patent Owner appears to rely upon an isolated portion of evidence without considering the overall teachings of the Hevehan reference. The combination of Schlegl and Hevehan does not teach a TPR of zero; to the contrary, we find it teaches additional points within the broad range of claim 1.

(b) incubating the refold mixture; and

Petitioner asserts that Schlegl describes "[c]omplete refolding, including formation of disulfide bonds, proline isomerization and domain pairing may take hours and up to several days" of further incubation in the

refolding tank to allow complete refolding of the protein. Pet. 48 (citing Ex. 1003 ¶¶ 16, 60). Patent Owner does not significantly dispute this teaching.

(c) isolating the protein from the refold mixture.

Lastly, Petitioner asserts that Schlegl discloses isolation of the protein from the refold mixture as a final step in the disclosed refold method, including via dialysis, filtration, extraction, precipitation and chromatography. Pet. 48 (citing Ex. 1003 ¶¶ 39, 65). Patent Owner does not significantly dispute this teaching.

On consideration of the evidence presented at trial, including Patent Owner's evidence to the contrary, we find Petitioner to have met its burden of proof. We conclude that claim 1 is unpatentable as obvious over Schlegl and Hevehan.

Claim 2

Claim 2 depends from claim 1, and further recites that the final thiol-pair ratio is selected from the group consisting of 0.05 to 50, 0.1 to 50, 0.25 to 50, 0.5 to 50, 0.75 to 40, 1.0 to 50 and 1.5 to 50, 2 to 50, 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50 or 40 to 50. Ex. 1001, 17:60–18:2.

Petitioner asserts that Schlegl describes contacting the protein with a refold buffer with a thiol-pair ratio of 2. Pet. 49 (citing Ex. 1003 at ¶ 75). Hevehan is said to describe a thiol pair ratio of 0.3 to 9. *Id.* (citing Ex. 1004, 5). Patent Owner does not separately argue claim 2.

As the evidence shows that the final TPR in Schlegl and Hevehan fall within several of the claimed ranges of claim 2, we are persuaded that Petitioner has demonstrated that challenged claim 2 is unpatentable as obvious over Schlegl and Hevehan.

Claim 3

Claim 3 depends from claim 1 and further recites that the thiol-pair buffer strength is selected from the group consisting of greater than or equal to 2.25 mM, 2.5 mM, 2.75 mM, 3 mM, 5 mM, 7.5 mM, 10 mM and 15mM. Ex. 1001, 18:3–6.

Petitioner asserts that the example in Schlegl describes a redox buffer strength of 6 mM. Pet. 49 (citing Ex. 1003 ¶ 75). Hevehan is also said to describe a redox buffer strength of 5 to 19 mM, with an optimum 10 to 16 mM. *Id.* (citing Ex. 1004, 5). Both disclosures are urged to fall within the scope of claim 3. Patent Owner does not separately argue claim 3.

As the final RBS in Schlegl and Hevehan appear to fall within the claimed range of claim 3, we are persuaded that Petitioner has demonstrated that challenged claim 3 is unpatentable as obvious over Schlegl and Hevehan.

Claims 4 and 5

Claim 4 depends from claim 1, and further recites that the protein is present in the volume in a non-native limited solubility form. Ex. 1001, 18:7–8. Claim 5 depends from claim 4, and further recites that the form is an inclusion body. *Id.* 18:9–10.

Petitioner asserts that Schlegl discloses that the protein is deposited in the cells in a paracrystalline form, in so-called "inclusion bodies," also termed "refractile bodies." Pet. 52–53 (citing Ex. 1003 ¶ 6). Hevehan is said to describe that the "[a]ctive protein can be recovered by solubilization of inclusion bodies followed by renaturation of the solubilized (unfolded)

protein." *Id.* (citing Ex. 1004, Abstract). Patent Owner does not separately argue claims 4 or 5.

As the evidence of record establishes that the final inclusion bodies in Schlegl and Hevehan fall within the non-native limited solubility form of claim 4, and the inclusion body of claim 5, we are persuaded that Petitioner has demonstrated that challenged claims 4 and 5 are unpatentable as obvious over Schlegl and Hevehan.

Claim 6

Claim 6 depends from claim 1, and recites that the protein is present in the volume in a soluble form. Ex. 1001, 18:11–12.

Petitioner asserts that Schlegl describes a method of refolding a protein, where that protein before refolding is dissolved as a protein solution. Pet. 53 (citing Ex. 1003 ¶¶ 16, 63). Patent Owner does not significantly argue claim 6.

As the evidence of record establishes that the protein solution in Schlegl falls within the soluble form of claim 6, we are persuaded that Petitioner has demonstrated challenged claim 6 is unpatentable as obvious over Schlegl and Hevehan.

Claims 7-11

Claim 7 depends from claim 1, and further recites that the protein is recombinant. Ex. 1001, 18:13–14. Claim 8 depends from claim 1 and further recites that the protein is an endogenous protein. *Id.* 18:15–16. Claim 9 depends from claim 1, and further recites that the protein is an antibody. *Id.* 18:17–18. Claim 10 depends from claim 1, and further recites that the protein is a complex protein. *Id.* 18:19–20. Claim 11 depends from

claim 1, and further recites that the protein is a multimeric protein. *Id.* 18:21–22.

Petitioner asserts, alternatively, that Schlegl discloses a method of refolding the various proteins identified in claims 7-11, and that one of ordinary skill in the art would immediately recognize that the methods of Schlegl could be applied. Pet. 53–54. Petitioner points to Schlegl's description that the methods can be applied to "any protein, protein fragment or peptide that requires refolding upon recombinant expression in order to obtain such protein in its biologically active form" *Id.* (citing Ex. 1003 ¶ 31). Petitioner observes that Schlegl describes the refolding of bovine α-lactalbumin, a protein containing 123 amino acid residues and four disulfide bonds, while Hevehan describes refolding hen egg white lysozyme having 129 amino acids and four disulfide bonds. Pet. 54 (citing Ex., 1003, 1004).

Dr. Robinson testifies that a person of skill in the art would immediately recognize that the methods taught by Schlegl could be applied to each of these types of proteins, and in particular multimeric proteins, such as antibodies. Ex. 1002, ¶ 145 (citing Ex. 1006 at 281).

Patent Owner does not separately argue claims 7 and 8. Patent Owner, however, provides contrary arguments for claims 9, 10, and 11.

Patent Owner urges that none of the refolded proteins of Schlegl and Hevehan are complex proteins as recited in claim 10. Resp. 42–44. More specifically, Patent Owner asserts that there is no empirical evidence that a person of ordinary skill in the art would have had a reasonable expectation of success of refolding complex proteins, antibodies, or multimeric proteins. Resp. 43. Dr. Willson testifies that neither Schlegl nor Hevehan "teach or

suggest" the proteins required by claims 9, 10, and 11. Ex. 2020, ¶ 94. Dr. Willson concedes that Schlegl broadly states that its method can be used on "any protein, protein fragment or peptide that requires refolding upon recombinant expression in order to obtain such protein in its biologically active form." Ex. 1003, ¶ 31. However, he would require an experimental showing to support this assertion, not the model protein example actually conducted. Patent Owner also observes that refolding complex proteins can be "extremely difficult" and "challenging." Resp. 43.

We accept that refolding proteins is difficult and challenging. However, the person of ordinary skill in the art is highly skilled. The Petition asserts that one of ordinary skill in the art would immediately recognize that the methods of Schlegl could be applied to those types of molecules, and Dr. Robinson's testimony supports the statement made in Schlegl. Ex. 1002, ¶ 145.

Dr. Robinson relies in part on Ex. 1006, which is a publication from "mAbs" journal in 2009.[7] We also take into account her cross-examination testimony in which she stated:

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[7] Patent Owner asserts that Ex. 1006 is not prior art to their claims. They have provided the declaration testimony of Dr. Roger A. Hart (Ex. 2021), and internal Amgen presentations (Ex. 2022 and Ex. 2024) which are considered to be confidential and subject to protective order. Exhibits 2022 and 2024 discuss protein AMG 745 and Exhibits 2023 and 2025 indicate the documents were created in 2009 and 2008 However, other than the code names of the proteins, no real identification of the type of protein that designation reflects is made in the contemporaneous documents. Dr. Hart testifies that AMG 745 falls within the scope of, *e.g.,* claims 1, 7, 10, 11, and 12(Ex. 2021, ¶ 35) , identification would have been unnecessary on

> Q So let me ask the question this way:
> Generally speaking, would you expect the refolding of multimeric proteins, antibodies and FC-protein conjugates to be more complex than the refolding of hen egg white lysozyme?
>
> A So again, I think it's protein-dependent. I think some complex proteins refold easily and some -- some multimeric proteins refold readily, I guess I should say, and some don't. So I don't think there's a hard and fast rule.

Ex. 2019, 61:21–25, 62:1–5.

We accept her testimony such that, even not considering Ex. 1006, we find that one of ordinary skill would recognize that the methods of Schlegl could be applied to those and various types of protein molecules.

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internal documents (Ex. 1054, 68:13–20; 95:12–17; and 102:11–103:3), and as such the invention was reduced to practice prior to the publication of Ex. 1006(Ex. 2021, 16). However, the documentation relied upon to identify AMG 745 with reasonable precision is from 2014, as discussed infra. Ex. 2026. Our careful review of the evidence leads us to observe that about the closest the contemporaneous documents come is an undescribed molecular schematic labeled AMG 745 (Ex. 2022, 16) which Patent Owner characterizes as "resembl[ing] an antibody." Resp. 52. We therefore agree with Petitioner that documents relied upon to teach a specific type of protein should, in this instance, give a more credible identification of what the protein is if the antedating effort is to be persuasive. Reply 24–25. Testimony from 2017 and a document by others from 2014 are fairly well removed from the events of 2008 and 2009, and not sufficiently persuasive.

Consequently, as the proteins described in Schlegl fall within the types recited by these claims, Petitioner has demonstrated that challenged claims 7–11 are unpatentable as obvious over Schlegl and Hevehan.

Claim 13

Claim 13 depends from claim 1, and recites that "the non-mammalian expression system is one of a bacterial expression system and a yeast expression system." Ex. 1001, 18:24–26.

Petitioner asserts that Schlegl describes microorganisms such as bacteria, yeast or fungi, or from animal or plant cells to produce a protein of interest. Pet. 54–55 (citing Ex. 1003 at ¶ 4). Patent Owner does not separately argue claim 13

We find that Schlegl describes various conventional non-mammalian systems. Ex. 1003, *passim.*

Petitioner has demonstrated that challenged claim 13 is unpatentable as obvious over Schlegl and Hevehan.

Claim 14

Claim 14 depends from claim 1, and recites that "the denaturant is selected from the group consisting of urea, guanidinium salts, dimethyl urea, methylurea and ethylurea." Ex. 1001, 18:28–30.

Petitioner asserts, and we find, that Schlegl teaches the use of components that promote the solubilization of inclusion bodies, e.g. chaotropic agents such as urea, guanidinium chloride (GdmCl), sodium and/or potassium thiocyanate. Pet. 49 (citing Ex. 1003 ¶ 64). Patent Owner does not separately argue claim 14

Petitioner has demonstrated that challenged claim 14 is unpatentable as obvious over Schlegl and Hevehan.

Claim 15

Claim 15 depends from claim 1, and recites that "the protein stabilizer is selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes." Ex. 1001, 18:31–35.

Petitioner asserts that Schlegl shows refolding buffers were known in the art and commercially available; typical buffer components are guadinium chloride, dithiothreitol (DTT) and optionally a redox system (e.g. reduced glutathione GSH/oxidized glutathione GSSG), EDTA, detergents, salts, and refolding additives like L-arginine. Pet. 50 (citing Ex. 1003 ¶¶ 36, 41). Patent Owner does not separately argue claim 15

As Schlegl describes arginine, which falls within the stabilizers recited by this claim, Petitioner has demonstrated that challenged claim 15 is unpatentable as obvious over Schlegl and Hevehan.

Claim 16

Claim 16 depends from claim 1, and further recites that "the aggregation suppressor is selected from the group consisting of arginine, proline, polyethylene glycols, nonionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes." Ex. 1001, 18:36–41.

Petitioner asserts that Schlegl describes arginine. Pet. 50. Patent Owner has not separately challenged this.

As we find that Schlegl describes arginine, which falls within the aggregation suppressors recited by this claim, Petitioner has demonstrated that challenged claim 16 is unpatentable as obvious over Schlegl and Hevehan.

Claim 17

Claim 17 depends from claim 1, and recites that the thiol-pairs comprise at least one component selected from the group consisting of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and betamercaptoethanol. Ex. 1001, 18:42–45.

Petitioner asserts Schlegl describes the use of a refold buffer containing refolding additives including as examples L-arginine, Tris, detergents, redox systems like GSH/GSSG, ionic liquids like N'-alkyl and N'-(omega-hydroxy-alkyl)-N-methylimidazolium chlorides. Pet. 51–52, citing Ex. 1003 ¶ 41. Patent Owner does not separately argue claim 17.

As Schlegl describes GSH, which is glutathione-reduced and GSSG, which is glutathione-oxidized arginine, it discloses a thiol pair that falls within that recited by this claim, Petitioner has demonstrated that challenged claim 17 is unpatentable as obvious over Schlegl and Hevehan.

Claim 18

Claim 18 depends from claim 1, and recites that "the incubation is performed under non-aerobic conditions." Ex. 1001, 18:46–47.

Petitioner asserts that one of ordinary skill knew at the time of the invention that aerobic conditions could impact the redox chemistry of the refolding reaction, as testified to by Dr. Robinson. Pet. 55, citing Ex. 1002 ¶ 148. Petitioner also observes that Hevehan describes solutions of reduced

DTT that were prepared immediately prior to each experiment to minimize air oxidation. Pet. 55 (citing Ex. 1004 at 2; Ex. 1028 (fermentation); Ex. 1020, 3 (also fermentation)).

Patent Owner urges that Petitioner incorrectly asserts that the combination of Schlegl and Hevehan teaches that "incubation is performed under non-aerobic conditions." Resp. 47. According to Patent Owner, Dr. Robinson testified during her deposition that Schlegl is "silent on the presence or absence of oxygen." *Id.* (citing Ex. 2019 at 54:20-55:2). Moreover, it is urged that Schlegl's figures make abundantly clear that the refolding tanks are open to air, i.e., under aerobic conditions. *Id.* (citing Ex. 1003 at Figures 1–3.

As for Hevehan, Patent Owner urges that the minimization of oxidation of DTT, a reductant, does not indicate that the refolding of the protein occurred under anaerobic conditions. Reply 47–48 (citing Ex. 2019 at 82:17-20). We agree with Patent Owner, the evidence cited in the Petition does not support a finding that Schlegl or Hevehan describe anaerobic conditions. In its Reply, Petitioner asserts that as DTT oxidation should be minimized, a person of ordinary skill in the art would have been motivated to eliminate oxygen from the refolding reaction. Reply 17. Petitioner fails however to address the open-tank reactors of Hevehan and Schlegl, which substantially undercuts its position.

Consequently, as Hevehan and Schlegl fail to describe anaerobic conditions for folding, we are not persuaded that Petitioner has demonstrated that challenged claim 18 is unpatentable as obvious over Schlegl and Hevehan.

Claims 19–24

Claim 19 depends from claim 1, and recites that the isolation comprises contacting the mixture with an affinity separation matrix. Ex. 1001, 18:48–49. Claim 20 depends from claim 19, and recites that the affinity separation matrix is a Protein A resin. Ex. 1001, 18:50–51. Claim 21 depends from claim 19, and further recites that the affinity resin is a mixed mode separation matrix. Ex. 1001, 18:52–53. Claim 22 depends from claim 1, and further recites that "the isolating comprises contacting the mixture with an ion exchange separation matrix." Ex. 1001, 18:54–56. Claim 23 depends from claim 1, and recites that "the isolating further comprises a filtration step." Ex. 1001, 18:57–58. Claim 24 depends from claim 23, and further recites that "the filtration step comprises depth filtration." Ex. 1001, 18:58–59.

Petitioner asserts that Claims 19–24 are directed to particular isolation methods, each of which were well known in the art at the time of the invention. . Pet. 55–56 (citing Ex. 1002 at ¶ 149). Petitioner urges that these standard methods and their usage are the result of routine optimization, and thus are not patentably distinguishing claim elements. *Id.* Additionally, Petitioner observes that Schlegl describes that protein is separated and purified according to methods known in the art, including, but not limited to, dialysis, filtration, extraction, precipitation and chromatography techniques. Pet. 56 (citing Ex. 1003 ¶ 65). Patent Owner does not meaningfully separately argue claims 19–24.

As Schlegl describes customary known isolation methods, which fall within the methods recited by these claims, and Dr. Robinson has testified to

these being known methods, we are persuaded that Petitioner has demonstrated that challenged claims 19–24 are unpatentable as obvious over Schlegl and Hevehan.

b. Obviousness of Claim 12 in View of Schlegl, Hevehan, and Hakim

Claim 12 depends from claim 1, and further recites that the protein is an Fc-protein conjugate. Ex. 1001, 18:23–24.

Petitioner asserts that a person of ordinary skill at the time the invention was made would have understood Hakim to teach that that the methods of Schlegl and Hevehan could be applied to an Fc-protein conjugate. Pet. 56–58, citing Dr. Robinson's testimony. Ex. 1002 ¶ 151.

Petitioner also observes that Hakim describes a method for producing a full-length antibody fusion protein using an E. coli expression system. Ex. 1006, Abstract.

Because Hakim was able to successfully obtain a full-length antibody fusion protein using an E. coli expression system, Petitioner concludes, based upon Dr. Robinson's testimony, that a person of ordinary skill in the art would have had a reasonable expectation of success in using the method described by Schlegl and Hevehan to produce a fusion protein with an antibody fragment because the Fc region is a smaller portion of a heavy chain, and an Fc-conjugate represents a polypeptide linkage between the Fc region and another protein. Pet. 57 (citing Ex. 1002 ¶ 152).

Patent Owner argues this ground separately. First, it urges that Patent Owner has antedated the Hakim reference. Resp. 50–60. Hakim is relied upon for the teaching of a fusion protein. Ex. 1006, Abstract. Thus, as Petitioner correctly observes, the Patent Owner's evidence of antedating

must credibly establish that AMG 745 is a fusion protein. We are told, that AMG 745 is an Fc protein conjugate. Resp. 52. We are pointed to a passage in Ex. 2026:

> Antimyostatin peptibody (AMG 745) is a novel antimyostatin peptibody. Structurally, it is a fusion protein with a human Fc at the N terminus and a myostatin-neutralizing bioactive peptide at the C terminus.

Ex. 2026, 2.

This description is contained in a 2014 journal article written by researchers who are not the listed inventors of the instant claimed invention. Dr. Hart also testifies that "the AMG 745 identified in the presentations is a protein (Claim 1) and is also a recombinant protein (Claim 7), a 'complex protein' (Claim 10), a multimeric protein (Claim 11), and an Fc-protein conjugate (Claim 12)." Ex. 2021 ¶ 35. He points us to Ex. 2024 at page 5 and Ex. 2022 at page 24. Neither of those exhibits appear to explain what AMG 745 actually is, and the origin and likely continuity of the nomenclature from 2008-2014. We have not been pointed to, nor found, persuasive testimony on this point. We have carefully reviewed the 2008 presentation (Ex. 2024) and are unable to discern sufficient description of AMG 745. Ex. 2022 is somewhat better, giving a model (Ex. 2022, 16) that resembles an antibody, but again no persuasive example of precisely what AMG 745 is.

We are cognizant of Dr. Hart's later testimony (Ex. 2021), and have carefully considered it in its entirety, including paragraphs 33 *et seq.* which

attempt to fill in the gaps of the documentary evidence. However, his testimony is somewhat conclusory. See, *e.g*. paragraphs 35 and 36.

We therefore are unpersuaded that the description in a later publication is sufficient to establish what AMG 745 was in 2008-2009.

In any event, we remain of the viewpoint that Dr. Robinson's testimony (e.g. Ex. 1002 ¶ 151; Ex. 2019, 61–62) is credible. Therefore, even if we do not consider Ex. 1006, we find one of ordinary skill would recognize that the methods of Schlegl could be applied to these types of protein molecules. Schlegl's own description that the methods can be applied to "any protein, protein fragment or peptide that requires refolding upon recombinant expression in order to obtain such protein in its biologically active form" Ex. 1003 ¶ 31 is very direct on this point and consequently very persuasive, despite Patent Owner's characterization of it as overbroad.

We therefore determine that Petitioner has demonstrated that claim 12 is unpatentable as obvious over Schlegl, Hevehan, and Hakim

IV. THE MOTIONS

Paper 17, a joint motion for protective order, requests entry of a protective order slightly modified relative to the Board's protective order. We have reviewed the motion and modified protective order, and find that the modifications are reasonable. Accordingly, the joint motion is GRANTED.

Paper 16 is Patent Owner's Motion to seal exhibits 2021, 2022, and 2024. Patent Owner asserts these are confidential business documents. We have reviewed the documents and, based upon Patent Owner's

representation, agree that their disclosure is not necessary. Accordingly, Patent Owner's Motion to Seal is GRANTED.

Paper 27 is Petitioner's Motion to seal portions of Ex. 1054, which is Dr. Hart's deposition transcript, and portions of the Reply (Paper 26) which rely upon the transcript. We have reviewed the transcript, and based upon Patent Owner's representation, agree that portions asserted to contain confidential information are not necessary to be disclosed. Accordingly, Petitioner's Motion to Seal is GRANTED.

Paper 31 is Patent Owner's Motion to Submit Supplemental Information. As the decision today does not rely upon the supplemental information, the motion is dismissed as moot. Paper 33, a motion to seal the supplemental information that would be submitted if Paper 31 were granted, is also DISMISSED as moot. The papers will remain in the file in confidential status until such time as the Board grants a request for expungement from Patent Owner, following the expiration of any appeal period.

Additionally, we have considered Patent Owner's Motion to seal portions of Exhibit 2059, 2061 (Paper 40), Amgen's Motion for Observations (Papers 41/42) and Amgen's Motion to Exclude (Papers 43/44). We have reviewed these documents, and based on Patent Owner's representation, we agree that the asserted confidential information is not necessary to be disclosed. Accordingly, Patent Owner's Motion to Seal is GRANTED.

Paper 50 is Patent Owner's Motion to Seal its Opposition to Petitioner's Motion to Exclude (Paper 51/52). We have reviewed these

documents and, based on Patent Owner's representation, we agree that the portions redacted in the public version (Paper 52) need not be disclosed. Accordingly, Patent Owner's Motion to Seal is GRANTED.

Paper 44 is Patent Owner's Motion to Exclude several items: (1) portions of Dr. Hart's deposition testimony concerning metadata. (Exhibit 1054) as irrelevant and prejudicial; (2) a construction of "non-aerobic conditions" after Petitioner's Reply as inadmissible; (3) arguments relating to human tissue-type plasminogen activator as new, irrelevant, and misleading; (4) Apotex's arguments and evidence regarding Hevehan's mention of HTTPA as new; (5) Apotex's reliance on Hevehan's mention of L-Arginine in relation to HTTPA as irrelevant, misleading, and confusing; and (6) Apotex's arguments as to the undesirability of oxygen during protein refolds in relation to Claim 18 as new. As we did not rely upon any of the foregoing in rendering this decision, this motion is DISMISSED as moot.

V. CONCLUSION

For the foregoing reasons, we determine that Petitioner has demonstrated that challenged claims 1–17 and 19–24 are unpatentable Petitioner, however, has not demonstrated that challenged claim 18 is unpatentable.

V. ORDER

Accordingly, it is ORDERED that:

Claims 1-11, 13-17 and 19-24 under 35 U.S.C. § 103(a) are unpatentable over Schlegl and Hevehan

Claim 12 under 35 U.S.C. § 103(a) is unpatentable over Schlegl, Hevehan, and Hakim.

IPR2016-01542
Patent 8,952,138 B2

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Exhibit 12

UNITED STATES PATENT AND TRADEMARK OFFICE

_____

BEFORE THE PATENT TRIAL AND APPEAL BOARD

_____

APOTEX INC. and APOTEX CORP.,
Petitioner

v.

AMGEN INC. and AMGEN MANUFACTURING LIMITED,
Patent Owner

_____

CASE IPR2016-01542
Patent 8,952,138

_____

APOTEX'S REQUEST FOR REHEARING UNDER 37 C.F.R. § 42.71(d)

Mail Stop PATENT BOARD
Patent Trial and Appeal Board
U.S. Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

I. INTRODUCTION

Petitioner respectfully requests rehearing under 37 C.F.R. § 42.71(d) of the Board's Final Written Decision ("Decision", Paper 60) finding that claim 18 is not unpatentable.

The Decision contains errors of law based on misapprehending or overlooking the record in the case as it relates to the construction of "non-aerobic conditions" in claim 18, and the application of that construction to find claim 18 not unpatentable. A proper understanding of the record, combined with application of appropriate claim-construction principles, would have provided ample basis to find that Petitioner has carried its burden of showing that claim 18 is unpatentable. 35 U.S.C. § 316(e).

Petitioners respectfully submit that the Board misinterpreted "non-aerobic conditions" in claim 18 by overlooking the express definition of that term in the specification of the '138 Patent. Decision at 40. That express definition is in the "Definitions" section of the '138 Patent specification:

> As used herein, the term "non-aerobic condition" means any reaction or incubation condition that is performed *without intentional aeration* of the mixture by mechanical or chemical means.

Ex. 1001 at 7:20-37 (emphasis added). Rather than applying this broadest reasonable interpretation consistent with the specification, the Board overlooked

that definition and instead applied an inconsistent definition – "in the absence of oxygen". As a result, the Board incorrectly found that claim 18 is not obvious over Schlegl and Hevehan.

The construction of terms not explicitly construed by Petitioner, including "non-aerobic conditions", was previously addressed in the Petition at page 20. Petitioners and their expert, Dr. Robinson, acted accordingly and gave the term "non-aerobic conditions" its broadest reasonable construction in light of the specification in the Petition, Reply, and Dr. Robinson's two Declarations. Pet. (paper 2) at 55; Reply (paper 26) at 17; Ex. 1002 at ¶¶ 147-148; Ex. 1056 at ¶ 67. The express definition of "non-aerobic conditions" was previously addressed in Petitioner's Opposition to Patent Owner's Motion to Exclude at 8-9.

When the proper definition is applied to "non-aerobic conditions", Petitioners have met their burden of demonstrating that claim 18 is unpatentable as obvious. The Final Written Decision on claim 18 should be reconsidered using the explicit definition of "non-aerobic conditions" in the specification, and the Board should conclude that claim 18 is unpatentable over Schlegl and Hevehan.

II. LEGAL STANDARD

"A party dissatisfied with a decision may file a request for rehearing, without prior authorization from the Board." 37 C.F.R. § 42.71(d). "The burden of showing a decision should be modified lies with the party challenging the

teach the intentional addition of oxygen. Consequently, the record supports the finding that claim 18 is unpatentable as obvious over Schlegl and Hevehan.

IV. CONCLUSION

The term "non-aerobic conditions" should have been construed in a manner consistent with the express definition and consistent usage in specification: "any reaction or incubation condition that is performed without intentional aeration of the mixture by mechanical or chemical means." When that broadest reasonable interpretation in light of the specification is applied, the prior art renders claim 18 of the '138 Patent obvious.

In view of the foregoing, Petitioner respectfully requests that the Board grant rehearing and modify its Decision to find that Apotex has shown, by a preponderance of the evidence, that claim 18 is obvious over Schlegl and Hevehan.

Date: March 16, 2018

Respectfully submitted,

/Teresa Stanek Rea/

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IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF NEW JERSEY

AMGEN INC. and AMGEN MANUFACTURING, LIMITED, Plaintiffs, v. ADELLO BIOLOGICS, LLC, AMNEAL PHARMACEUTICALS, LLC, and AMNEAL PHARMACEUTICALS, INC. Defendants.	Civil Action No. 2:18-cv-03347 (CCC-MF)

DECLARATION OF RICHARD C. WILLSON, Ph.D. IN SUPPORT OF AMGEN'S OPENING CLAIM CONSTRUCTION BRIEF FOR U.S. PATENT NOS. 8,940,878 AND 9,643,997

TABLE OF CONTENTS

Page

I, RICHARD C. WILLSON, hereby declare:

I. INTRODUCTION

1. I have been retained as an independent expert consultant by counsel on behalf of Amgen Inc. and Amgen Manufacturing, Limited (collectively, "Amgen") in connection with *Amgen Inc. et al. v. Adello Biologics, LLC et al.*, Case No. 18-cv-3347 (CCC-MF).

2. The patents in this case about which I have been asked to opine are: U.S. Patent Nos. 8,940,878 (the "'878 Patent") and 9,643,997 (the "'997 Patent") (collectively, the "Asserted Patents").

3. For my work on this matter, I am being compensated at my current standard rate of $600 per hour. My compensation is not dependent on the opinions I express herein or on the outcome of this matter.

4. The opinions expressed herein are based on my education and professional experience, which spans 30 years in biochemical engineering including protein purification. A copy of my curriculum vitae ("CV") is attached hereto as **Exhibit A**.

5. In the past four years, I have provided expert testimony in six matters by way of declarations, depositions or at trial as listed in **Exhibit B** hereto.

6. In connection with the preparation of this declaration, I have reviewed the materials listed in **Exhibit C** hereto.

7. I reserve the right to respond to positions taken by Defendants Adello Biologics, LLC., Amneal Pharmaceuticals, LLC and Amneal Pharmaceuticals, Inc. (together, "Adello") and their experts in connection with Adello's opening claim construction brief.

II. PROFESSIONAL BACKGROUND AND QULIFICATIONS

8. My CV lists the publications I have authored or co-authored during the previous ten years. Over the course of my career I have published approximately 130 papers in peer-reviewed journals pertaining to my research.

9. In 1981 and 1982, respectively, I received B.S. (honors) and M.S. degrees in Chemical Engineering from the California Institute of Technology.

10. In 1988, I received a Ph.D. from the Massachusetts Institute of Technology ("MIT") in Chemical Engineering, with a minor in Microbiology. The title of my thesis was "Fermentation Product Recovery by Supercritical Fluid Extraction: Microbiological and Phase Equilibrium Aspects."

11. I am currently the Huffington-Woestemeyer Professor of Chemical and Biomolecular Engineering and Biochemistry at the University of Houston, a position I have held since 2013. I joined the faculty of University of Houston in 1988 as an Assistant Professor, and became a tenured Associate Professor in 1994 and a tenured Professor in 2003.

12. Over the course of my career, I have taught undergraduate and graduate courses in chemical and biomolecular engineering and biochemistry, and have overseen a laboratory conducting research in these areas. I have taught courses in chromatography, protein manufacturing and bioseparations and basic biochemical engineering courses that have included lessons on protein refolding and protein purification. My laboratory's research and certain of our publications have, from time to time, addressed and/or involved expressing proteins in non-mammalian expression systems, such as *E. coli*, and subsequent downstream processing, including protein refolding and purification on a variety of separation matrices, including ion exchange, hydrophobic interaction, and immobilized-metal affinity matrices. Many of my former students work in downstream processing and biologics manufacturing in the pharmaceutical industry, and several have prominent positions in the industry.

13. During the course of my career, I have received numerous awards and have become a member of many scientific associations. Some of these awards include the van Lanen Award from the Biochemical Technology Division of the American Chemical Society (2001), the UH Cullen College of Engineering Senior Faculty Research Award (2005), the Fluor-Daniel Award of the UH Cullen College of Engineering (2009) and the Pierce Award in Affinity Technology from the International Society for Molecular Recognition (2013) among others. I have

been a Fellow of the American Institute of Medical and Biological Engineering since 1999, a member of Phi Kappa Phi, a Fellow of the American Association for the Advancement of Science since 2011, and a Fellow of the American Chemical Society since 2014, among others. I have served as a member on various federal review committees for the National Science Foundation, National Institutes of Health, and Department of Defense.

14. I regularly review scientific papers submitted for publication in various highly ranked scientific journals. These journals include *Journal of Molecular Recognition*, *Journal of Chromatography, International Journal of Biochromatography*, *Biotechnology Progress*, *Biotechnology & Bioengineering*, and *PLOS One*, among others.

15. I am currently a primary organizer of *Highland Games*, a global industry benchmarking competition on prediction of biophysical and purification (including chromatography) properties of candidate monoclonal antibody pharmaceuticals.

III. ISSUES ADDRESSED

16. I understand that this case has progressed to the stage at which the Court will construe the claims of the Asserted Patents. I understand that both patents are owned by Amgen and have been asserted in this case against Adello.

17. I have been informed that the claims of a United States patent are to be interpreted from the vantage point of a person of ordinary skill in the art ("POSITA") to which the patent pertains, in light of the teaching of the patent and the prosecution history as of the priority date of the patent.

18. I have been asked to provide background information in the art of protein purification, which is the art to which the Asserted Patents pertain. This declaration summarizes that information.

19. To the extent I am asked to testify at the Claim Construction hearing, I may provide additional background on the science and technology underlying the Asserted Patents. I reserve the right to use visual aids to illustrate my testimony.

20. I understand that discovery is still ongoing and that the issues in dispute may change as a result. I reserve the right to modify or supplement my opinions as appropriate.

IV. THE PERSON OF ORDINARY SKILL IN THE ART

21. It is my opinion that the person of ordinary skill in the art of protein purification would have a Ph.D. in biochemical engineering, biomedical engineering, biochemistry, or a related discipline, with at least two years of work experience in the field of protein chromatography as of the priority date of the Asserted Patents. Additional training or study could substitute for additional work

experience and additional work experience or training could substitute for formal education.

22. Based on my education and professional experience, I believe I have the appropriate background to discuss the knowledge of a POSITA as of the priority date of the Asserted Patents.

V. TECHNOLOGY OVERVIEW

A. The Asserted Patents

23. The ’878 Patent is generally directed to improved methods for purifying proteins expressed in non-mammalian cells and, in certain embodiments, to the purification of proteins expressed as insoluble inclusion bodies in recombinant bacteria. *E.g.*, ’878 Patent, 11:55-17:4. Prior to the invention of the ’878 Patent, it was believed in the art that certain of the specialized chemical compounds used to process inclusion bodies (so that the proteins in them can be solubilized and subsequently recovered in biologically active form) had to be diluted or reduced or removed prior to the application of a refold solution to a separation matrix to achieve purification. *Id.* at 12:16-20. The conventional wisdom before the ’878 Patent was that these specialized chemical compounds in the refold solution could prevent or disrupt the interactions with a separation matrix necessary to achieve purification. *Id.* at 15:29-37. Thus, in the prior art, processing steps, such as a dilution, intervened between protein refolding and application to a first chromatographic separation matrix. *Id.* at 15:25-29.

24. The '878 Patent invention reflects the inventors' insight that protein purification can be achieved by ***directly*** applying a refold solution to a separation matrix, without intervening processing. *Id.* at 11:58-63, 15:25-42. Such additional processing can be costly and time-consuming, particularly at a large manufacturing scale. *Id.* at 11:58-63, 12:21-26, 15:30-42. The invention is applicable whether the first separation matrix to which the refold solution is directly applied captures an impurity protein or the protein of interest (*i.e.*, the desired protein at the end of the purification process). *Id.* at 14:65-15:5. Whatever the function of the first separation matrix in the overall purification scheme, the patent teaches eliminating processing steps typically used in the prior art after protein refolding and prior to application to a first separation matrix.

25. The '997 Patent is also generally directed to improved methods for purifying proteins expressed in non-mammalian cells. The inventors of the '997 Patent discovered that proteins could be purified by applying a refold solution to a separation matrix without diluting such refold solution and without subjecting the refold solution to the steps of dialysis, precipitation or centrifugation, after protein refolding but before purifying the protein. *See* '997 Patent at col. 4:52-57, 12:14-20; Exhibit 5 of the Sandel Declaration, '997 Patent Prosecution History, at 8-9 (10/2/2015 Office Action at 6-7) and at 22-23 (3/1/2016 Response to Office Action at 11-12). Prior to this invention, these intervening processing steps had been used

in the prior art. This invention thus provides a more efficient means of purifying proteins, eliminating these steps from the purification process. *See id.* at col. 12:14-20.

B. Recombinant Proteins

26. A review of the basic structure and function of DNA is helpful to understanding the process of recombinant protein expression. As shown below, DNA is a long molecule made up of just four different kinds of building blocks, or nucleotides, called A, C, T, and G. The order in which these nucleotides are strung together carries genetic information. DNA usually exists in a form where two such strands of nucleotides are wound around one another to create the famous double helix. With few exceptions, opposing nucleotides in the double helix obey precise pairing rules. Specifically, an A in one strand pairs with a T in the complementary strand, and a G in one strand pairs with a C in the complementary strand.

27. For a cell to reproduce, it must copy and transmit all of its DNA, which contains its genetic information, to its progeny. The two opposing strands of DNA facilitate this process, with each strand acting as a template for the synthesis of a new complementary DNA molecule.

Figure 1. Basic Structure of DNA

28. In addition to its role in reproduction, DNA also provides the cell with a blueprint for protein expression. Each protein is encoded by a unique sequence of nucleotides, which is called a gene. Cells use the information stored in the gene to produce proteins following a two-step process. In the first step, "transcription," the cell transcribes the gene sequence into a single-stranded molecule called mRNA (messenger ribonucleic acid), which is chemically similar to DNA and contains the same information encoded in a similar, though slightly different, way. The second step of the process is called "translation." During this step, ribosomes—protein-synthesizing machines within the cell—read the mRNA and use that information to select and connect together amino acids to make protein molecules (polypeptides). Specifically, three nucleotides code for one amino acid. The structure and function of a protein molecule is determined by its precise sequence of amino acids.

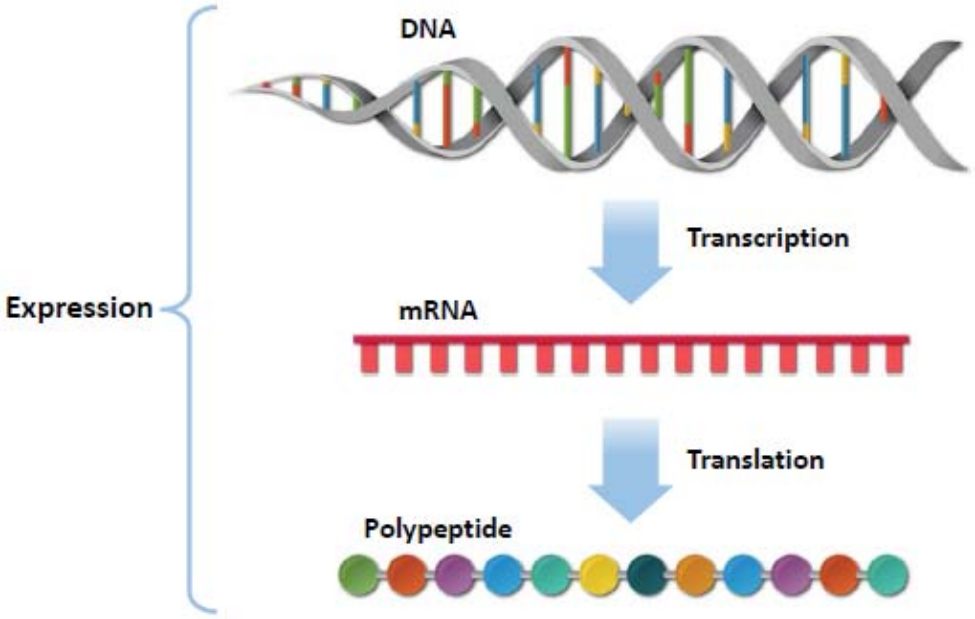

Figure 2. Expression of Proteins

29. Granulocyte - colony stimulating factor ("G-CSF") is an example of a protein. Every cell contains thousands of different proteins which work together to perform virtually every process within the cell, including cellular functions such as metabolic reactions, signaling, sensing, and growth. Proteins are made up of one or more chains of amino acids, the sequence of which is encoded in an organism's DNA as described above. These chains of amino acids are also called "polypeptides" because the amino acids making up the chains are connected by chemical bonds called peptide bonds. The way a linear polypeptide chain folds into its three-dimensional structure gives a protein its characteristic shape and function.

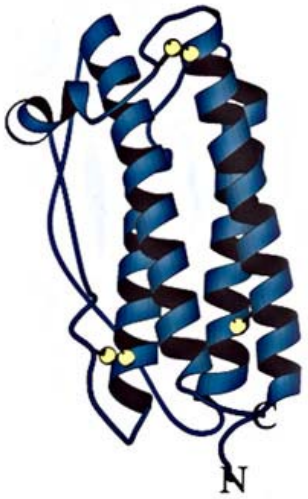

Figure 3. G-CSF[1]

30. For a cell to produce a biologically-active protein, several things must happen—the cell must make the correct sequence of amino acids and must also ensure that the protein is properly folded, processed, and in some cases, transported. This complex series of processes must be replicated on a large scale in order to harness cells' ability to synthesize useful quantities of therapeutically-valuable proteins.

31. Human cells that naturally produce a protein like G-CSF usually do so in very small quantities such that harvesting the product would be prohibitively expensive. Recombinant DNA technology was a breakthrough that allowed for the

[1] Figure 3 illustrates the tertiary structure of G-CSF. Courtesy of Professor Donald Metcalf, a*vailable at* http://archive.maas.museum/australia_innovates/view/dsp_image0523.html?image=/media/client/ACF15E.jpg

production of larger amounts of useful proteins. Recombinant DNA technology typically refers to the process of combining DNA from two or more sources.

32. Recombinantly produced G-CSF is used to stimulate the production of granulocytes (a type of white blood cell) in patients undergoing therapy that will cause low white blood cell counts. This medication is used to prevent infection and neutropenic (low white blood cells) fevers caused by chemotherapy.

33. G-CSF is composed of a single chain of 175 amino acids. In order to express a recombinant protein, a cell must be genetically engineered using recombinant DNA technology. Recombinant DNA technology uses host cells, such as *E. coli* bacteria, to produce proteins foreign to that cell through a process called "recombinant expression" of the protein. For example, human DNA can be inserted into the DNA of the host cell, such as *E. coli* bacteria. *E. coli* bacteria are an example of a non-mammalian expression system.

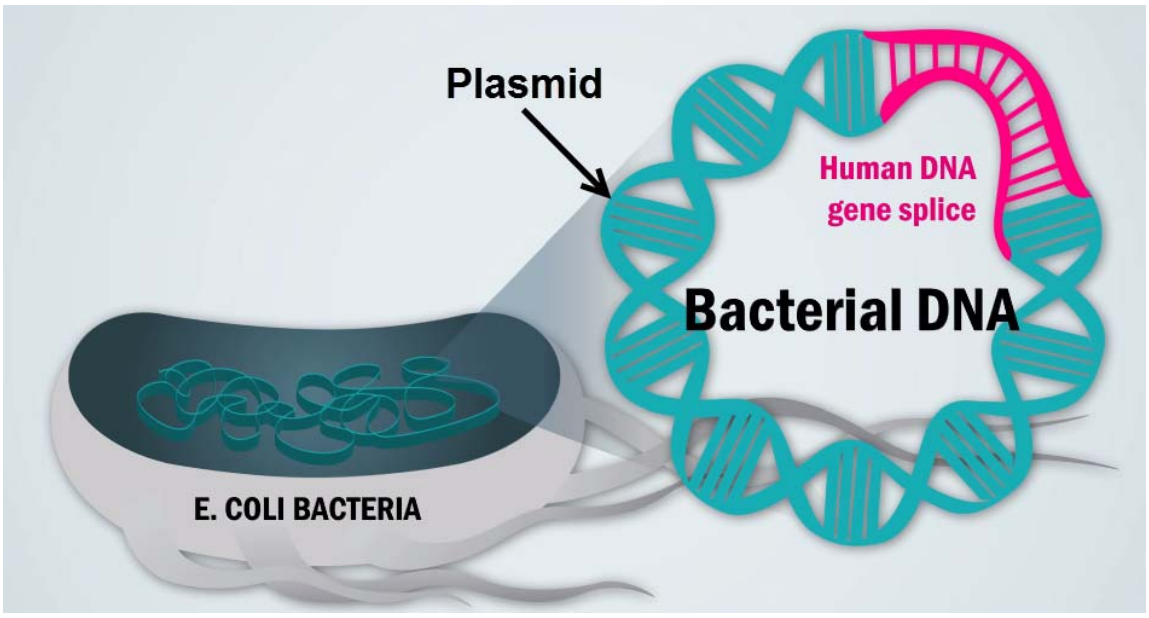

Figure 4. Recombinant DNA Technology

34. Recombinant protein expression is a multi-step process. First, the gene encoding the desired protein (for example, the gene encoding human G-CSF) must be identified and sequenced. Then, that gene is introduced into a vector, such as a circular loop of DNA called a "plasmid," so that it can be inserted into the host cell. On the plasmid, the gene must also be linked to special sequences instructing the host cell to read the gene and produce the desired protein from it. After the plasmid is introduced into the host cell, the hope is that the host cell will read the newly inserted gene and "express" the desired protein in sufficient quantities so that it can then be purified, and put to use.

C. Protein Structure and Function

35. Amino acids (except proline), which determine the structure and function of a protein molecule, have the generic structure shown below. The

amino substituent (H_2N– or –NH_2), at left, is called the "alpha"-amino group. The "R" substituent is a side-chain that varies with the type of amino acid, and gives each amino acid its unique identity. For example, in the amino acid cysteine, the R group is –CH_2–SH. Lastly, at right, is the carboxyl group (which may also be depicted as –COOH).

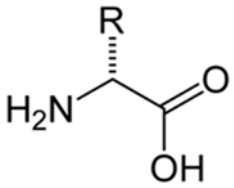

Figure 5. Amino Acid Structure

36. In a protein molecule, where the amino acids are connected in a sequence by peptide bonds, one end of the protein molecule retains the unreacted "alpha"-amino group, referred to as the "N-terminus" while the other end retains the unreacted carboxyl group, referred to as the "C-terminus".

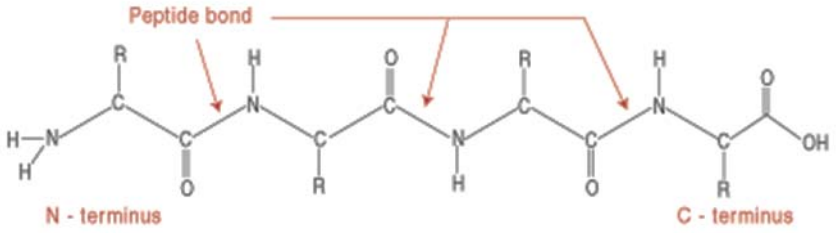

Figure 6. Protein Molecule, or Polypeptide Chain

37. The structure of a protein molecule can be described in three ways: its primary structure, secondary structure, and tertiary structure.[2]

[2] Some proteins can also be described in terms of their quaternary structure, which refers to the structure formed by the interaction of two or more protein chains (as

38. The primary structure of a protein refers to its amino acid sequence, which is generally written from the N-terminus to the C-terminus (this is the way polypeptides are normally synthesized in the body). This sequence dictates how the initially linear protein molecule folds up on itself to take on a unique three-dimensional conformation that, in turn, confers the protein's unique function.

39. The secondary structure refers to the three-dimensional structure of local segments of the protein. These local substructures often have distinctive shapes, such as alpha helices and beta sheets, which are formed by hydrogen bonding between different segments of the protein's peptide backbone. For an example, see Figure 7 below. Different secondary structures often are present in different parts of the same protein molecule.

opposed to the structure of a single protein chain) to form a single multi-chain assembly. G-CSF has a single chain.

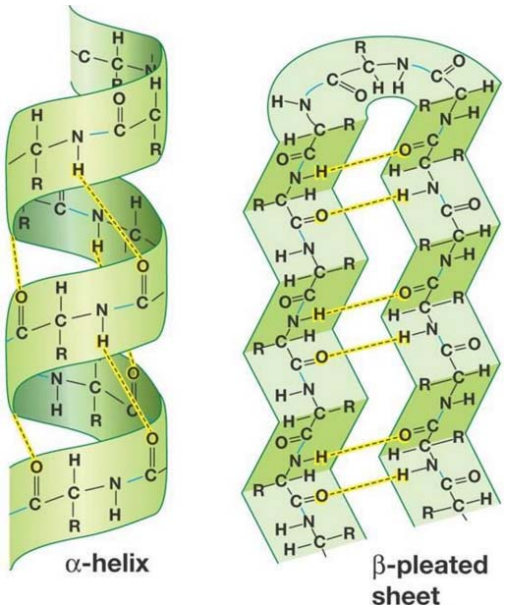

Figure 7. Secondary Structures of Polypeptides Formed by Hydrogen Bonds

40. A protein's tertiary structure is the overall three-dimensional shape into which it is folded, *i.e.*, the spatial relationship of the secondary structures to one another. It controls the basic function of the protein.

41. The tertiary structure is generally stabilized by nonlocal interactions between amino acids not adjacent to one another in the primary sequence, most commonly the formation of a hydrophobic core (hydrophobic ("greasy") side chains located in the interior of a protein away from water), but also through interactions of charged groups, hydrogen bonds, and disulfide bonds between cysteines. The amino acid cysteine contains a "—S-H" or "thiol" side chain group. Each cysteine residue in a protein is capable of forming a disulfide bridge ("—S-S—") with other cysteine residues, both within the same protein chain and with

other protein chains, depending on the conditions. Disulfide bonds can help stabilize the three dimensional structure of cysteine-containing proteins

D. Protein Purification

42. There are a variety of different methods used to purify a protein of interest after refolding. Chromatography is the purification method that is the focus of this declaration.

43. Chromatography is a method of separating molecules, such as protein molecules, in a solution (the "mobile phase") based on differences in their chemical or physical interactions with a solid, or stationary, matrix (the "stationary phase").

44. The stationary phase often is in the form of particles, or "resins," packed inside a tube, or "column." Chromatography resins are most commonly made of natural or synthetic polymeric materials and may have certain functional groups (also called "ligands") attached that facilitate binding.

45. One or more solutions may pass through the column in chromatography, including, for example, solutions to prepare the resin for use, solutions containing the molecule or protein of interest to be purified, solutions to wash undesired proteins or contaminants from the resin, solutions to elute, *i.e.*, release, the protein from the matrix, and a single solution may serve multiple functions. These solutions can be referred to in different ways. For example, a

solution used to prepare the resin for use may be called an "equilibration buffer" or "equilibration solution," among other names. Practitioners may ascribe specific meanings to these terms depending on the characteristics and purpose of the solutions, or they may use the terms interchangeably. For example, the word "buffer" refers to a solution that resists changes in pH, but in the context of chromatography the same term is also commonly used in the art to refer to liquid preparations generally, regardless of whether such a preparation resists pH changes.

46. During chromatography, one or more solutions are added to the column such that no part of the stationary matrix is allowed to run dry at any point in time because doing so leads to poor separation of the protein of interest from contaminants.

47. Chromatographic separations of proteins can be monitored and displayed visually in a "chromatogram," a plot of some measured property of the proteins in the liquid exiting the chromatography column as a function of time. Measured properties include the degree of absorbance of ultraviolet light, *e.g.*, at 205 nm or 280 nm wavelength which correlates with protein concentration. Proteins in particular are known for their ability to absorb light at these wavelengths. Absorbance at 280 nm ("UV_{280} nm") can be plotted on a strip chart

recording or electronic display, generating a graphic representation of the separated proteins.

48. The liquid exiting the chromatography column may also be monitored for pH, conductivity (a measure of salt concentration) and the like, by various devices and in-line probes. Traces of such measurements can also be plotted on the chromatogram as a function of time.

49. There are two basic mechanisms of chromatography: adsorption and non-adsorptive. In the case of adsorption, sample molecules can interact with, be adsorbed onto, or bind to, the stationary phase. The types of adsorption-based chromatography differ in the nature of the interaction between the sample molecules and the stationary phase. Examples of adsorption-based chromatography include ion-exchange chromatography and hydrophobic interaction chromatography. Non-adsorptive chromatography, such as size exclusion chromatography, relies on interactions between the sample molecules and the stationary phase, but not binding. In the case of size exclusion chromatography, the stationary phase is porous. Small molecules can enter the pores and take longer to exit the column while larger molecules can only enter the void spaces around the particles, and move past the stationary phase and exit the column sooner. Hence, the molecules separate on the basis of size.

I declare under penalty of perjury under the laws of the United States that the foregoing is true and correct. Executed on April 15, 2019, in Houston, Texas.

Richard C. Willson

Dated: April, 15, 2019

Exhibit A

Exhibit A: Curriculum Vitae

Richard C. Willson

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University of Houston
4826 Calhoun Rd.
Houston, TX USA 77204-4004
832 455-1428; Willson@uh.edu

Training:

B.S.	Chemical Engineering (1981; honors), California Institute of Technology.
M.S.	Chemical Engineering (1982), California Institute of Technology.
Ph.D.	Chemical Engineering, Minor: Microbiology (1988), Massachusetts Institute of Technology.
Postdoctoral	Biology (1988), Massachusetts Institute of Technology

Professional Experience:

1988-1994	Assistant Professor of Chemical Engineering, University of Houston
1993-1994	Assistant Professor of Biochemistry, University of Houston
1994-2003	Associate Professor, Chemical Engineering and Biochemistry, University of Houston
2003-2010	Professor of Chemical & Biomolecular Engineering and Biochemistry, University of Houston
2007-present	Senior Affiliate Member, Houston Methodist Research Institute
2008-present	Affiliate, Structural and Computational Biochemistry and Molecular Biology Program, Baylor College of Medicine
2008-present	Theme Leader, Diagnostics, NIH Western Regional Center of Excellence in Biodefense and Emerging Infectious Diseases
2010-2013	John & Rebecca Moores Professor of Chemical & Biomolecular Engineering and Biochemistry, University of Houston
2013-present	Huffington-Woestemeyer Professor of Chemical & Biomolecular Engineering and Biochemistry, University of Houston
2015-2016	Interim Associate VP Technology Transfer, Division of Research, University of Houston

Professional Activities/Honors:

1990-1995	NSF Presidential Young Investigator
1996-1998	Editorial Board, *Journal of Molecular Recognition*
1998-2002	Editorial board, *International Journal of Biochromatography*
1999	Chair, ACS Division of Biochemical Technology
1999-present	Fellow, American Institute of Medical and Biological Engineering
2001	van Lanen Award, Biochemical Technology Division, American Chemical Society
2001-present	Editorial board, *Journal of Molecular Recognition*
2003-present	Editorial board, *Biotechnology Progress*
2003-present	President, past President, International Society for Molecular Recognition
2004-present	Editorial board, *Faculty of 1000*
2005	UH Cullen College of Engineering Senior Faculty Research Award
2007-present	Senior Affiliate Member, Houston Methodist Research Institute
2008-present	Editorial Board, *Journal of Biophysics*
2008-present	Editorial Board, *PLOS One*
2009	Fluor-Daniel Award of the UH Cullen College of Engineering
2011-present	Fellow, American Association for the Advancement of Science
2014-present	Member, Phi Kappa Phi
2014-present	Fellow, American Chemical Society
2014-present	Fellow, US National Academy of Inventors
2015	Pierce Award in Affinity Technology, International Society for Molecular Recognition
2017-present	Co-organizer, *Highland Games*, global benchmarking competition on prediction of biophysical/purification properties of candidate monoclonal antibody pharmaceuticals
2018-present	Research Committee, DHS S&T Directorate Borders, Trade and Immigration Institute

Recent Consulting Activities

Technical Advisory Board, Moderna, Inc., Cambridge, MA

Recent Refereed Publications:

Chen H, Crum M, Chavan D, Vu B, Kourentzi K, Willson RC. Nanoparticle-Based Proximity Ligation Assay for Ultrasensitive, Quantitative Detection of Protein Biomarkers. ACS Appl Mater Interfaces. 2018 Sep 26;10(38):31845-31849.

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Supervision of Research:

Chemical Engineering: R. Kimura, M.S., 1990; E. Thorne, M.S., 1993; B. Cornut, Ph.D. 1993; D. Gill, Ph.D. 1993; K. Hibbits, Ph.D. 1994; D. Roush, Ph.D., 1994; K. Maillard, Ph.D., 1996; A. Xavier, Ph.D., 1996; R. Rippley, Ph.D., 1996 (with C. Stokes); Sheryl Horton-King, M.S., 1997; N. Singh, M.S., 1997; N. Offringa, Ph.D., 1998; Robert Wagner, M.S., 1998; Jason Murphy, M.S., 1998; C. Uehara, M.S., 1999; J. Murphy, Ph.D., 2000; D. Hill, M.S., 2001; K. Kourentzi, Ph.D., 2002; D. Jewell, M.S., 2002; J. Strauch, M.S., 2002; C. Uehara, Ph.D., 2003; T. Cano, Ph.D., 2005; GW Jackson, Ph.D., 2006, A. Potty, Ph.D. 2008; S. Kemper, Ph.D., 2008; J. Fu, Ph.D., 2009; M. Anez, Ph.D., 2010; E. Cacao, Ph.D., 2011; W. Chen, Ph.D. 2012; B. Raja, Ph.D., 2014; G. Garvey, Ph.D., 2016; Patterson, A., Ph.D. 2016; Kim, J., Ph.D. 2016; Yang, Z., M.S., 2016; Luo, D., Ph.D., 2018; Hlavinka, V., M.S. 2018.

Biochemical and Biophysical Sciences: P. Patel, M.S. 1995; J. Marshall, M.S., 1995; Auguste Nioupin, M.S., 1995; J. Wibbenmeyer, Ph.D. 1997; S. Balan, Ph.D. 2005; X. Zhang, Ph.D., 2008, C. Gupta, Ph.D., 2010; I. Kanakaraj, Ph.D., 2012; M. Adhikari, Ph.D., 2013; MV. Poongavanam, Ph.D., 2014; H. Chen, Ph.D. 2017.

Bioengineering: U. Prasad, M.S. 1994; R. Walia, M.S., 1998; P. Gibbs, Ph.D. 2000; A. Tang, M.S., 2003; D. Patel, M.S., 2004; J. Litvinov, Ph.D., 2013.

Exhibit B

Exhibit B: Prior Testimony

1. *Amgen Inc. and Amgen Manufacturing Limited v. Apotex Inc. and Apotex Corp.*, Case No. 15-cv-61631-JIC/BSS, Southern District of Florida.

2. *Apotex Inc. and Apotex Corp. v. Amgen Inc. and Amgen Manufacturing Limited*, Inter Partes Review No.: IPR2016-01542.

3. *Amgen Inc. and Amgen Manufacturing Limited v. Sandoz Inc., Sandoz International GmbH, and Sandoz GmbH*, Case No. 3:14-cv-04741, Northern District of California.

4. *Amgen Inc. and Amgen Manufacturing Limited v. Sandoz Inc., Sandoz International GmbH, and Sandoz GmbH, Lek Pharmaceuticals d.d.*, Case No. 3:16-cv-02581, Northern District of California.

5. *Amgen Inc. and Amgen Manufacturing Limited v. Mylan Inc., Mylan Pharmaceuticals Inc., Mylan GmbH, Mylan N.V.*, Case No. 2:17-cv-01235-MRH, Western District of Pennsylvania.

6. *Immunomedics, Inc. v. Roger Williams Medical Center, Richard P. Junghans, M.D., Ph.D., Steven C. Katz, M.D.*, Case No. 2:18-cv-00407-JLL-SCM, Northern District of California.

Exhibit C

Exhibit C: List of Materials Considered

Patents

U.S. Patent No. 9,643,997
U.S. Patent No. 8,940,878

Other Documents

D.I. 101, Joint Claim Construction and Prehearing Statement (Mar. 22, 2019)
U.S. Application No. 14-599,336: Prosecution History for U.S. Patent No. 9,643,997
U.S. Application No. 12-822,990: Prosecution History for U.S. Patent No. 8,940,878

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF NEW JERSEY

AMGEN INC. and AMGEN MANUFACTURING, LIMITED, Plaintiffs, v. ADELLO BIOLOGICS, LLC, AMNEAL PHARMACEUTICALS, LLC, and AMNEAL PHARMACEUTICALS, INC. Defendants.	Civil Action No. 2:18-cv-03347 (CCC-MF)

DECLARATION OF RICHARD C. PAGE, Ph.D. IN SUPPORT OF AMGEN'S OPENING CLAIM CONSTRUCTION BRIEF FOR U.S. PATENT NO. 9,856,287

TABLE OF CONTENTS

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I, RICHARD C. PAGE, hereby declare:

I. INTRODUCTION

1. I have been retained as an independent expert consultant by counsel on behalf of Amgen Inc. and Amgen Manufacturing, Limited (together, "Amgen") in connection with *Amgen Inc. et al. v. Adello Biologics, LLC et al.,* Case No. 18-cv-3347 (CCC-MF).

2. I have been informed that Amgen sued Adello Biologics, LLC., Amneal Pharmaceuticals, LLC and Amneal Pharmaceuticals, Inc. (together, "Adello") for, among other things, infringement of U.S. Patent No. 9,856,287, which I will refer to as the '287 Patent.

3. For my work on this matter, I am being compensated at my current standard rate of $480 per hour. My compensation is not dependent on the opinions I express herein or on the outcome of this matter.

4. The opinions expressed herein are based on my education and professional experience, which spans 13 years in biochemistry and biophysics. A copy of my curriculum vitae ("CV") is attached hereto as Exhibit A.

5. In connection with the preparation of this declaration, I have reviewed the materials listed in Exhibit B hereto.

6. I reserve the right to respond to positions taken by Adello and its experts in connection with Adello's opening claim construction brief.

II. PROFESSIONAL BACKGROUND AND QULIFICATIONS

7. My CV lists all publications I have authored or co-authored, up to November 2018, during the previous 13 years. Over the course of my career, I have published approximately 42 papers in peer-reviewed journals pertaining to my research.

8. In 2002, I received B.S. in Biochemistry from California Polytechnic State University, San Luis Obispo.

9. In 2008, I received a Ph.D. from Florida State University in Biochemistry. My Ph.D. work was to develop methodologies to express, purify and biophysically characterize membrane proteins and transmembrane peptides by solution and solid state nuclear magnetic resonance ("NMR") under the mentorship of Prof. Timothy Cross. The title of my thesis was "Structural Characterizations of Integral Membrane Proteins: The Nexus of Solution and Solid State NMR Spectroscopy."

10. Between 2008 and 2013, I was a post-doctoral fellow at the Cleveland Clinic and utilized NMR and X-ray crystallography to uncover the molecular basis for regulating CHIP mediated ubiquitination, a key protein quality control pathway. These studies examined the role of the ubiquitin ligase CHIP, and the chaperone Hsp70 in connecting protein refolding and protein degradation pathways within cells. Specifically, we sought to determine how Hsp70 identifies misfolded

proteins, how Hsp70 acts to refold misfolded proteins back to their native conformation, and how CHIP seeks to direct misfolded proteins to proteasomal degradation by hijacking the ability for Hsp70 to recognize and recruit misfolded proteins. My post-doctoral advisor was Dr. Saurav Misra at the Department of Molecular Cardiology.

11. I am currently the Robert H. and Nancy J. Blayney Associate Professor and the Assistant Department Chair at the Department of Chemistry and Biochemistry at Miami University, Ohio, a position I have held since 2018. I joined the faculty of Miami University in 2013 as an Assistant Professor and became a tenured Associate Professor in 2018.

12. Over the course of my career, I have taught undergraduate and graduate courses in biophysical chemistry (thermodynamics), membrane proteins, and signal cascades. My course on biophysical chemistry focuses on the thermodynamics of biological systems and places particular focus on proteins. In addition to conventional thermodynamics topics, this course covers the energetics (Gibbs free energy, enthalpy, and entropy) of protein folding, the thermodynamics of oxidation and reduction chemistry, and techniques for analyzing the folding state and stability of proteins. The membrane proteins and signal cascades courses include extensive discussions of protein structure and the relation between protein structure and biological function. My laboratory's research and certain of our

publications have, from time to time, addressed and/or involved the thermodynamic analyses of protein folding, the protein folding, misfolding, and refolding, and structural characterizations of proteins including 21 protein structures deposited in the Protein Data Bank. While all of my projects are centered on the structure and biophysics of proteins, my project on protein quality control by the CHIP/Hsp70 complex focuses on the refolding of misfolded proteins and the biochemical, biophysical, and structural mechanisms that govern the interplay between protein refolding and degradation, termed protein quality control. This project is funded by an R35 grant from the National Institutes of Health entitled "Triage mechanisms for directing protein refolding and degradation" and was previously funded by a CAREER grant from the National Science Foundation entitled "Dynamic Regulation of Protein Quality Control."

13. During the course of my career, I have received numerous awards and have become a member of many scientific associations. Some of these awards include the Miami University Distinguished Junior Faculty Scholar Award, and the F. Merlin Bumpus Junior Investigator Award. I have been a member of the American Society for Biochemistry and Molecular Biology, American Chemical Society, International Society for Magnetic Resonance, American Association for Cancer Research, American Association for the Advancement of Science, and the Biophysical Society. In 2015, I was elected to serve on the Public Affairs

Advisory Committee for the American Society for Biochemistry and Molecular Biology. I have served as a member on various federal review committees for the National Science Foundation, National Institutes of Health, the National Sciences and Engineering Council of Canada, Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), and the Agence Nationale de la Recherche (ANR, French National Research Agency).

14. I regularly review scientific papers submitted for publication in various highly ranked scientific journals. These journals include *Nature Communications*, *Biochemistry*, *Methods in Enzymology*, *PLoS ONE*, *Nature Chemical Biology*, *Protein Science*, and *Biomacromolecules*, among others.

III. ISSUES ADDRESSED

15. I have been informed by Amgen's counsel that under a section of the Patent Act, 35 U.S.C. § 112 ¶ 2, the claims of a patent must "particularly point[ ] out and distinctly claim[ ] the subject matter which the applicant regards as [the] invention," and that the U.S. Supreme Court has interpreted Section 112 to require that patent claims, read in light of the specification and prosecution history, inform persons skilled in the art about the scope of the claimed invention with reasonable certainty. I further understand that if a patent claim does not meet this standard, it is invalid for indefiniteness.

16. I understand from counsel that the claims of a patent are written to those of skill in the art in the field at the time of the invention and that the meaning of the claims should be understood in light of the patent specification and the patent prosecution history.

17. I have been asked to provide background information in the art of protein refolding, which is the art to which the ’287 Patent pertains. I have also been asked to provide my opinions as to the definiteness of certain claim terms of the ’287 Patent.

18. To the extent I am asked to testify at the Claim Construction hearing, I may provide additional background on the science and technology underlying the ’287 Patent. I reserve the right to use visual aids to illustrate my testimony.

19. I understand that discovery is still ongoing and that the issues in dispute may change as a result. I reserve the right to modify or supplement my opinions as appropriate.

IV. PERSONS OF ORDINARY SKILL IN THE ART

20. It is my opinion that persons of ordinary skill in the art (POSITA) of the ’287 Patent would have a Ph.D. in biochemistry, biochemical engineering, molecular biology, or a related biological/chemical/engineering discipline, or a master’s degree in such disciplines and several years of industrial experience

producing proteins in non-mammalian expression systems, as of the '287 Patent priority date of June 22, 2009.

21. Based on my education and professional experience, I believe I have the appropriate background to discuss the knowledge of POSITA as of the priority date of the '287 Patent.

V. TECHNOLOGY OVERVIEW

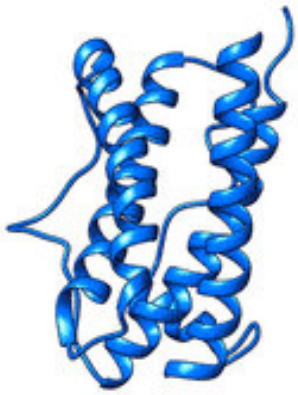
22. The '287 Patent discloses and claims a methodology for refolding proteins under certain chemical conditions that allow for proper refolding of a desired protein. Using their redox chemistry-based approach, the inventors of the '287 Patent discovered that the distribution of properly and improperly refolded species of a protein of interest was influenced by a relationship between thiol-pair ratio and thiol-pair buffer strength, both of which were dependent on the absolute and relative concentrations of oxidants and reductants used to perform the refold. With this insight, the inventors could select redox conditions that would optimize the yields of the desired properly refolded protein specie in the refold mixture.

A. Protein Expression

23. Proteins play crucial roles in virtually all biological processes. Every cell contains numerous different proteins working in concert during all these processes, *e.g.*, all chemical reactions in cells, transport and storage, and control of growth and differentiation. One example of a protein is granulocyte-colony

stimulating factor or "G-CSF". In humans, G-CSF is one of the hematopoietic growth factors that plays an important role in stimulating proliferation, differentiation, and functional activation of white blood cells. Cancer patients undergoing chemotherapy frequently develop neutropenia, a condition marked by abnormally low levels of certain white blood cells. Administration of G-CSF to such patients alleviates neutropenia. Human G-CSF has been produced recombinantly, in genetically engineered *Escherichia coli* ("*E. coli*"). It is referred to as "rhu-G-CSF" or "filgrastim."

24. Proteins are biological polymers made up of one or more chains of monomer units called amino acids. Filgrastim, for example, is composed of a single chain of 175 amino acids. In nature, DNA encodes the sequence of each protein. A protein molecule is initially produced as a linear chain of amino acids, the amino acid sequence and the surrounding environment dictates folding into the three-dimensional shape the protein ultimately takes. Depicted below is what is called a ribbon model of the three-dimensional structure of filgrastim.



filgrastim (G-CSF)
(18.8 kDa)

Figure 1. Filgrastim[1]

25. The properties and function of a protein are largely determined by its three-dimensional structure, or how it is "folded." Proteins such as G-CSF function by binding to specific receptors in the body. The interaction of the protein with its receptor is sort of like a lock and key where only a key (or protein) with the right shape will fit in the lock. Thus, whether or not a protein will serve the biological function it is supposed to serve—in other words, whether or not the protein is "biologically active"—largely depends on how the protein is folded.

26. A cell, such as a human cell, produces protein through careful coordination of various steps. First, the cell needs to produce a protein with the correct linear amino acid sequence. This is accomplished when a gene (DNA) encoding the protein is "transcribed" into an intermediary nucleic acid called

[1] Figure taken from Japelj et al., Scientific Reports, 6:32201 (2016).

"mRNA", which in turn is "translated" into a linear chain of amino acids, the protein molecule. The sequence of the amino acids is directed by the encoding DNA sequence through the so-called "genetic code."

27. Second, the linear protein molecule needs to be folded properly and, if necessary, transported to a particular location within the cell to be biologically active. When proteins are produced natively—that is by the cell type that makes the protein in nature—the cell's own machinery is capable of folding the protein into its correct and active conformation. However, as discussed below, the same is not always true for recombinant proteins that are produced in non-native host cells.

28. Human cells typically produce proteins in far too small quantities for them to be useful in making therapeutic products. However, recombinant DNA technology allows for heterologous expression or production of human proteins in host cells, such as *E. coli* bacteria, in large quantities. Such bacterial host cells are referred to as "heterologous expression systems" or "non-mammalian expression systems." They have been genetically engineered to contain DNA encoding a human protein of interest. When the protein of interest, encoded for by the genetically engineered DNA, is "expressed," the bacteria serve as living "factories" to produce the desired protein. *E. coli* can be grown at industrial scale, making it possible to mass-produce therapeutic proteins of interest.

29. Production of a recombinant protein in *E. coli* requires coordination of multiple steps. First, one needs to introduce the gene encoding the protein into *E. coli*. One simple and common approach is to integrate the gene, once it is identified, into a circular DNA construct called a "plasmid" that is physically separate from chromosomal DNA, that can replicate independently and that contains special DNA sequences allowing the gene to be "expressed" in an *E. coli* cell. As noted above, the expectation is that *E. coli* cells containing this plasmid will turn into living factories and mass-produce or "overexpress" the protein.

30. Over expresssion of recombinant proteins in *E. coli* often results in misfolded and/or aggregated proteins. These proteins are stored within the bacterial cells in limited solubility, inactive forms referred to as "inclusion bodies" (step 1 below). These inclusion bodies are isolated by breaking open or lysing the bacterial cells circular DNA construct (step 2). Next, to isolate the protein, the inclusion bodies are solubilized and the protein along with other contaminants are unfolded linearized (denatured and reduced) (step 3). Then, under appropriate conditions, including those taught by the '287 Patent, which involve the use of redox chemistry (discussed below), the unfolded proteins can be refolded properly into their biologically active three-dimensional structure or "conformation" (step 4), isolated and purified (step 5), and put to use.

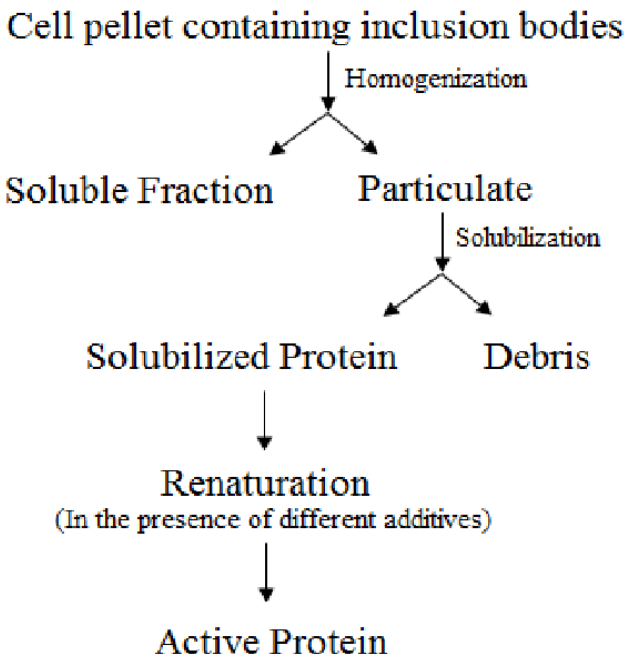


Figure 2. Protein Refolding[2]

B. Protein Structure and Folding

31. Amino acids are the basic structural units of proteins. An individual amino acid consists of an amino group, a carboxylic acid group, a hydrogen atom, and a distinctive R group bonded to a carbon atom. An R group is referred as a side chain.

[2] Figure taken from Gerami et al., African Journal of Biotechnology Vol. 10(53), 10811-10816 (2011).

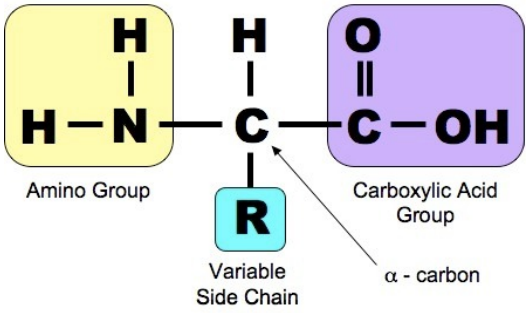


Figure 3. Structure of an amino acid[3]

32. In proteins, the carboxylic acid group of one amino acid is joined to the amino group of another amino acid by an amide bond, also referred to as a "peptide bond." Many amino acids are sequentially joined by peptide bonds to form a polypeptide chain. An amino acid unit within a polypeptide is called a "residue." A polypeptide chain has direction because its building blocks have different ends—namely, the amino group and the carboxyl group. By convention, the amino terminus is taken to be the beginning of a polypeptide chain. The sequence of amino acids in a polypeptide chain is written starting with the amino-terminal residue.

33. A polypeptide chain consists of a regularly repeating part, called the main chain, and a variable part, comprising the distinctive side chains. The main chain is sometimes termed the backbone.

[3] Figure taken from http://www.vce.bioninja.com.au/aos-1-molecules-of-life/biomolecules/proteins.html.

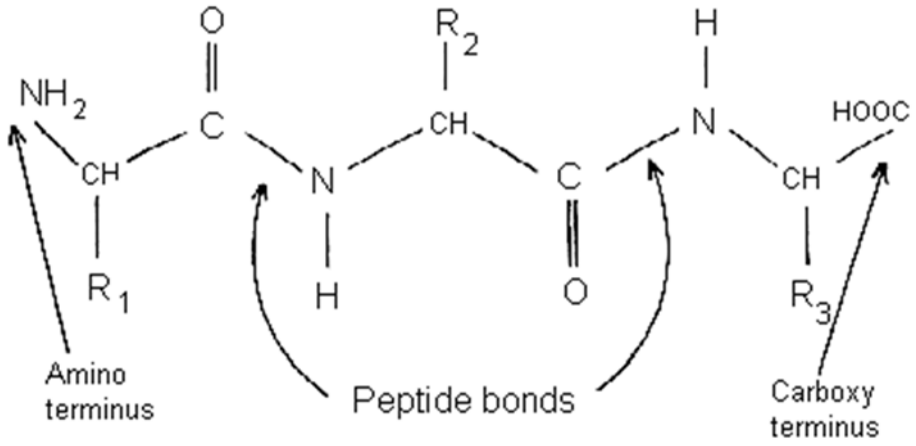


Figure 4. Protein Molecule, or Polypeptide Chain[4]

34. Many proteins contain residues of the amino acid cysteine. The side chain of cysteine contains a sulfhydryl group (-SH), also called a "thiol." Thiols can react with other thiols to form bonds and play an important role in protein folding. As a linear chain of amino acids fold into the three-dimensional shape of the protein, cysteine residues can become oxidized as the sulfhydryl groups can oxidize to form a disulfide (-S-S-) bond. Such disulfide bonds can stabilize the protein molecule by forming covalent bonds between amino acid residues from different regions of the protein. The cross-linking of portions of the protein chain by disulfide bonds is one way in which three-dimensional structure is conferred to the protein. Each disulfide bond requires two cysteine residues. If a protein has more than two cysteine residues then there are multiple possible pairings of

[4] Figure taken from http://www.cryst.bbk.ac.uk/PPS95/course/3_geometry/peptide2.html.

cysteines only one of which will allow the protein to fold into its proper, biologically active, conformation. *See* ¶ 37, *infra*.

35. In addition to disulfide bonds, there are other types of bonds and forces that keep a protein in its correct conformation. For example, hydrogen bonds form between a hydrogen atom and an electronegative atom such as nitrogen or oxygen. Some amino acids have positively or negatively charged side chains. Positively charged side chains are attracted to negatively charged side chains and can form electrostatic bonds. In addition, some amino acids have side chains that are hydrophobic (repel water) and some are hydrophilic (water liking). Proteins in aqueous environments will often fold such that the hydrophobic side chains are toward the inside of the protein rather than on the surface where they would be exposed to water.

36. In discussing the architecture of proteins, it is common to refer to four levels of structure. Primary structure is simply the linear sequence of amino acids made upon translation of mRNA. Secondary structure refers to the pattern of backbone torsion angles, or rotations about the bonds along the protein backbone, that result in the formation of regular structural units. These structural units give rise to periodic structures such as the alpha helix and the beta strand which result in hydrogen bonding between amino acid residues of the protein's peptide backbone. The tertiary structure refers to the steric relationship of amino acid residues that are

far apart in the linear sequence. In order to be biologically active a protein must adopt the correct secondary and tertiary structure. Proteins that contain more than one polypeptide chain display an additional level of structural organization, namely quaternary structure, which refers to the way in which the chains are packed together. Each polypeptide chain in such a protein is called a subunit. In some proteins, subunits are held together by covalent disulfide (-S-S-) bonds formed between cysteine residues of different chains.

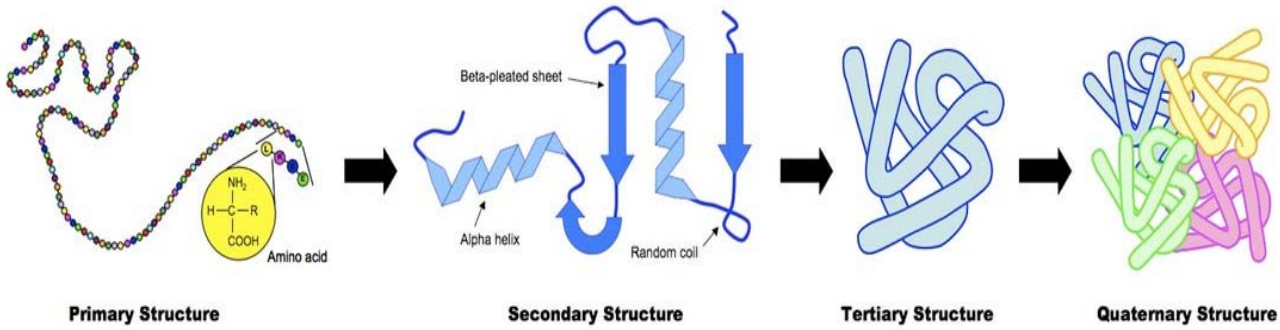


Figure 5. Protein Structure[5]

37. As discussed above, cysteine residues within a protein can form disulfide bonds and these bonds are essential for proper protein folding. For proteins that contain more than two cysteine residues, the proper formation of disulfide bonds becomes more challenging because it is not readily apparent that the correct cysteine residues will pair to form disulfide bonds. With each additional cysteine residue in a protein molecule, the possibility of mismatched

[5] Figure taken from http://www.old-ib.bioninja.com.au/higher-level/topic-7-nucleic-acids-and/75-proteins.html.

disulfide bonds increases semifactorially according to the equation (2*n*-1)!!, where 2*n* is the number of cysteine residues. Rubinstein and Fiser, Bioinformatics, 24:498-504 (2008) (attached as Exhibit C); Zhao et al., Bioinformatics, 21:1415-20 (2004) (attached as Exhibit D). For example, a protein with two cysteines can only pair in one way, but a protein with six cysteines has 15 possible disulfide bonds and a protein with eight cysteines has 105 possible pairings. This situation becomes more complex when proteins isolated from inclusion bodies are refolding because cysteine residues from two protein molecules can form disulfide bonds leading to protein aggregations.

38. Filgrastim is one such protein with disulfide bonds that affect its three-dimensional structure and its biological function. Filgrastim contains five cysteine residues at positions 18, 37, 43, 65 and 75. The properly folded filgrastim proteins has two disulfide bonds—residues 37 and 43 form one disulfide bond and residues 65 and 75 form the second one as depicted in Figure 6 below. Studies have demonstrated that these cysteine residues are essential for functional filgrastim protein. Molineux, Current Pharm. Design 10:1235-1244 (2004) (attached as Exhibit E).

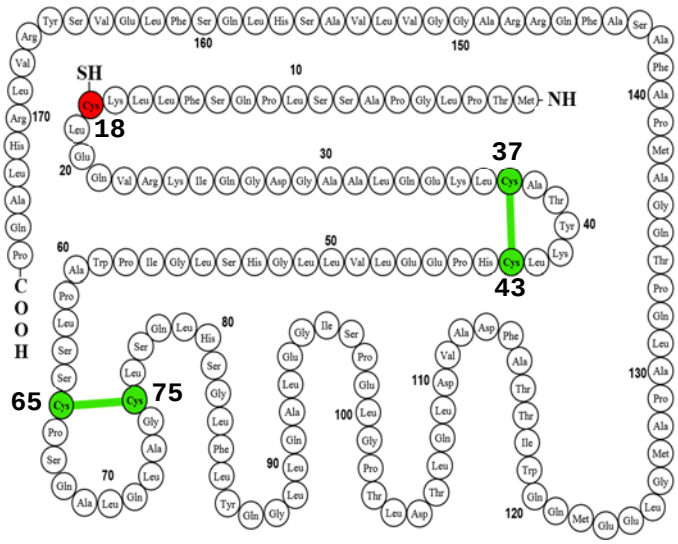


Figure 6. Two-Dimensional Schematic of Correctly Folded Filgrastim

39. A hypothetical example of misfolded protein when improper disulfide bonds form in filgrastim is depicted below. In this hypothetical protein, two disulfide bonds are formed between residues 43 and 65 and 37 and 75 rather than the properly formed bonds between residues 37 and 43 and residues between 65 and 75. As demonstrated, the protein is structurally different than the protein with proper cysteine bonds. Compare Figures 6 and 7.

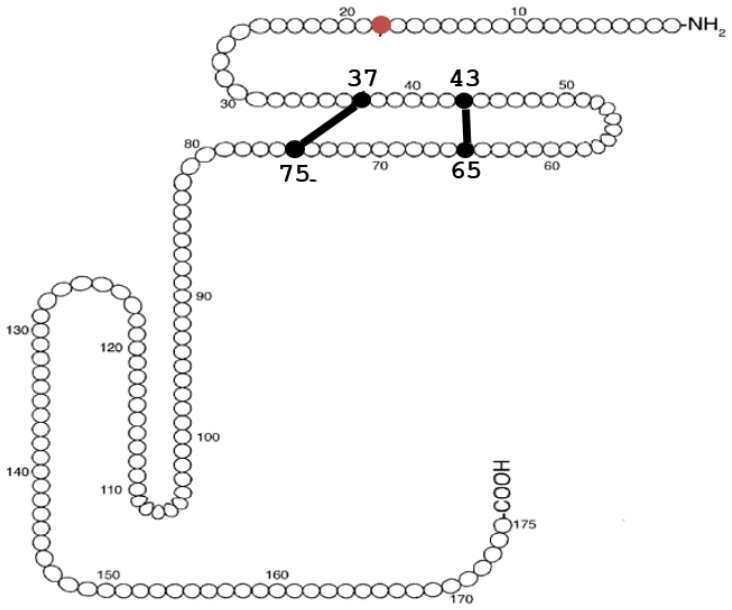


Figure 7. Two-Dimensional Schematic of Incorrectly Folded Filgrastim

C. Redox Chemistry

40. In oxidation/reduction reactions, there is a transfer of electrons from one species to another. Oxidation is defined as an increase in oxidation number (loss of electrons) and reduction as a decrease in oxidation number (gain of electrons). If one substance gains electrons and is thereby reduced, another substance must lose electrons and thereby be oxidized. Oxidation and reduction occur simultaneously; there cannot be one without the other. In discussing oxidation-reduction reactions, the substance causing oxidation is the oxidant. Oxidants possess an affinity for electrons. Similarly, a substance that causes reduction is called a reductant. Its tendency is to give up electrons.

41. As discussed above, disulfide (-S-S-) linkages between two cysteine residues are an integral component of the three-dimensional structure of many

proteins. The interconversion between thiol (-SH) and disulfide (-S-S-) groups is a redox reaction: the thiol is the reduced state, and the disulfide is the oxidized state.

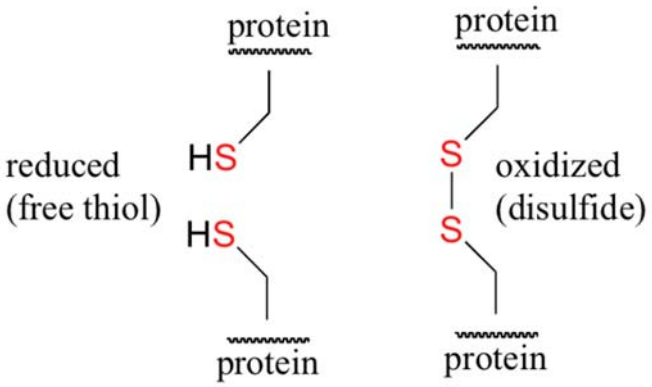


Figure 8. Thiol and disulfide groups[6]

42. Two commonly used reductants and oxidants that facilitate these thiol-disulfide exchange reactions are cysteine and cystine. In solution, the reductant cysteine is in equilibrium with the oxidant cystine as illustrated below. Together, they form a "redox" pair, with cysteine being a reductant (capable of giving up hydrogen ions) and cystine being an oxidant (capable of accepting hydrogen ions). Further, unlike some other oxidants and reductants, cysteine and cystine can be re-used repeatedly in redox reactions because, as explained in more detail below, in redox reactions, cysteine and cystine cycle back and forth.

[6] Figure taken from http://www.wou.edu/chemistry/courses/online-chemistry-textbooks/ch105-consumer-chemistry/chapter-10-compounds-sulfur-phosphorous-nitrogen/.

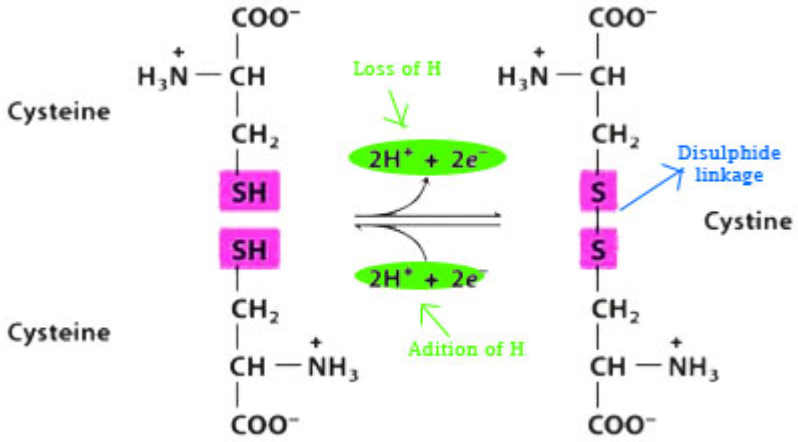


Figure 9. Conversion of cysteine to cystine[7]

43. Thiol-disulfide exchange reactions are central to oxidative protein folding. During refolding, oxidants can react with cysteine residues of a protein (containing thiols), and facilitate formation of disulfide bonds in a protein, affecting the tertiary structure of the protein. Conversely, reductants can disrupt these disulfide bonds in a protein. Disulfide bonds can also "reshuffle", meaning that disulfide bonds can form, break, and then reform between different cysteine residues. This is sometimes called disulfide bond "interchange" or "reshuffling." To achieve acceptable yields of a properly refolded protein, while minimizing formation of improperly refolded species, one would want disulfide bonds to reshuffle until the correct cysteine residue pairs are formed.

[7] Figure taken from https://bioch3mworld.wordpress.com/2013/03/30/review-of-amino-acid-protein-lecture-1/.

44. In this process, it is not clear how the correct disulfide bonds form when a protein contains multiple cysteine residues. It is thought that two cysteine molecules can react with a disulfide bond between thiol residues in protein to break the disulfide bond and create a cystine molecule. The cystine molecule can then react with thiol residues in protein to form a disulfide bond and two cysteine molecules. This process of breaking and forming disulfide bonds, often termed "shuffling," can continue until the correct (presumably, most stable) disulfide bonds are formed such that the protein is properly refolded. Eliana De Bernardez Clark, Current Opinion in Biotechnology, 12:202 (2001) (attached as Exhibit F).

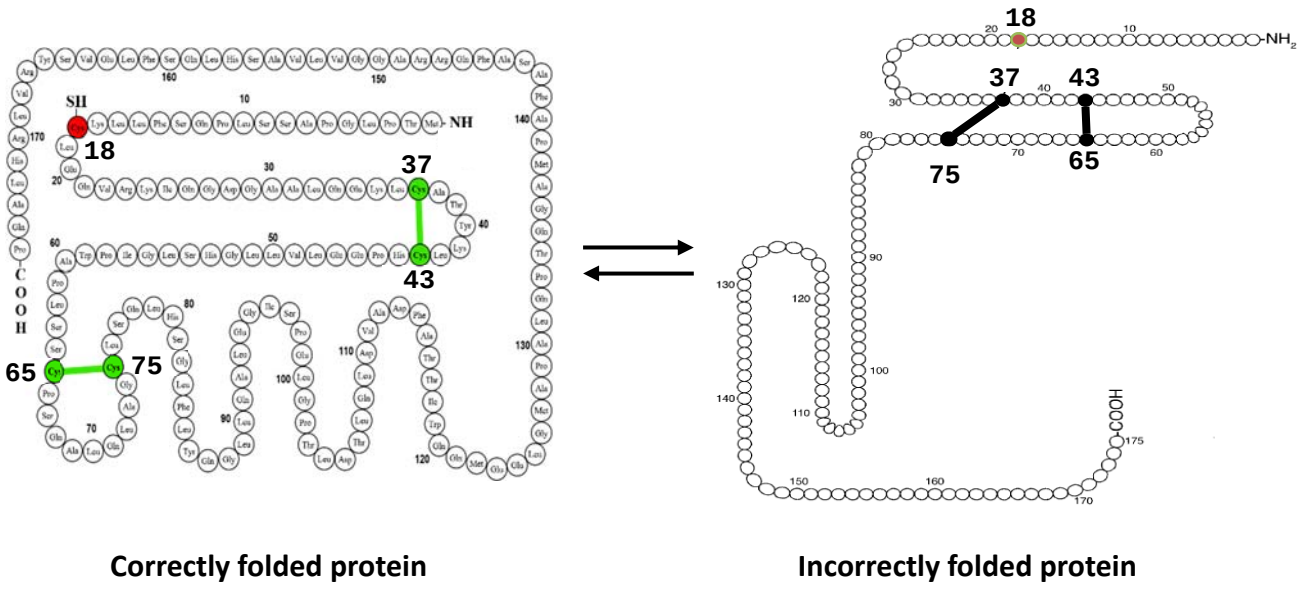


Figure 10. Disulfide bond shuffling

45. Depending on the redox conditions, the process of disulfide bond shuffling may lead to formation of incorrectly folded protein species rather than

the one correctly folded protein specie that is the desired end product of the refolding reaction. Consequently, methodology that improves the distribution of refolded protein species in the direction of the desired, biologically active protein is very important in the production of therapeutically valuable proteins.

VI. THE '287 PATENT

46. The '287 Patent is entitled "Refolding Proteins Using a Chemically Controlled Redox State." The invention disclosed in the patent is an improved, redox chemistry-based methodology to rationally select redox conditions that would optimize the yields of the desired properly refolded protein specie in the refold mixture.

47. The inventors of the '287 Patent addressed the problem of properly refolding proteins isolated from inclusion bodies made in non-mammalian expression systems, such as *E. coli*, which had long been a challenging problem in the art. Their systematic approach, generally applicable to cysteine-containing proteins, improves design of conditions for efficiently refolding proteins, that is, conditions under which protein molecules in the desired, biologically active conformation, predominate. They did so through the use of oxidation and reduction reactions that can reshuffle disulfide bonds until the correct bond is formed—in addition to the use of certain chemicals that can assist with denaturing and stabilizing proteins and preventing them from aggregating. Significantly, their

approach to protein refolding gave higher yields of the desired protein species, the properly refolded protein.

48. The theory underlying the methodology is explained in the '287 Patent starting at column 8 line 25. Specifically, the most important factor affecting the efficiency of refolding is the redox state of the refold system. More particularly, the inventors found a relationship between two parameters referred to as "thiol-pair ratio" and "thiol-pair buffer strength," both of which are based on the amount of oxidant and reductant used to refold the protein. Depending on the amount of oxidant and reductant, the values of these parameters vary and influence the distribution of properly/incorrectly refolded protein species in the refold mixture. In addition, it was found that thiol-pair buffer strength also relates to protein concentration in the refold mixture which also may be taken into account to achieve optimal refold conditions. '287 Patent column 4 line 52 to column 5 line 10.

49. The '287 Patent teaches that the thiol-pair ratio, thiol-pair buffer strength, and protein concentration can be varied, along with the inclusion of one or more chemicals that serve as a denaturant, aggregation suppressor, or protein stabilizer, to optimize the process of refolding cysteine-pair-containing proteins, either by maximizing the proportion of the desired, properly refolded and biologically active protein species, while minimizing the undesired, misfolded protein species, or by purposefully shifting the resultant undesired product-related

species to a form that is most readily removed in the subsequent purification steps. '287 Patent column 9 lines 32 to 60.

50. The thiol-pair ratio and thiol-pair buffer strength relationship disclosed in the patent takes into account the complex chemistry associated with disulfide bond reshuffling. Thus, for example, the thiol-pair ratio is not a simple ratio of amount of reductant to amount of oxidant—it is instead the square of the amount of reductant to the amount of oxidant (Equation 1). Equations 3 and 4 provide the relationships between the concentrations of the oxidant and reductant and the thiol-pair ratio and thiol-pair buffer strength. Equation 3 expresses the concentration of the reductant as a function of the thiol-pair ratio and thiol-pair buffer strength. Equation 4 expresses the concentration of the oxidant as a function of the thiol-pair ratio and the concentration of the reductant.

Calculation of the Reduced Redox Species with Regard to a Defined Redox Buffer Strength (*BS*) and buffer Redox Potential — Equation 3

$$\text{Concentration of Reduced Redox Component} = \frac{\left(\sqrt{bufferTPR^2 + 8 * bufferTPR * BS}\right) - bufferTPR}{4}.$$

Calculation of the Oxidized Redox Species with Regard to a Defined Redox Buffer Strength (*BS*) and Buffer Redox Potential — Equation 4

$$\text{Concentration of Oxidized Redox Component} = \frac{(\text{Concentration of Reduced Redox Component})^2}{TPR}.$$

The patent defines "redox component" as "any thiol-reactive chemical or solution comprising such a chemical that facilitates a reversible thiol exchange with another thiol or the cysteine residues of a protein" ('287 Patent column 7 lines 20 to 23), in other words, chemicals that facilitate disulfide bond reshuffling. Concentrations of oxidants and reductants are thus related through the complex "thiol-pair ratio" and "thiol-pair buffer strength" parameters disclosed in the patent, when they provide the redox conditions for disulfide bond reshuffling.

51. The patent teaches contacting proteins with a refold buffer (called a preparation in claims 1 and 10) that supports the renaturation of at least one of the proteins to a biologically active form. The refold buffer includes certain ingredients, including an amount of oxidant and an amount of reductant. These amounts provide the redox conditions for disulfide bond reshuffling during refolding of the protein, and are thus related through the "thiol-pair ratio" and "thiol-pair buffer strength" parameters. Claim 1 recites that the thiol-pair ratio should be in the range of 0.001-100, and the thiol-pair buffer strength should maintain the solubility of the preparation.

VII. TERMS OF THE CLAIMS OF THE '287 PATENT

1. "thiol-pair ratio"

52. I understand that "thiol-pair ratio" ("TPR") is defined in the specification by the equation $\frac{[reductant]^2}{[oxidant]}$, *i.e.*, the square of the concentration of the reductant divided by the concentration of the oxidant, at column 6 lines 50 to 55.

53. I understand that Adello argues that the term is indefinite because no unit is provided. As I understand it, Adello believes that a person of skill in the art would not be able to understand the claim with reasonable clarity. In my opinion, the term is not indefinite because POSITA would understand that the unit for the concentrations of the oxidant and reductant is millimolar (mM) and that numerical values to be used in the TPR equation are such mM amounts.

54. The claims and the specification of the '287 Patent consistently and exclusively use the unit millimolar for the concentrations of oxidants and reductants. Millimolar is a common scientific notation of concentration expressing the number of millimoles of a substance in a defined volume of solution. A "mole" of a substance is its molecular weight in grams. By way of example, the molecular weight of glucose is 180, so 1 mole of glucose is 180 g of glucose. One "millimole" is a thousandth of a mole. A "1 M" (one molar) solution of glucose has a concentration of 180 grams per liter, while a "1 mM" (one millimolar) solution of glucose has a concentration of 0.18 grams per liter.

55. Claims 4, 12, 19, and 29 recite that the thiol-pair buffer strength is "2 mM or greater." The specification also uses only the unit millimolar for thiol-pair buffer strength. '287 Patent figures 1a-1f; column 2 line 67 to column 3 line 1; column 3 lines 9 to 18; column 9 lines 18 to 22; column 10 lines 58 to 67; column 11 lines 24 to 34; column 11 lines 47 to 56; column 12 lines 11 to 20; column 15 lines 5 to 6. The thiol-pair buffer strength is defined by the equation $2[oxidant] + [reductant]$. '287 Patent column 6 lines 56 to 67. POSITA would understand that, because the sum of twice the concentration of the oxidant and the concentration of the reductant is reported in the unit millimolar, the concentrations of the oxidant and the reductant also have the unit millimolar. Because there is no indication that different units should be used for the concentrations of the reductant and the oxidant in the thiol-pair ratio equation, POSITA would use the same unit, *i.e.*, millimolar.

56. The specification reports concentrations of oxidants and reductants in millimolar only. For example, the specification reports amounts of cysteine (a reductant) and cystamine (an oxidant) in millimolar:

> In one embodiment the final composition is 1-4 M urea, 5-40% glycerol or sucrose, 25-500 mM arginine, **0.1-10 mM cysteine** and **0.1-10 mM cystamine**.

'287 Patent column 13 lines 18 to 21.

> The Protein A column elution pool was mixed at a ratio of 8 parts Protein A elution material to 2 parts of a refold

> buffer containing urea (10 M), arginine-HCI (2.5 M), Tris at pH 8.5 (1050 mM), and **cysteine (10 mM, 5 mM, or 4 mM)** and **cystamine (4 mM)**.

'287 Patent column 15 lines 44 to 48.

> The final component concentrations are 4 M urea, 150 mM arginine HCI, 20.9% (w/v) glycerol, **2.03 mM cysteine**, and **2.75 mM cystamine**.

'287 Patent column 16 lines 28 to 30.

57. Having read the claims and the specification, POSITA would understand to use the numerical millimolar values for the concentrations of the oxidant and reductant in the equation. POSITA would not use a different unit value than the unit value consistently and exclusively used for the concentrations of oxidants and reductants in the claims and specification. Thus, the thiol-pair ratio is determined using millimolar (mM) for the concentrations, and the specification so informs a person of ordinary skill with far-more-than-reasonable certainty.

2. "wherein the thiol-pair buffer strength maintains the solubility of the preparation"

58. I understand that Adello also argues that this term is indefinite. I disagree that there is anything indefinite about maintaining the solubility of a solution like the preparation disclosed in the claims.

59. In simplest terms, solubility is the ability of a component to be dissolved. A common example is dissolving sugar in water. There is a maximum

amount of sugar you can dissolve in water while preparing simple syrup. This is known as the solubility of sugar. If you exceed that solubility, the excess sugar will not dissolve in the water, and you will have sugar precipitating "out of solution." If the amount of sugar does not exceed its solubility, all of the sugar will be "in solution" and the solubility of sugar in the solution is maintained.

60. As recited in claims 1 and 10, the preparation comprises at least one of a denaturant, an aggregation suppressor, and a protein stabilizer; an amount of oxidant; and an amount of reductant. The preparation does not contain protein, and it is contacted with proteins to form a refold mixture.

61. The patent defines the thiol-pair buffer strength by a relationship between an oxidant and a reductant. POSITA would understand that all the ingredients of the preparation would have to be in solution in order to serve as oxidants, reductants, denaturants, aggregation suppressors, or protein stabilizers.

62. POSITA would understand that the amounts of oxidant and reductant must be such that the components in the preparation stay in solution. For example, a commonly used oxidant is cystine. Cystine has limited solubility in aqueous solutions when its concentration is above 0.112 mg/ml. L-Cystine Product Information Sheet, Sigma-Aldrich (attached as Exhibit G). POSITA would understand that its limited solubility needs to be accounted for in the preparation

because at thiol-pair buffer strengths that are too high, its solubility will not be maintained.

I declare under penalty of perjury under the laws of the United States that the foregoing is true and correct. Executed on April 15, 2019, in Oxford, Ohio.

Richard C. Page

Exhibit A

Richard C. Page, Ph.D.
CURRICULUM VITAE

CONTACT INFORMATION

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LAB:	Department of Chemistry and Biochemistry Miami University 651 E. High Street Oxford, OH 45056 pagerc@miamioh.edu (513)529-2281

EDUCATION AND TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
California Polytechnic State University, San Luis Obispo	B.S.	1998-2002	Biochemistry
Florida State University	Ph.D.	2002-2008	Biochemistry
Cleveland Clinic		2008-2013	Biochemistry/Biophysics

A. Employment Summary

2013-present Robert H. and Nancy J. Blayney Associate Professor, Assistant Department Chair
Department of Chemistry and Biochemistry, Miami University, Oxford, OH. *Developed an externally funded research program in biophysical chemistry with an emphasis on mentorship and teaching of graduate and undergraduate students.*

2008-2013 Postdoctoral Research Fellow
Advisor: Dr. Saurav Misra, Associate Staff, Department of Molecular Cardiology, Cleveland Clinic, Cleveland, OH. *Utilized NMR and X-ray crystallography to uncover the molecular basis for regulating CHIP mediated ubiquitination, a key protein quality control pathway.*

2002-2008 Graduate Research Assistant
Advisor: Dr. Timothy Cross, Professor and NMR Program Director, Department of Chemistry and Biochemistry, Florida State University and the National High Magnetic Field Laboratory, Tallahassee, FL. *Developed methodologies to express, purify and biophysically characterize membrane proteins and transmembrane peptides by solution and solid state NMR.*

1998-2002 Undergraduate Research Assistant
Advisor: Dr. Nanine Van Draanen, Professor and Chair, Department of Chemistry and Biochemistry, California Polytechnic State University, San Luis Obispo, CA. *Examined the regioselectivity of o-thioalkylation reactions, a key synthetic step for many pharmaceutical precursors produced via [2,3]-sigmatropic rearrangements.*

2001 National Science Foundation Undergraduate Research Fellow
Advisor: Dr. Atom Yee, Professor and Dean, Department of Chemistry, College of Arts and Sciences, Santa Clara University, Santa Clara, CA. *Analyzed the photoisomerization of diphenylbutadiene as a photochemical analog of retinol isomerization in vision.*

B. Personal Statement

My core research area seeks to determine the biophysical and biochemical underpinnings of triage mechanisms used by cells to assess the fidelity of protein folding. I apply a combination of structural and biophysical tools including NMR, small angle X-ray scattering, and EPR to interpret biochemical observations with an eye toward the role of dynamic motions of proteins in eliciting specific biological outcomes. I have authored 42 publications on biochemical and biophysical analyses of protein structure and function. Throughout my research efforts I have also sought to emphasize training of graduate and undergraduate students along with service contributions that improve training environments and advocate for basic biomedical research. My laboratory aims to make significant impacts in the areas of protein quality control, protein-polymer bioconjugates, discovery of novel β-lactamase inhibitors, development of hybrid methods for structural characterization of dynamic systems, and invention of new biochemical and biophysical assays for studying ubiquitination and protein refolding.

My training and mentorship of graduate and undergraduate researchers has resulted in 26 publications at Miami University. To date, I have trained thirty-three undergraduate students and eight graduate students in

biochemistry, biophysics and structural biology by X-ray crystallography and NMR. Undergraduates in my laboratory have received recognition for our research efforts including individual research fellowships from Miami University and external scholarships such as the Barry M. Goldwater Scholarship, the Astronaut Foundation Scholarship, and the Beckman Scholars Award. I have published papers with twenty-five Miami University undergraduate students as co-authors and five of these students were first authors. My former undergraduate students are now enrolled in STEM graduate programs at Case Western Reserve University, Vanderbilt University, the University of California, Berkeley, and Yale University. One of my undergraduate students received an NIH Postbaccalaureate Intramural Research Training Award to conduct research at the National Cancer Institute, and one received a Summer Undergraduate Laboratory Internship from the US Department of Energy to conduct research at Lawrence Berkeley National Laboratory.

C. Positions and Honors

Positions and Employment

2018-present	Assistant Department Chair, Department of Chemistry and Biochemistry, Miami University
2018-present	Associate Professor, Department of Chemistry and Biochemistry, Miami University
2013-2018	Assistant Professor, Department of Chemistry and Biochemistry, Miami University
2008-2013	Postdoctoral Research Fellow, Department of Molecular Cardiology, Cleveland Clinic
2002-2008	Graduate Research Assistant, National High Magnetic Field Laboratory
2001	Undergraduate Fellow, National Science Foundation Research Experience for Undergraduates program, Santa Clara University

Professional Service

2017-present	Panelist, National Institutes of Health Macromolecular Structure and Function B (MSFB) Study Section, February 2017 and October 2018
2015-2018	Elected Member, Public Affairs Advisory Committee, American Society for Biochemistry and Molecular Biology
2014-2017	Elected Director for Research, Board of Directors, Cleveland Clinic Alumni Association
2014-present	Panelist, National Science Foundation Graduate Research Fellowship Program

University, College, and Departmental Service

2017-present	High-Performance Computing Committee, Miami University
2017-present	Committee on Faculty Research, Miami University
2016-present	Chair, Graduate Recruitment Committee, Department of Chemistry and Biochemistry, Miami University
2016-present	Pre-Health Advisory Committee, Miami University
2016	Committee on FLSA Overtime Rule for Postdoctoral Fellows, Miami University
2015	Interim Chair, Graduate Advising Committee, Department of Chemistry and Biochemistry, Miami University
2014-present	Mentor and Advisory Board Member, Louis Stokes Alliance for Minority Participation Program, Miami University
2014-present	Cell, Molecular and Structural Biology (CMSB) Graduate Admissions Committee
2014-present	Member, Senior Staff Appointments Committee, served as a member of four tenure-track search committees
2014-present	Instrumentation Laboratory Advisory Committee, College of Arts and Science, Miami University

Honors

2016-2019	Robert H. and Nancy J. Blayney Professorship
2016	Miami University Distinguished Junior Faculty Scholar Award
2012	Cleveland Clinic Caregiver Excellence Award, Cleveland Clinic, Cleveland, OH
2012-2013	National Institutes of Health Ruth L. Kirschstein National Research Service Award Postdoctoral Training Fellowship (T32 HL007914)
2010	F. Merlin Bumpus Junior Investigator Award, Cleveland Clinic, Cleveland, OH
2009-2011	American Heart Association Postdoctoral Fellowship (AHA 09POST2010041)
2007	Joseph M. Schor Fellowship in Biochemistry, Florida State University, Tallahassee, FL
2006-08	American Heart Association Predoctoral Fellowship (0615223B)

Other Experience and Professional Memberships

2014-present	Member, American Chemical Society
2014-present	Member, American Association for the Advancement of Science
2013-present	Member, American Society for Biochemistry and Molecular Biology
2013-2016	Member, American Association for Cancer Research
2013-2016	Member, American Heart Association
2009	Cold Spring Harbor Laboratory Course: X-ray Methods in Structural Biology
2003-present	Member, Biophysical Society
2003-2008	Member, International Society for Magnetic Resonance

D. Publications

Publication and Citation Metrics (as of 10/16/2018)			orcid.org/0000-0002-3006-3171
Peer-reviewed publications	42	First and ¶co-first author publications	11
Length of publication record	13 years	*Corresponding author publications	20
Publications at Miami University	26	Miami University graduate and undergraduate co-authors	51

Summary of Publications by Topic

1. **Structural and biophysical insights into protein folding and degradation.** The interactions of co-chaperones with Hsp70 and homologs regulate protein homeostasis and defects in these pathways are implicated in wide range of diseases. Thus, explorations of these interactions may provide therapeutically accessible points of modulation. I have spearheaded multiple studies focusing on the interaction between Hsp70 or Hsp70-homologs and the ubiquitin-proteasome system that impact several key checkpoints. Taken together, these studies have provided the field with a more complete picture of the biophysical and biochemical basis for how the Hsp70/CHIP chaperoned ubiquitination complex is regulated and how co-chaperones influence this complex. I served as the first author in two of these studies and a corresponding author in four of the studies. These studies have been cited 113 times.
2. **Biophysical studies of protein-polymer bioconjugates.** Situated at the interface between biochemistry, biophysics, and organic chemistry, our studies of protein-polymer bioconjugates have identified bioconjugates that elicit increased thermal stability, increased resistance to proteolytic digestion, and increased resistance to unfolding by denaturants. Our studies with lysozyme delineated key differences between conjugation site and varied polymers. Our cellulase-polymer conjugates have enabled the production of highly stable enzymes for biofuels production. These studies have been cited 115 times.
3. **Structural studies of membrane proteins.** My first publications laid the groundwork for structural characterizations of membrane proteins by nuclear magnetic resonance spectroscopy (NMR). These studies presented the first detailed explorations of sample preparation techniques and strategies for NMR characterization of membrane proteins. These publications also introduced novel data interpretation approaches that increase the quantity and value of information obtained from NMR spectroscopy of detergent-solubilized or lipid bilayer-embedded membrane proteins. The advances presented in these publications enabled NMR studies of membrane proteins to rapidly extract structural and dynamic data in native or near-native environments. I served as the first author in all of these studies. These studies have been cited 243 times.
4. **Structural biology of metal-centered proteins.** I have collaborated with the Stuehr, Crowder and Tierney laboratories to carry out structural, biochemical and biophysical characterizations of metal-centered enzymes. Our work on the metallo-β-lactamase VIM-2 has identified the role of key active site residues and explains the behavior of a key family of metallo-β-lactamases including VIM-2, IMP-1, and BcII that exhibit markedly different mechanisms from the structurally similar metallo-β-lactamases NDM-1 and CcrA. Our study of MMP7 identifies key differences with the related matrix metallo proteinase MMP16 and uncovers key biophysical considerations for the design of MMP inhibitors. Our study of inducible Nitric Oxide Synthase (iNOS) determined a novel intermediate that was stabilized in an active site mutant. This study has enabled subsequent kinetic exploration of iNOS activity, including a novel study of electronic effects of heme substitution upon iNOS reaction kinetics. These studies have been cited 72 times.
5. **Structural basis for the regulation of angiogenesis, inflammation, and cytoskeletal interactions.** Angiogenesis, inflammation, and cytoskeletal rearrangements rely upon spatial and temporal regulation of

protein-protein interactions initiated by interactions with external factors. Our results identified a new model for transmembrane communication in integrin/filamin focal adhesion complexes, the role of phosphorylation in mediating Act1 interactions with TRAF2 and TRAF5, and the first structural evidence for dynamic interactions between thrombospondin-1 and CD36. These studies have been cited 245 times.

Participation of Undergraduates in Publications

Since joining Miami University in August 2013, I have published 24 papers with Miami University undergraduate co-authors and five of these papers featured undergraduate students as first authors. These publications have applied NMR and crystallography in combination with biochemical and biophysical approaches to produce insights into CHIP/Hsp70 interactions, the structure and activity of protein-polymer conjugates, and the development of small molecule inhibitors of STAT3. The training I provide undergraduate researchers increases the cost-effectiveness of my lab and combines cutting-edge research with high quality research training of junior scientists in my laboratory. These studies have been cited 126 times.

List of Publications at Miami University (26 peer reviewed publications, 8/2013-Present, ‡Miami undergraduate students, †Miami graduate students, ¶co-first author, *corresponding author):

1. Amick, J., Schlanger, S., Wachnowsky, C., †Moseng, M.A., ‡Emerson, C.C., Dare, M., Luo, W., Ithychanda, S., Nix, J.C., Cowan, J.A., ***Page, R.C.**, and *Misra, S., *Crystal structure of the nucleotide binding domain of Mortalin, the mitochondrial Hsp70 chaperone*, Protein Science, 23(6), 833-842 (2014). Impact factor: 2.735.
2. †Aitha, M., †Marts, A.R., †Bergstrom, A., ‡Moller, A.J., ‡Moritz, L., ‡Turner, L., Nix, J.C., Bonomo, R.A., **Page, R.C.**, Tierney, D.L., and *Crowder, M.W., *Biochemical, mechanistic, and spectroscopic characterization of metallo-β-lactamase VIM-2*, Biochemistry, 53(46), 7321-7331 (2014). Impact factor: 3.377.
3. †Falatach, R., ‡McGlone, C., Al-Abdul-Wahid, M.S., Averick, S., ***Page, R.C.**, *Berberich, J.A., and *Konkolewicz, D., *The best of both worlds: active enzymes by grafting-to followed by grafting-from a protein*, Chemical Communications, 51(25), 5343-5346 (2015). Impact factor: 6.378.
4. †¶Zhang, H., ¶Amick, J., Chakravarti, R., Schlanger, S., ‡McGlone, C., Dare, M., Nix, J.C., Scaglione, K.M., Stuehr, D.J., *Misra, S., and ***Page, R.C.**, *A bipartite interaction between Hsp70 and CHIP regulates ubiquitination of chaperoned client proteins*, Structure, 23(3), 472-482 (2015). Impact factor: 5.618.
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List of Publications Prior to Miami University (16 peer reviewed publications, ¶co-first author, *corresponding author):

1. **Page, R.C.**, Moore, J.D., Nguyen, H.B., Sharma, M., Chase, R., Gao, F.P., Mobley, C.K., Sanders, C.R., Ma, L., Sönnichsen, F.D., Lee, S., Howell, S.C., Opella, S.J., and *Cross, T.A., *Comprehensive evaluation of solution nuclear magnetic resonance spectroscopy sample preparation for helical integral membrane proteins*. Journal of Structural and Functional Genomics, 7(1), 51-64 (2006). Impact factor: 1.482.
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5. Xu, Z., **Page, R.C.**, Gomes, M.M., Kohli, E., Nix, J.C., Herr, A.B., Patterson, C., and *Misra, S., *Structural basis of nucleotide exchange and client binding by the novel Hsp70-cochaperone Bag2*. Nature Structural and Molecular Biology, 15(12), 1309-1317 (2008). Impact factor: 11.902. *Article recommended by Ulrich Hartl and Andreas Bracher on F1000Prime.*
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Complete List of Published Work in NCBI MyBibliography:
http://www.ncbi.nlm.nih.gov/sites/myncbi/richard.page.1/bibliography/43575619/public/?sort=date&direction=descending

E. Research Support

Ongoing External Research Support

NIH MIRA R35GM128595 Page (PI) 09/01/2018-06/30/2023 Awarded $1,796,635
Triage mechanisms for directing protein refolding and degradation
The goal of this grant is to discover the mechanistic details that underlie triage of misfolded proteins to either refolding or degradative pathways will enhance our fundamental knowledge of protein quality control pathways and identify avenues that may be exploited for future therapeutic targeting.
Role: PI

G03061, Allecra Therapeutics Page (PI) 08/01/2018-12/31/2019 Awarded $78,030
Evaluation of a novel β-lactamase inhibitor, AAI101
This contract work seeks to determine the effect of tazobactam and AAI101 on the dynamics and structure of KPC-2 and CTX-M-15 serine β-lactamases.
Role: PI

G03061, Merck & Co., LTD Page (PI) 10/01/2018-09/30/2021 Awarded $53,938
KPC-2 interactions with relebactam
This contract work seeks to determine the extent of interactions with relebactam with the serine β-lactamase KPC-2 and the resulting alterations in dynamics and structure of KPC-2.
Role: PI

NSF MRI CHE-1725502 Lorigan (PI) 08/15/2017-07/31/2020 Awarded $1,194,390
MRI: Acquisition of a Q-band pulsed EPR spectrometer
This Major Research Instrumentation grant supports acquisition of an X-/Q-band pulsed electron paramagnetic resonance (EPR) spectrometer.
Role: Co-PI

Completed External Research Support

NSF CAREER MCB1552113 Page (PI) 03/01/2016-09/30/2018 Awarded $923,934
Dynamic Regulation of Protein Quality Control
The goal of this grant is to determine the dynamics that regulate ubiquitination and protein refolding by the CHIP/Hsp70 complex. The integrated educational objective seeks to increase the participation of underrepresented minorities in STEM.
Role: PI

R01 GM111926 Fast (PI) 04/01/2015-09/30/2018 Awarded $262,309
Developing Metallo-Beta-Lactamase Inhibitors

The goal of this grant to harness powerful biochemical, microbiological, structural, and biophysical resources in an effort to identify chelator-fragment inhibitors of metallo-beta-lactamases that can be used as a scaffold for developing novel antibiotics.
Role: Subcontractor

AHA SDG 16SDG26960000 Page (PI) 01/01/2016-02/29/2016 Awarded $308,000
Protein quality control mechanisms in the heart
This grant sought to determine the role CHIP and Hsp70 protein quality control system in the response to ischemia. This grant was forfeited as a condition of receiving the NSF CAREER.
Role: PI

A15-0745, Ono Pharmaceutical, LTD Schisler (PI) 08/01/2015-07/31/2016 Awarded $132,940
Co-factor validation and activity assay development for CHIP
This contract work sought to validate co-factors targeted by the CHIP/Hsp70-axis and develop robust assays to be used to screen for inhibitors of CHIP.
Role: Subcontractor

1014031, Burroughs-Wellcome Fund Page (PI) 07/15/2014-12/31/2015 Awarded $8,161
Functional implications for restrained ubiquitin ligase interactions with Hsp70
The goal of this collaborative research travel grant was to visit the ALS-SIBYLS beamline 12.3.1 and collect small angle X-ray scattering (SAXS) data.
Role: PI

Ongoing Internal Research Support

Blayney Professorship Page (PI) 07/01/2016-06/30/2019 Awarded $42,000
The goal of the Robert H. and Nancy J. Blayney Professorship is to support excellence in scholarship at Miami University.
Role: PI

Completed Internal Research Support

Committee on Faculty Research Page (PI) 06/01/2014-12/31/2015 Awarded $31,106
Support for Page Laboratory research efforts
The goal of these startup funds was to provide funds for one graduate assistant to jumpstart research into the biophysical mechanisms underlying the CHIP/Hsp70 protein quality control system.
Role: PI

Startup Funds Page (PI) 07/01/2013-06/30/2018 Awarded $400,000
Support for Page Laboratory setup and initial research efforts
The goal of these startup funds was to provide the financial resources to develop a fully functional, modern structural biology laboratory with resources for cloning, protein expression and purification, biochemical and biophysical assay capabilities and equipment for NMR and X-ray crystallographic studies.
Role: PI

Funding for Teaching and Related Activities

1. Miami University Student Technology Fee Proposal, PI: R Page. *Acquisition of an isothermal titration calorimetry instrument for advanced undergraduate lecture and laboratory courses, undergraduate independent study, and graduate student research*. Co-PI: Michael Kennedy. 7/1/2015–6/1/2016. Awarded $60,515.
    a. This internal competitive award provided funds to purchase a TA Instruments Nano ITC instrument utilized for a 400-level undergraduate laboratory course and for graduate and undergraduate research.
2. NSF: Major Research Instrumentation (MRI) Program, Co-PI: R Page. *Acquisition of a Q-band pulsed EPR spectrometer*. Award No. 1725502. PI: Gary Lorigan. 7/01/2017–7/01/2020. Awarded: $1,194,390
    a. This NSF MRI award also included funding for a Bruker EMXnano benchtop electron paramagnetic resonance (EPR) spectrometer. The EMXnano EPR will be utilized for 400-level undergraduate laboratory courses and for undergraduate research.

Exhibit B

Exhibit B: List of Materials Considered

Patents and File Histories

U.S. Patent No. 9,856,287
U.S. Application No. 15/422,327: Prosecution History for U.S. Patent No. 9,856,287

Publications

Eliana De Bernardez Clark, Current Opinion in Biotechnology, 12:202 (2001)
L-Cystine Product Information Sheet, Sigma-Aldrich
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Rubinstein and Fiser, Bioinformatics, 24:498-504 (2008)
Zhao et al., Bioinformatics, 21:1415-20 (2004)

Other Documents

D.I. 101, Joint Claim Construction and Prehearing Statement (Mar. 22, 2019)

Exhibit C

BIOINFORMATICS ORIGINAL PAPER

Vol. 24 no. 4 2008, pages 498–504
doi:10.1093/bioinformatics/btm637

Sequence analysis

Predicting disulfide bond connectivity in proteins by correlated mutations analysis

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Received on August 14, 2007; revised on December 24, 2007; accepted on December 25, 2007

Advance Access publication January 18, 2008

Associate Editor: Limsoon Wong

ABSTRACT

Motivation: Prediction of disulfide bond connectivity facilitates structural and functional annotation of proteins. Previous studies suggest that cysteines of a disulfide bond mutate in a correlated manner.

Results: We developed a method that analyzes correlated mutation patterns in multiple sequence alignments in order to predict disulfide bond connectivity. Proteins with known experimental structures and varying numbers of disulfide bonds, and that spanned various evolutionary distances, were aligned. We observed frequent variation of disulfide bond connectivity within members of the same protein families, and it was also observed that in 99% of the cases, cysteine pairs forming non-conserved disulfide bonds mutated in concert. Our data support the notion that substitution of a cysteine in a disulfide bond prompts the substitution of its cysteine partner and that oxidized cysteines appear in pairs. The method we developed predicts disulfide bond connectivity patterns with accuracies of 73, 69 and 61% for proteins with two, three and four disulfide bonds, respectively.

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1 INTRODUCTION

The disulfide bond is the most frequent naturally occurring covalent cross-link in proteins. It is derived from the oxidation of the thiol groups of two cysteine residues. Proteins with disulfide bonds are usually secreted and rarely found in the cytoplasm, which has a reducing environment and lacks enzymes that promote disulfide bond formation (Kadokura *et al*., 2003). However certain archea are rich in cytoplasmic proteins with disulfide bonds (Mallick *et al*., 2002). A number of studies have linked disulfide bonds to protein stability and to folding rate (Wedemeyer *et al*., 2000). It has been suggested that disulfide bonds stabilize the protein's folded state by restricting the protein's conformation, thereby reducing the entropy of the unfolded state (Harrison and Sternberg, 1994; Poland and Scheraga, 1965). Meanwhile, disulfide bonds increase the enthalpy of the folded state by stabilizing local interactions (Wedemeyer *et al*., 2000). Furthermore, disulfide bonds increase the protein's half-life by enhancing protein protection against proteases by maintaining the integrity of protein structure against local unfolding events. Disulfide bonds have also been observed to contribute to protein function regulation (Hogg, 2003).

Disulfide bonds constrain the conformation of the protein structure and thus, knowledge of their location can facilitate protein structure prediction. In addition, disulfide bond connectivity patterns can be used to discriminate between protein folds and to accurately superimpose protein structures (Chuang *et al*., 2003; Gupta *et al*., 2004; Mas *et al*., 1998). The underlying assumption in these methods is that similar disulfide bond connectivity patterns place similar spatial constraints on proteins, resulting in similar protein structures. Finally, variation in disulfide bridge patterns may be used to infer variation of protein function (Cao *et al*., 2007).

There are two distinct steps in the process of predicting disulfide bond connectivity patterns. The first is the classification of bound (oxidized) and unbound (reduced) cysteines. The second is the correct pairing of all bound cysteines. Muskal and colleagues (1990) published the first method to identify bound and free cysteines by utilizing a neural network and reported a prediction accuracy of 82%. Fiser and colleagues (1992) observed that the sequence environments of bound and free cysteines have different compositions and they subsequently introduced a method to calculate disulfide-bond forming potential that is based on the amino acid composition of the sequential environment of cysteines. Later, Fiser and Simon analyzed the apparent difference between the conservation level of oxidized and reduced cysteines and developed a method to predict the oxidation states of cysteines from the conservation analysis of multiple sequence alignments. This simple approach reached a prediction accuracy of 82% (Fiser and Simon, 2000). In the same study it was noted that it is rare for a protein to have cysteines with mixed oxidation states. Mucchielli-Giorgi *et al*. (2002) developed a cysteine oxidation state predictor based on Fiser and Simon's finding (Fiser and Simon, 2000) and on the global amino acid composition of proteins and attained an 84% prediction accuracy. Chen *et al*. (2004) trained a support vector machine on the local environment of cysteines as well as on global information of the protein and reported a 90% prediction accuracy. Given these high prediction accuracies for cysteines oxidation state prediction our current study focuses on the second step in prediction of disulfide bond

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connectivity; the challenging problem of identifying the correct pairing of bound cysteines.

Given $2n$ cysteines that form n disulfide bonds, the number of possible connectivity patterns of all $2n$ cysteines is $(2n-1)!!$. The number of possible disulfide bond connectivity patterns (cysteine pairings) increases rapidly with the number of bound cysteines (e.g. for proteins with four, six and eight disulfide bonds there are 105, 10 395 and $\sim 2 \times 10^6$ possible connectivity patterns, respectively). An exhaustive search for optimal pairing of cysteines is possible only when the number of bound cysteines is small. To overcome the combinatorial explosion problem, the problem of pairing bound cysteines was translated into the problem of finding the perfect match in a complete weighted and undirected graph (Fariselli and Casadio, 2001), which can be solved in polynomial time using the Edmund–Gabow algorithm (Gabow, 1976). In their approach, graph vertices, edges and the weights of edges represent bound cysteines, potential connectivity between two cysteines and confidence scores for the pairing of two cysteines, respectively (Fariselli and Casadio, 2001).

Most current methods that predict disulfide bond connectivity use graph representation. These methods typically differ in the way in which the weights of the edges are calculated. Fariselli and Casadio (2001) assigned contact potentials to edge weights based on the assumption that the nearest sequential neighbors of the paired cysteines were also in contact. Their calculation was limited to protein queries with up to five disulfide bridges as the process of calculating the contact potential employed time consuming Monte Carlo and simulated annealing procedures. In a more recent work, the authors increased the speed of the contact potential calculation by employing a neural network (Fariselli *et al*., 2002). Vullo and Frasconi (2004) were able to significantly increase the accuracy of prediction by incorporating evolutionary information. The authors utilized a recursive neural network to score disulfide connectivity patterns. Ferre and Clote (2005) utilized a neural network with a unique hidden layer intended to examine bi-residue information. They incorporated evolutionary information in the form of Position Specific Scoring Matrices (PSSM) and also added secondary structure information. Tsai *et al*. (2005) used a Support Vector Machine (SVM) with evolutionary information and protein sequence separation of cysteines pairs as inputs. Cheng *et al*. (2006) created a complete platform for disulfide bridge prediction by predicting both the bound state of the cysteines and the disulfide bond connectivity pattern. The authors utilized kernel methods to predict the bound state of cysteines and a recursive neural network to predict disulfide bond pairing. The input to the neural network included evolutionary information, sequence separation of cysteine pairs, and solvent accessibility. Zhao *et al*. (2005) approached the problem of identifying the correct pairing of bound cysteines from a global perspective. Rather than scoring each possible pair of cysteines, the authors compared the query cysteine sequence separation profile to a database of similar profiles of proteins with known disulfide bonds. The limitation of the approach is that a novel cysteine pattern cannot be found. Chen and Hwang (2005) incorporated both local evolutionary information, in the form of PSSM, and global information, in the form of cysteine separation profile, as inputs for a SVM. Lu *et al*. (2007) used a genetic algorithm to improve the optimal selection of input sources for disulfide bond prediction. Chen *et al*. (2006) utilized global and local information as inputs for a two layer SVM. The first layer had a SVM that utilized local information as the input. The inputs for the second layer were scores from the first layer of SVM along with global information such as the protein length, the cysteine separation profile and the disulfide connectivity frequency.

Fiser and Simon (2000) showed that it is possible to discriminate between the different oxidation states of cysteines based solely on the conservation analysis of the cysteines. However, this analysis cannot predict the correct pairing of oxidized cysteines because all the oxidized cysteines are expected to have a similar level of conservation (Fiser and Simon, 2002). Two cysteine residues in a disulfide bond form a strong interaction, which, in many cases, maintains both the protein's structure and function. Such a strong interaction is expected to lead to interdependency between the two positions, which could be traced through evolution. In addition, it is difficult to maintain different redox conditions for the same protein environment, i.e. if a bridge forming an oxidized cysteine is mutated, its reduced cysteine partner will be under pressure to mutate as well. Correlated mutation algorithms aim to identify residue–residue linkages through identifying patterns of concerted variations in different positions in a multiple sequence alignment. A variety of correlated mutation algorithms have already been utilized for predicting residue contacts in a protein 3D structure (Dekker *et al*., 2004; Gobel *et al*., 1994; Hamilton *et al*., 2004; Larson *et al*., 2000; Neher, 1994; Shindyalov *et al*., 1994), but there has not yet been an attempt to utilize correlated mutation algorithms to automatically identify disulfide bond connectivity.

Thornton (1981) analyzed 15 cases of non-conserved disulfide bonds and observed that when a disulfide bond is not conserved both cysteines are mutated in concert. Kreisberg *et al*. (1995) examined the trypsin-like serine proteases and phospholipase A2 protein families and demonstrated that cysteines forming disulfide bonds mutate in a correlated pattern. The authors noted that this correlated pattern could be used to predict disulfide bonds in proteins. These two studies observed trends in cysteine mutations with respect to conservation of disulfide bonds, although they utilized very small databases.

This study uses a large set of protein families to analyze non-conserved disulfide bonds. Subsequently, we introduce a novel method that predicts disulfide bond connectivity pattern using a correlated mutation algorithm.

2 METHODS

2.1 Conservation analysis of disulfide bonds

2.1.1 Data set In order to assess the conservation pattern of disulfide bonds, we examined protein domains from (SCOP) structural classification of proteins (Murzin *et al*., 1995) that had at least one disulfide bond. Our operational definition for a disulfide bond occurrence is when Sulfur gamma (SG) atoms of two cysteine residues fall within a 2.5 Å distance of each other. The expected SG–SG distance for disulfide bond is ~2 Å but this more generous definition accounts for inaccuracies in experimental data. We removed redundancy of

proteins at a 90% sequence identity level using CD-HIT (Li and Godzik, 2006).

2.1.2 Generating multiple structural alignments Multiple structural alignments of each SCOP family were generated with multiple structural alignment algorithm (MUSTANG) (Konagurthu *et al.*, 2006). Since MUSTANG aims to optimize alignment of all residues in the proteins, yet we were looking specifically for optimal alignments of disulfide bonds, we realigned the cysteine pairs for disulfide bonds that were found to be misaligned. A disulfide was assumed to be misaligned if cysteines were found within five alignment positions off of a common disulfide bond position.

2.2 Disulfide bond prediction

2.2.1 Data set In order to benchmark the predictive power of our method we used the same version of annotated protein sequences of Swiss-Prot as other studies: release 39 (1999) (Boeckmann *et al.*, 2003). Protein sequences were filtered by two requirements as described previously (Fariselli and Casadio, 2001). First, only proteins with known 3D structures were considered. Second, disulfide bonds annotation could not contain the words 'by similarity', 'probable' or 'potential'. The test set had 435 proteins that were grouped by the number of disulfide bonds.

2.2.2 Generating multiple sequence alignment For each query protein, evolutionary related sequences were extracted from NR (Wheeler *et al.*, 2007) by running five rounds of PSI-BLAST (Altschul *et al.*, 1997). A representative multiple sequence alignment was generated by filtering the sequences from the PSI-BLAST output using BlastProfiler (Rai *et al.*, 2006) with the following parameters: minimum *e*-value lower than 0.0001, hit-query alignment sequence identity of at least 15%, hit-query alignment coverage of at least 30% and 90% maximum sequence identity between any two hits.

2.2.3 Scoring scheme Our scoring scheme is based on the correlated substitution pattern observed for positions participating in disulfide bonds. In most cases of non-conserved disulfides both cysteines are substituted (see Section 3). Since it is unlikely that all disulfide bonds will always mutate simultaneously, we search for a simple correlation pattern of concerted appearing and disappearing of cysteines in order to predict disulfide bonds. Given a multiple sequence alignment, we examined only those sequence positions (columns) that correspond to disulfide forming cysteine positions in the query. For each sequence in the alignment, we divided the examined positions into two sets based on their amino acids composition; the first set is composed of positions with cysteine residues, while the second set is composed of positions with a gap or any residue other than a cysteine. The score for each possible pair in a sequence is a number between zero and one, and corresponds to our expectation that this pair of positions form a disulfide bond in the query protein based solely on the current sequence examined. If two positions are part of different sets (only one position is a cysteine) then the correlation score is zero, because our observation demonstrated that it is unlikely that only one of the positions that formed a disulfide bond in the query is substituted. If two positions are part of the same set (either a set of all cysteines or a set of anything but cysteines) then the score for a pair of such positions is 1/(size of set −1), which is the probability of selecting the correct position pair randomly and with equal chance, assuming that pairing is possible between only those positions that are part of the same set. Those sequences were ignored in the alignment that contained either completely conserved or completely varied all cysteines as no correlated mutation information can be extracted from these. Also, sequences with an odd number of cysteines, at the sequence positions examined, were removed as they were assumed to be a product of a misalignment. Scores for pairing all possible combinations of all positions for each of the aligned sequences were collected in a matrix that represented all possible disulfide bond combinations in the query. Averaging the scores in all matrices generate a final scoring matrix. Next, we exhaustively generated all possible disulfide bond connectivity patterns and scored them by summing up the scores of the individual disulfide bonds using values from the final scoring matrix. A global score is reported for each possible disulfide bond connectivity pattern.

The steps described are formalized below:

$A = \{$Alignment positions (columns) corresponding to the query bound cysteines$\}$

$I^j = \{i \mid i \in A$& Position i in sequence j is a cysteine$\}$

$II^j = \{i \mid i \in A$& Position i in sequence j is not a cysteine$\}$

$$M^j_{n,m} = \begin{Bmatrix} 0, n \in I^j \,\&\, m \in II^j \\ 0, n \in II^j \,\&\, m \in I^j \\ \frac{1}{|I^j|-1}, n, m \in I^j \\ \frac{1}{|II^j|-1}, n, m \in II^j \end{Bmatrix}$$

$$M_{n,m} = \frac{\sum_j^N M^j_{n,m}}{N}$$

where N is the number of sequences in the alignment.

2.2.4 Retrospective prediction of disulfide connectivity for year 1999 Multiple sequence alignments were generated as described earlier. Then sequences were removed if, in the NCBI protein flat-file, the description of the year of creation was later than 1999.

3 RESULTS

3.1 Disulfide conservation analysis

3.1.1 Cysteines of disulfide bonds mutate in concert In order to analyze the conservation of disulfide bonds, we constructed multiple structural alignments of proteins of SCOP families that contained at least one disulfide bond. From 189 families 1363 such proteins were analyzed. We examined whether cysteines of non-conserved disulfides mutate in concert by comparing the number of times disulfides were substituted by two non-cysteines residues (or gaps) to the number of times only one of the cysteines was substituted. Disulfide bonds were observed to vary in 4288 cases and in 3463 (81%) of these cases both cysteines mutated in concert. However, after manual investigation it turned out that, in most of the cases, the non-concerted disulfide substitution was a consequence of either a misalignment or a protein structural divergence that the alignment program could not account for. Upon removing such disulfides from our conservation analysis 99% of the cases showed disulfides that mutated in concert.

3.1.2 Disulfide bond forming cysteines are not always conserved Proteins of the same SCOP family have obvious evolutionary relationships, usually sharing >35% sequence identity. Nevertheless, we observed that, even within the same SCOP family, disulfide bonds are not always conserved. Of the families, 60% had at least one non-conserved disulfide bond and, overall, we observed that 66% of all disulfide bonds are not conserved. These results are in agreement with recent findings, which show that variations in the number of disulfide bonds in proteins of the same structural family are not unusual (Cheek *et al.*, 2006).

Table 1. Summary of the accuracy [TP/(TP + FP)] and coverage (predicted queries/all queries) of disulfide *connectivity* predictions for proteins with two, three and four disulfides

Number of disulfides (number of query proteins)	Current 2007 NR		1999 NR
	Accuracy	Coverage	Coverage
2 (148)	0.73	0.68	0.56
3 (146)	0.69	0.33	0.14
4 (98)	0.61	0.18	0.11

Current NR and 1999 NR—sequences for the multiple sequence alignment are collected from the current (2007) or from the 1999 non redundant protein sequence database, respectively.

Table 2. Accuracy and coverage of *disulfide-bond* predictions for proteins with two, three, four and five disulfide-bonds

Number of disulfides (number of query bridges)	Current 2007 NR		1999 NR
	Accuracy	Coverage	Coverage
2 (296)	0.73	0.68	0.56
3 (438)	0.71	0.43	0.24
4 (392)	0.64	0.34	0.27
5 (215)	0.59	0.43	0.29

3.2 Prediction of disulfide bond connectivity pattern

3.2.1 Performance of the prediction method Our method can predict disulfide bonds in proteins with any number of bonds. However, results are reported only for proteins with 2–4 disulfide bonds because the prediction of proteins with one bond is trivial and sequence databases lack a sufficient number of protein sequences with five or more disulfide bonds for a statistically significant analysis.

Tables 1 and 2 summarize our results for predicting disulfide bond connectivity patterns and disulfide bonds, respectively. When reporting the prediction accuracy of disulfide bond connectivity patterns, we assess our success in predicting the entire disulfide bond connectivity pattern in the protein correctly. In contrast, when reporting on disulfide bonds prediction we measure our ability to correctly predict any disulfide bond in a protein. Tables 3 and 4 list results of predictions obtained in previous studies as well as results from two trivial predictors: a random predictor and a frequency predictor. A random predictor predicts disulfide connectivity by random, while a frequency predictor predicts bridges by relying on the most common connectivity pattern observed in the database. Comparing our results to the random and frequency predictors demonstrates that correlated mutations can capture the evolutionary signal generated by the disulfide bond interactions. The strength of using a correlated mutation analysis is most apparent when predicting connectivity patterns for proteins with four disulfide bonds (105 possible ways to combine four bonds). The method presented here is capable of predicting all four disulfide bonds with 61% accuracy and can predict a subset of bonds out of the four bridges with an accuracy of 64%. Because, we predicted a subset of the data set utilized by other studies any direct comparison is limited. Nevertheless, in order to obtain a general insight, we evaluated our results along with the results obtained from other methods. With the exception of one method (Lu *et al.*, 2007), our approach produces predictions with the highest accuracies for proteins with three and four disulfide bonds both in predicting disulfide bond connectivity and in predicting disulfide bonds.

Table 3. Accuracy of predicting disulfide bond connectivity by other methods

Methods	Number of disulfide bonds		
	2	3	4
Random	0.33	0.07	0.01
Frequency[a]	0.58	0.29	0.01
FC[b]	0.56	0.21	0.17
VF[a]	0.73	0.41	0.24
CSB[c]	0.74	0.51	0.27
FCL[d]	0.62	0.4	0.55
CSP[e]	0.74	0.44	0.26
TCCLK[f]	0.79	0.53	0.55
CH[g]	0.74	0.61	0.3
CTCK[h]	0.85	0.67	0.57
GASVM[i]	0.86	0.75	0.63

Results are taken from previous works and are reported for proteins with two, three and four disulfide bonds.
References for methods in Tables 3 and 4: (a) (Vullo and Frasconi, 2004), (b) (Fariselli and Casadio, 2001), (c) (Cheng *et al.*, 2006), (d) (Ferre and Clote, 2005), (e) (Zhao *et al.*, 2005), (f) (Tsai *et al.*, 2005), (g) (Chen and Hwang, 2005), (h) (Chen *et al.*, 2006) and (i) (Lu *et al.*, 2007).

Table 4. Accuracy of disulfide bond predictions by other methods

Methods	Number of disulfide bonds			
	2	3	4	5
Random	0.33	0.2	0.14	0.11
Frequency[a]	0.58	0.37	0.1	0.23
FC[b]	0.56	0.36	0.37	0.21
VF[a]	0.73	0.51	0.37	0.30
CSB[c]	0.74	0.61	0.44	0.41
CSP[d]	0.74	0.53	0.44	0.31
TCCHK[e]	0.79	0.62	0.70	0.71
CH[f]	0.74	0.69	0.4	0.31
GASVM[g]	0.86	0.80	0.77	0.71

Results are cited from previous works and reported for proteins with two, three, four and five bonds.

3.2.2 Predicting disulfide bonds of proteins with a mixed state of cysteines We also analyzed our prediction method using protein sequences with disulfide bonds but with an odd number of cysteines. In addition to providing information on the predicted disulfide pattern, we also identified the unbound cysteine.

The sequences we analyzed rarely had cysteines with mixed oxidation states in agreement with observations of earlier studies (Fiser and Simon, 2000). Out of the 137 cases, we studied only 12 could be confirmed to have both oxidized and reduced cysteine, for 38 it was not possible to identify the origin of the extra cysteine (not even after consulting the original literature) and 87 cases came from separating cysteines into intra and inter domain disulfide bonds. In these cases the cysteine of the interdomain disulfide bond shows as an unbound one because the crystal structure presents the monomeric state only. This latter set presents a more difficult task to the prediction algorithm, as the conservation levels of intra and inter disulfide bond forming cysteines are rather similar and these differ in their correlation pattern only. Our results for proteins with three, five and seven cysteines (i.e. proteins with one, two and three disulfide bonds and one free cysteine, respectively) demonstrate 91, 55 and 24% prediction accuracy, respectively. In terms of possible number of connectivity combinations, these numbers can be compared to the prediction accuracies of 73, 69 and 61% of the connectivity patterns for four, six and eight cysteines, respectively, when all cysteines are known to be in disulfide bonds. This suggests that with an increasing number of combinations the accuracy of prediction is getting worse, possibly due to an extra task of identifying free/interdomain cysteines. However, the accuracies remain significant and comparable to our earlier results.

3.2.3 Applicability of the method Prediction of the disulfide bond's connectivity pattern using the correlated mutation algorithm presented here requires that all but one disulfide bond is not fully conserved. Our method cannot predict disulfide connectivity patterns of proteins that do not follow this requirement. We evaluated the applicability of our algorithm by measuring the coverage (the number of predicted proteins divided by the number of proteins tested, or the number of disulfide bond predictions divided by the number of disulfide bonds tested) (Tables 1 and 2). The number of predicted proteins with 2–4 disulfide bonds was high enough for a statistically significance analysis. However, there were only nine predicted proteins with five disulfide bonds, which limited the reliability of statistical analysis, and therefore, we did not analyze the accuracy of disulfide bond connectivity predictions for proteins with five or more disulfide bonds. When we tested the performance of the current algorithm using a sequence database from 1999, we found a considerable decrease in coverage, sometimes by half (Tables 1 and 2). This implies that our algorithm applicability will further increase in the future, as sequence databases expand.

3.2.4 Illustration of the prediction method The first example suggests that, in order to accurately predict all disulfide bonds in a query protein, our algorithm may require only a very few evolutionary related sequences to the query as long as they are sufficiently diverse in their disulfide bond patterns. Pepsin-A precursor (pepa_human) is a human protein that belongs to the peptidase A1 family. This protein has three disulfide bonds located at the query sequence positions of 107–112, 268–272 and 311–344. We automatically generated a multiple sequence alignment with 152 sequences but only two of the 152 sequences could be used for correlated mutation analysis because, in the

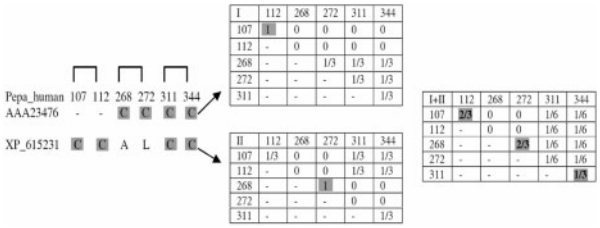

Fig. 1. Pepsin-A precursor (pepa_human) has three disulfide bonds 107–112, 268–272, and 311–344. The alignment positions of pepa_human disulfides with two protein sequences with non-conserved disulfides, and the correlated mutation score matrices corresponding to each sequence is shown. Matrix I+II is the final correlated mutation score matrix, which is obtained by summing and normalizing the two sequence specific scoring matrices (I, and II). The correlation scores of two cysteines that allow unambiguous pairing are highlighted in the matrices.

rest of the cases, all three disulfide bonds were completely conserved. However, the alignment with the two remaining protein sequences (AAA23476, and XP_61523) was sufficient to decipher the connectivity of all three disulfide bonds in the query protein. Each protein sequence in the alignment had a different non-conserved disulfide bond, which is the minimum required information to predict disulfide patterns properly in a protein with three disulfide bonds (Fig. 1).

A second example highlights the correlated mutation pattern observed in disulfide bond positions. β-lactamase (hcpB) from Helicobacter pylori has four disulfide bonds formed between cysteines at sequence positions of 22–30, 52–60, 88–96 and 124–132. Figure 2 illustrates the correlated mutation pattern observed in a multiple sequence alignment of hcpB proteins. The corresponding correlated mutation matrix scores generated by our algorithm highlight the simultaneous mutations of both cysteines in non-conserved disulfide bonds.

A third example illustrates the fact that a correlated mutation signal can drastically reduce the problem of disulfide connectivity prediction even if the prediction is partially ambiguous. Proproteinase E precursor (Cac3_bovine) is a bovine protein with 10 cysteines involved in five disulfide bonds located at query sequence positions 41–57, 100–103, 140–206, 171–187 and 196–227. A multiple sequence alignment reveals that two of the five disulfide-bonds are completely conserved (171–187 and 196–227). However, three disulfide bonds can be accurately predicted based on their correlated pattern of conservation. Although our algorithm cannot fully predict all the disulfide bonds of Cac3_bovine, it produces valuable information as it reduces the complexity of prediction from 945 possible combinations of 10 cysteines to three possible combinations of four cysteines. The multiple sequence alignment of Cac3_bovine is composed of 171 sequences of which nine are completely conserved and 16 have an odd number of cysteines at the examined positions (these were ignored as assumed to be product of misalignment). Out of the remaining 146 sequences 131 had one unconserved disulfide bond, which was always aligned with the query disulfide bond at sequence positions 100–103. Fifteen sequences had two unconserved disulfide bonds, of which 13 had neither the disulfides corresponding to the query sequence positions 100–103, nor to the sequence positions 140–206. Two sequences did not have

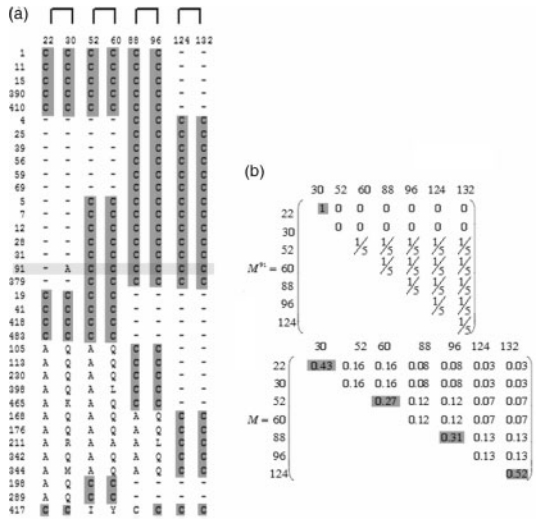

Fig. 2. (**a**) Columns of a multiple sequence alignment corresponding to bound cysteine positions of β-lactamase (hcpB) protein are shown. The horizontal numbers on the top are the sequence positions of eight cysteines in the hcpB protein that form four disulfide bonds (connectivity pattern is illustrated above). (**b**) The resulting scoring matrices when applying our correlated mutation algorithm (Section 2) for sequence 91 in the multiple sequence alignment and for all sequences are M^{91} and M, respectively. Based on the M^{91} matrix a prediction for only one bond is possible (22–30). In order to predict the other disulfide bonds at least two other sequences are required (e.g. sequences one and four). Highlighted correlation scores in the matrices allow unambiguous paring of cysteines.

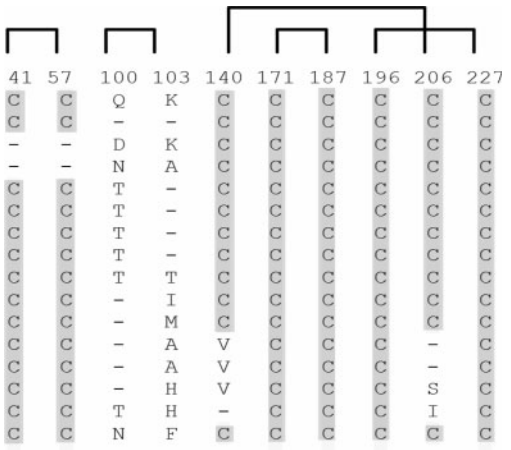

Fig. 3. Columns of a multiple sequence alignment that correspond to oxidized cysteine sequence positions of proproteinase E precursor (Cac3_bovine). Sequence positions that form disulfide bonds are shown above. Alignments positions are shown for a representative subset of sequences that are related to Cac3_bovine but have at least one non-conserved disulfide bond. Conserved cysteines are highlighted.

disulfide bonds corresponding to the query disulfide bonds at sequence positions 41–57 and 100–103 (Fig. 3).

4 DISCUSSION

In the current study, we developed a correlated mutation algorithm to identify disulfide bonds in proteins using sequence information alone. We assumed that correlated mutation analysis is a suitable technique to predict disulfide bonds, because disulfide bond is a well defined residue–residue interaction that plays an important role for the protein structure and function. When such a strong relationship exists between two sequence positions it is expected to result in the coevolution of these positions.

Two requirements have to be fulfilled in order to predict disulfide bonds with correlated mutation analysis. First, some disulfide bonds must be unconserved and second, cysteines of unconserved disulfide bonds have to substitute in a correlation manner. Our analysis of multiple structure alignments of proteins from the same SCOP family demonstrated that both conditions are met. In agreement with recent findings (Cheek *et al.*, 2006), we observed that the number of disulfide bonds varied between evolutionary related proteins. We also demonstrated that multiple sequence alignment columns corresponding to the query disulfide bonds showed a correlated pattern of conservation, i.e. the simultaneous appearance and disappearance of cysteines.

In the current analysis we assumed that the cysteines participating in a disulfide bond are known and, therefore we focused on identifying the correct pairing of these residues. The reason behind this feasible assumption is twofold: (i) the bound state of cysteines can be predicted by several methods with a high accuracy of around 90% (see Section 1), and (ii) <5% of proteins contain cysteines with mixed oxidation states (Fiser and Simon, 2000). Furthermore, past findings have shown that unbound cysteines are significantly less conserved than bound one (Fiser and Simon, 2000), and consequently these will have little or no correlation with bound cysteine. Therefore, any error in predicting the bound state of cysteines should affect only a small fraction of proteins. When we tested our algorithm on a set of proteins with cysteines in mixed oxidation states the predictive power was sustained.

A limitation of our algorithm is that if more than one fully conserved disulfide bond exists, we cannot predict all disulfide bonds of a protein unambiguously. We demonstrated that the recent expansion of sequence databases made our algorithm applicable to more proteins by an average factor of 1.5 since 1999, which suggests an increasing and wide applicability of this approach in the future. We also examined whether our approach provides a unique aspect of disulfide connectivity prediction, in comparison to other methods. Therefore, we compared the overlap between true positive predictions on the same test set using both our approach and a method developed by Cheng and colleagues (2006), which is one of the best method that is publicly available. The protein test set was composed of proteins sequences from a recent release of Swiss-Prot SP51 (2007) (Boeckmann *et al.*, 2003), filtered as described earlier. We retained only those proteins that shared <30% sequence identity with any other proteins from the training set that was used to train the neural network method developed by Cheng *et al.* (2006). The resulting test set was composed of 275 proteins with 2–4 disulfide bonds and was new to both methods. Our findings showed that our algorithm predicted 135 proteins, of which 83 were correct and 52 were incorrect predictions (61% accuracy). The method of Cheng *et al.*

correctly predicted 79 out of the 135 proteins (58% accuracy). When we compared the true positive predictions of both methods there were 57 overlapping cases. This indicates that an ideal combination of both methods could provide a maximum accuracy of 78%, suggesting a potential 17–20% increase over the current accuracies of these methods if used in combination.

Meanwhile it is very useful if one is able to accurately predict a subset of disulfide bonds that vary as this information can be used as a source input for meta predictors or as a complement for other indirect experimental studies that introduce cross-links. Disulfide bond prediction for proteins where only a subset of disulfides are unconserved has an important implication as it may suggest a structural/functional feature not shared by all members of a protein family. For instance, the T cell immunoglobulin mucin (TIM) protein family provides a recent and interesting example as these proteins were found to have two unique disulfide bonds on top of the canonical disulfide of the immunoglobulin domain. The two non-canonical disulfide bonds support the scaffold of a unique binding site in TIM proteins (Cao *et al*., 2007).

While past findings indicated that using multiple sequence alignment significantly increases the accuracies of disulfide bond prediction, it remained unclear as to how multiple sequence alignments support the prediction. In the current study we illustrated that part of the contribution of multiple sequence alignment is the identification of the correlated mutation patterns of the query-bounded cysteines. Future studies should evaluate the contribution of correlated mutation pattern of the sequence environment of bound cysteines to disulfide bond prediction.

ACKNOWLEDGEMENTS

We thank Joseph Dybas, Narcis Fernandez-Fuentes, Eduardo J. Fajardo, Dmitry Rykunov and Daniela Yaar for helpful discussions. Financials support was provided by NIH-NIAID HHSN266200400054C.

Conflict of Interest: none declared.

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Exhibit D

BIOINFORMATICS ORIGINAL PAPER

Vol. 21 no. 8 2005, pages 1415–1420
doi:10.1093/bioinformatics/bti179

Sequence analysis

Cysteine separations profiles on protein sequences infer disulfide connectivity

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Received on July 18, 2004; revised on October 29, 2004; accepted on November 23, 2004
Advance Access publication December 7, 2004

ABSTRACT

Motivation: Disulfide bonds play an important role in protein folding. A precise prediction of disulfide connectivity can strongly reduce the conformational search space and increase the accuracy in protein structure prediction. Conventional disulfide connectivity predictions use sequence information, and prediction accuracy is limited. Here, by using an alternative scheme with global information for disulfide connectivity prediction, higher performance is obtained with respect to other approaches.

Result: Cysteine separation profiles have been used to predict the disulfide connectivity of proteins. The separations among oxidized cysteine residues on a protein sequence have been encoded into vectors named cysteine separation profiles (CSPs). Through comparisons of their CSPs, the disulfide connectivity of a test protein is inferred from a non-redundant template set. For non-redundant proteins in SwissProt 39 (SP39) sharing less than 30% sequence identity, the prediction accuracy of a fourfold cross-validation is 49%. The prediction accuracy of disulfide connectivity for proteins in SwissProt 43 (SP43) is even higher (53%). The relationship between the similarity of CSPs and the prediction accuracy is also discussed. The method proposed in this work is relatively simple and can generate higher accuracies compared to conventional methods. It may be also combined with other algorithms for further improvements in protein structure prediction.

Availability: The program and datasets are available from the authors upon request.

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1 INTRODUCTION

A disulfide bond is a strong covalent bond between two cysteine residues in proteins. It plays a key role in protein folding and in determining the structure/function relationships of proteins (Abkevich and Shakhnovich, 2000; Wedemeyer *et al*., 2000; Welker *et al*., 2001). In addition, it is important in maintaining a protein in its stable folded state. A disulfide connectivity pattern can be used to discriminate the structural similarity between proteins (Chuang *et al*., 2003). In protein folding prediction, the knowledge of the locations of disulfide bonds can dramatically reduce the search in conformational space (Skolnick *et al*., 1997; Huang *et al*., 1999). Therefore, a higher performance in predicting disulfide connectivity pattern is likely to increase the accuracy in predicting the three-dimensional (3D) structures of proteins through the reduction of the number of steps during conformational space search.

Generally, the prediction of disulfide connectivity pattern in proteins consists of two consecutive steps. Firstly, the disulfide bonding state of each cysteine residue in a protein is predicted based on its amino acid sequence and evolutionary information using various algorithms, such as neural networks (Fariselli *et al*., 1999; Fiser and Simon, 2000), support vector machines (Chen *et al*., 2004) and hidden Markov models (Martelli *et al*., 2002). Secondly, the location of disulfide bonds is subsequently predicted based on the bonding state of each cysteine residue using algorithms such as Monte Carlo (MC) simulated annealing together with weighted graph matching (Fariselli and Casadio, 2001) and recursive neural networks with evolutionary information (Vullo and Frasconi, 2004). The prediction accuracy of the oxidation state of cysteine residues has reached 90% (Chen *et al*., 2004) and can be used confidently. However, the task of predicting disulfide connectivity remains challenging. The best prediction accuracy ever reported so far is only 44% (Vullo and Frasconi, 2004), in which recursive neural network was used to score connectivity patterns represented in undirected graphs. Such prediction accuracy is still far from being usable, although it is much higher than that by a random predictor.

In this work, cysteine separation profiles (CSPs) of proteins are adopted for the prediction of disulfide connectivity. It has been shown that proteins with similar disulfide bonding patterns also share similar folds (Chuang *et al*., 2003; van Vlijmen *et al*., 2004). Theoretical work has suggested that disulfide bonds may stabilize the structures of protein fragments between the connected cysteine residues (Abkevich and Shakhnovich, 2000); therefore, the separations between oxidized cysteine residues may be used in the task of predicting disulfide connectivity. Previous works on disulfide connectivity predictions have used graphs to represent disulfide connection patterns (Fariselli and Casadio, 2001; Vullo and Frasconi, 2004). Protein sequences, contact potentials and evolutionary information have been well used to score various connection patterns. The present approach encodes separations among cysteine residues into the form

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Table 1. Number of chains in the datasets, divided according to the number of disulfide bridges (B)

Datasets	$B = 2$	$B = 3$	$B = 4$	$B = 5$	$B = 2 \sim 5$
SP39	150	149	105	45	449
SP39-ID30	92	81	43	28	244
SP39-TEMPLATE	244	198	98	45	585
SP43	124	118	41	35	318

of vectors. The prediction of disulfide connectivity is based on the comparisons of vectors from testing and template dataset, in which similar vectors imply similar connection patterns. The method proposed here is much simpler than graph-based methods, and raises both efficiency and accuracy.

2 SYSTEM AND METHODS

2.1 Datasets

The datasets used to evaluate the predicting power of CSPs were constructed from SwissProt release No. 39 (Bairoch and Apweiler, 2000), including sequences with annotated disulfide bridges. Protein sequences in SwissProt release No. 39 are filtered according to procedures described in two previous works (Fariselli and Casadio, 2001; Vullo and Frasconi, 2004). This dataset is denoted as 'SP39'. Another dataset based on SP39 was also constructed; redundant sequences with pairwise sequence identity of more than 30% were removed. This non-redundant set is denoted as 'SP39-ID30'. SP39-ID30 is used to investigate the effects of sequence identities on the prediction accuracy of CSP.

Another dataset was further constructed to verify the predicting power of CSP. The same filter procedures were applied to sequences in SwissProt release No. 43, where sequences in release 39 were excluded. Thus it is possible to predict proteins newly added to SwissProt database between releases No. 39 and No. 43. This set is denoted as 'SP43'. Redundant sequences with pairwise sequence identity of more than 25% in SP43 were also removed. The template set used to predict disulfide connectivity in SP43 was constructed from SwissProt release 39. Sequences in this set were filtered as in SP39 and SP43, except for the PDB filter. Only sequences sharing less than 30% identity with those in SP43 were kept. This template set is denoted as 'SP39-TEMPLATE'.

The numbers of sequences divided according to the number of disulfide bridges in these datasets are summarized in Table 1.

2.2 Basic assumption

Similar disulfide bonding patterns infer similar protein structures regardless of sequence identity (Chuang *et al*., 2003). Figure 1 shows an example of two proteins with the same disulfide bonding patterns. Tick anticoagulant peptide (serine protease inhibitor, PDB id 1TAP) (Antuch *et al*., 1994) and cacicludine (calcium channel blocker, PDB id 1BF0) (Gilquin *et al*., 1999) exhibit the same disulfide connectivity [1–6, 2–3, 4–5], which means that the first oxidized cysteine is connected with the sixth one, the second with the third, and the forth with the fifth. These two proteins share sequence identity of only 18.2%, but with a C_α root-mean-square deviation (RMSD) of 3.6 Å (Chuang *et al*., 2003). Although the sequence identity is below the twilight zone, the structure and separations among cysteine residues are similar for these two proteins. The residue numbers for cysteines in the two proteins are [5, 15, 33, 39, 55, 59] and [7, 16, 32, 40, 53, 57], respectively. The positions and separations of cysteine residues are similar for these two proteins. It is likely that cysteine separations are related to disulfide connectivity patterns, and through the comparison of CSPs, the disulfide connectivity patterns may be inferred and predicted.

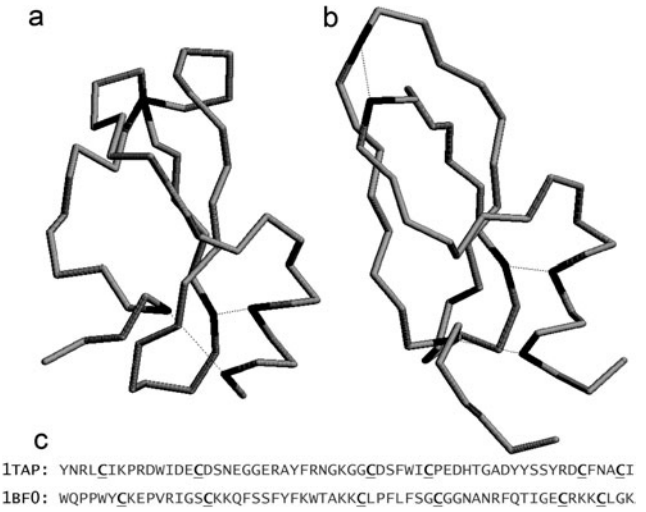

Fig. 1. The structures of two proteins with low sequence identity but sharing the same disulfide bonding patterns: (**a**) anticoagulant protein (PDB id 1TAP), (**b**) calcium channel blocker (PDB id 1BF0), and (**c**) the sequences of the two proteins, with cysteine residues highlighted with bold and underline. Both proteins have three disulfide bonds [1–6, 2–3, 4–5] and BPTI-like structures; the sequence identity is 18.2%.

2.3 CSP and evaluation of prediction accuracy

CSPs contain cysteine separation information. Protein x with n disulfide bonds and $2n$ cysteine residues has a cysteine separation profile (CSP^x) defined as

$$\begin{aligned} CSP^x &= (s_1, s_2, \ldots, s_{2n-1}) \\ &= (C_2 - C_1, C_3 - C_2, \ldots, C_{2n} - C_{2n-1}) \end{aligned}$$

where C_i is the position of ith cysteine residue in the given protein and s_i is the separation between cysteines C_i and C_{i+1}. By this definition, a protein with disulfide bonds will have a CSP.

The divergence, D, between two CSPs is defined as follows:

$$D = \sum_i |s_i^X - s_i^Y|$$

where s_i^X and s_i^Y are the ith separations for CSPs of two different proteins X and Y.

The CSP of a test protein was then compared with all CSPs of template proteins. The disulfide connectivity pattern of the test protein can be predicted as that of the template protein with the most similar CSP, i.e. with the smallest divergence value D. If the divergence D between two CSPs equals 0, the CSPs are termed 'matched profiles', otherwise they are 'mismatched profiles'. If more than one template proteins are matched, one of the templates is randomly selected for the prediction. The ambiguous situations are rare; only less than 2% are observed.

Our method is basically a nearest-neighbor (NN) approach. With only one template for each pattern, our method is essentially a 1-NN approach. We have tried k-NN method in our preliminary investigation. However, the prediction accuracy of k-NN is not significantly better than that of our current approach.

The prediction accuracy of our method was evaluated with Q_p and Q_c values, which are the fraction of proteins with correct disulfide connectivity prediction and are defined as:

$$Q_\mathrm{p} = \frac{C_\mathrm{p}}{T_\mathrm{p},} \qquad Q_\mathrm{c} = \frac{C_\mathrm{c}}{T_\mathrm{c}}$$

where C_p is number of proteins with all the disulfide connectivity correctly predicted; T_p is the total number of test proteins; C_c is the number of disulfide

Table 2. Comparison among different disulfide connectivity prediction algorithms

Algorithms	$B = 2$		$B = 3$		$B = 4$		$B = 5$		$B = 2 \sim 5$	
	Q_p (%)	Q_c (%)	Q_p (%)	Q_c (%)	Q_p (%)	Q_c (%)	Q_p (%)	Q_c (%)	Q_p (%)	Q_c (%)
Frequency[a]	58	58	29	37	1	10	0	23	29	32
MC graph-matching[b]	56	56	21	36	17	37	2	21	29	38
NN graph-matching[c]	68	68	22	37	20	37	2	26	34	42
BiRnn-1 sequence[d]	59	59	17	30	10	22	4	18	28	32
BiRnn-1 profile[d]	65	65	46	56	24	32	8	27	42	46
BiRnn-2 sequence[d]	59	59	22	34	18	30	8	24	31	37
BiRnn-2 profile[d]	73	73	41	51	24	37	13	30	44	49
CSP (SP39)[e]	89	89	81	84	81	85	51	60	81	81
CSP (SP39-ID30)[f]	74	74	44	53	26	44	18	31	49	52
CSP (SP43)[g]	71	71	49	58	30	40	28	33	53	53

[a]Prediction accuracy reported by Vullo and Frasconi (2004).
[b]Prediction accuracy reported by Fariselli and Casadio (2001).
[c]Prediction accuracy reported by Fariselli *et al*. (2002).
[d]Prediction accuracy reported by Vullo and Frasconi (2004).
[e]Prediction accuracy of CSP on SP39 with redundant sequences retained.
[f]Prediction accuracy of CSP with redundant sequences removed.
[g]Prediction accuracy of CSP on SP43 using SP39 as template set, with sequence identity less than 30%.

connectivity correctly predicted; and T_c is the total number of disulfide connectivity in test proteins.

3 RESULTS

3.1 Fourfold cross validation

In order to compare with other approaches for disulfide connectivity prediction, similar criteria were used to select our dataset. The same fourfold cross-validation has been applied to our datasets. The SP39 and SP39-ID30 datasets were divided into four subsets, and the disulfide connectivity prediction was repeated four times. For each prediction, one of the four subsets was used as the test set and the other three subsets were put together to form a template set. The final prediction accuracy was averaged over the four prediction results.

Table 2 summarizes the disulfide connectivity prediction results obtained from this study as well as those obtained from the previous works (Fariselli and Casadio, 2001; Vullo and Frasconi, 2004). 'Frequency' is a trivial method, where the prediction is based on most frequently observed pattern in the training set. 'MC graph-matching' and 'NN graph-matching' are both based on a graph representation of disulfide bonding patterns, using Monte Carlo and Neural Networks for pattern recognition, respectively (Fariselli and Casadio, 2001). The results termed BiRnn are obtained from recursive neural networks with sequence and evolutionary information (Vullo and Frasconi, 2004); the disulfide connectivity patterns are also represented using graphs. The prediction results from this work are termed CSP, with dataset noted in the parenthesis. The prediction results are divided according to the number of disulfide bridges.

The average value of Q_p using CSP is 0.81 for SP39. However, redundant sequences were observed in the SP39 dataset. There are 37.4% of matched profiles and 62.6% of mismatched profiles patterns. The number of matched profile patterns is high, and is likely to have resulted from redundant and homologous sequences in the SP39 dataset. The redundancy may have caused over-fitting in SP39, even with fourfold cross-validation. In order to control and test over-fitting, we extracted the sequences with pairwise sequence identities less than 30% from SP39 and then generated another dataset, SP39-ID30. The average value of Q_p ($B = 2 \sim 5$) using CSP is 49% for SP39-ID30. With redundant sequences removed, the fourfold cross-validation prediction accuracy of CSPs is higher than the best results ever reported from previous works.

The prediction accuracies for protein chains with different disulfide bridge numbers are all significantly higher for 'CSP (SP39)'. For proteins with two, four and five disulfide bridges, the prediction accuracies in 'CSP (SP39-ID30)' are higher than other works. The prediction accuracy for proteins with three disulfide bridges is 2% lower than that of 'BiRnn-1 profile', but is still significantly higher than those from other works.

3.2 Handout prediction of new sequences from SP43

We further validate CSP on a new dataset, SP43, which contains new sequences not seen in SwissProt release 39. We use SP39-TEMPLATE as the template set to predict disulfide connectivity patterns of new sequences in SP43. The pairwise identities of sequences in the template set and SP43 are less than 30%, with template sequences sharing higher identities with those in SP43 being removed. The overall prediction accuracy in SP43 dataset is 53%, which shows significant improvement over the prediction on the other dataset, SP39. The prediction results for SP43 are listed in Table 2. For proteins with three, four and five disulfide bridges, the prediction accuracies in the SP43 dataset are higher than those obtained with fourfold cross-validation in SP39-ID30 dataset. This implies that increasing even the number of non-redundant templates may improve the prediction accuracy of CSP.

3.3 Examples

Three examples of CSP matching are listed in Table 3. These examples are taken from the SSDB database (Chuang *et al*., 2003). The CSPs for template and query protein sequences, as well as their divergence score D, disulfide connectivity patterns and sequence

Table 3. Examples of CSP

Template PDB id	Template CSP	Query PDB id	Query CSP	Disulfide connectivity pattern	Divergence (D)	Sequence identity (%)
1TAP	(10, 18, 6, 16, 4)	1BF0	(9, 16, 8, 13, 4)	[1–6, 2–3, 4–5]	8	18.2
1GPS	(11, 6, 4, 10, 7, 2, 4)	1BRZ	(12, 6, 4, 11, 10, 2, 3)	[1–8, 2–5, 3–4, 6–7]	6	18.8
1TN3	(10, 17, 75, 16, 8)	1C3A:A	(11, 17, 72, 17, 8)	[1–2, 3–6, 4–5]	6	17.7

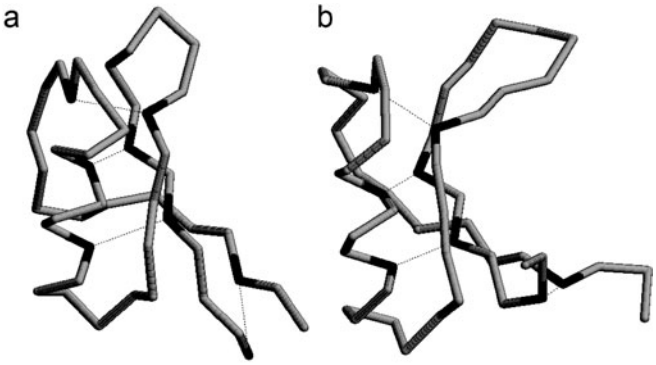

Fig. 2. The structures of (**a**) thionin, toxic arthropod protein (PDB id 1GPS), (**b**) brazzein, thermostable sweet-tasting protein (PDB id 1brz), and (**c**) their sequences with cysteine highlighted. The divergence score D between these two protein sequences is 6. Both proteins have disulfide connectivity [1–8, 2–5, 3–4, 6–7] and their sequence identity is 18.8%.

identities, are shown in Table 3. In the three examples, the divergence scores are all smaller than 10, implying that they share similar disulfide positioning and connectivity patterns. The sequence identities in the three examples are all lower than 20%, thus structure similarity from sequence homology can be ruled out.

The structures and sequences of these examples are illustrated in Figures 1–3. The first example is shown in Figure 1. Tick anticoagulant peptide (serine protease inhibitor, PDB id 1TAP) (Antuch *et al*., 1994) and cacicludine (calcium channel blocker, PDB id 1BF0) (Gilquin *et al*., 1999) have a divergence score $D = 8$; their disulfide connectivity pattern is [1–6, 2–3, 4–5]. Example 2 is illustrated in Figure 2. Thionin (toxic arthropod protein, PDB id 1GPS) (Bruix *et al*., 1993) and brazzein (thermostable sweet-tasting protein, PDB id 1brz) (Caldwell *et al*., 1998) share 18.8% sequence identity. Their divergence score D is 6, and the disulfide connectivity pattern is [1–8, 2–5, 3–4, 6–7]. The third example (Fig. 3), C-type lectin carbohydrate recognition domain of human tetranectin (PDB id 1TN3) (Kastrup *et al*., 1998) and flavocetin-A from Habu snake venom (PDB id 1C3A:A) (Fukuda *et al*., 2000) also have a divergence score of $D = 6$. Their sequence identity is 17.7% and the connectivity pattern is [1–2, 3–6, 4–5]. For all proteins, the oxidized cysteine residues are indicated in black. Cysteine residues on sequences are highlighted in bold and underline. In each case, the cysteine residues are positioned in similar sites along the sequence, and the separations among these cysteine residues are nearly identical.

4 DISCUSSIONS

The number of possible disulfide connectivity patterns increases rapidly with the number of disulfide bridges. For a protein with n disulfide bridges ($n * 2$ oxidized cysteines), the number of possible disulfide connectivity patterns N_p can be formulated as follows:

$$N_p = \frac{\binom{2n}{2}\binom{2n-2}{2}\binom{2n-4}{2}\cdots\binom{2}{2}}{n!} = (2n-1)!! = \prod_{i\leq n}(2i-1).$$

Table 4 lists the number of possible disulfide connectivity patterns for proteins with different disulfide bridge numbers. The use of CSPs may be obscure at first, since the rapidly increasing number of patterns cannot be covered exhaustively. However, the observed numbers of patterns in PDB peak at five disulfide bridges, and decline afterward. Only 45 patterns are observed for protein chains with five disulfide bridges, as opposed to the possible 945 patterns expected. These results imply that the disulfide connectivity pattern of a protein sequence can be predicted from a limited set of templates.

One limitation of our approach is that a pattern not presented in the training set cannot be predicted correctly. Other machine-learning approaches have to enumerate all possible patterns to obtain a prediction with the maximum score (Vullo and Frasconi, 2004); therefore it is possible to correctly predict a pattern never seen in the training set. However, evaluation of all possible patterns is expensive (Vullo and Frasconi, 2004); our approach can achieve comparable prediction performance in a much simpler and faster algorithm.

The prediction accuracies for protein chains with different divergence coverage are shown in Figure 4. The divergence coverage means that a profile matches with a divergence score smaller than or equal to that specified. For example, divergence coverage 5 means profiles matched with a divergence score $\leq$5. Prediction results of the three datasets are illustrated in Figure 4. As can be seen, when divergence coverage is 0, which means the profiles are 'matched profiles', the prediction accuracy is 100% for all datasets. The prediction accuracies become lower as divergence coverage increases. For divergence coverage 50, the prediction accuracy is slightly higher than the overall accuracy. Thus divergence coverage can be used as an index for adoption of CSP or other machine-learning approaches to predict disulfide connectivity. However, these divergence scores are not normalized according to the number of disulfide bridges and the lengths of protein sequences. Several complex factors should be considered in the normalization of divergence score; this is one of the objectives currently undertaken in our group. Sequences with low divergence coverage in a dataset (e.g. 5 for Q_p 0.8) can be predicted by CSP proposed in this work with high accuracy; otherwise, the

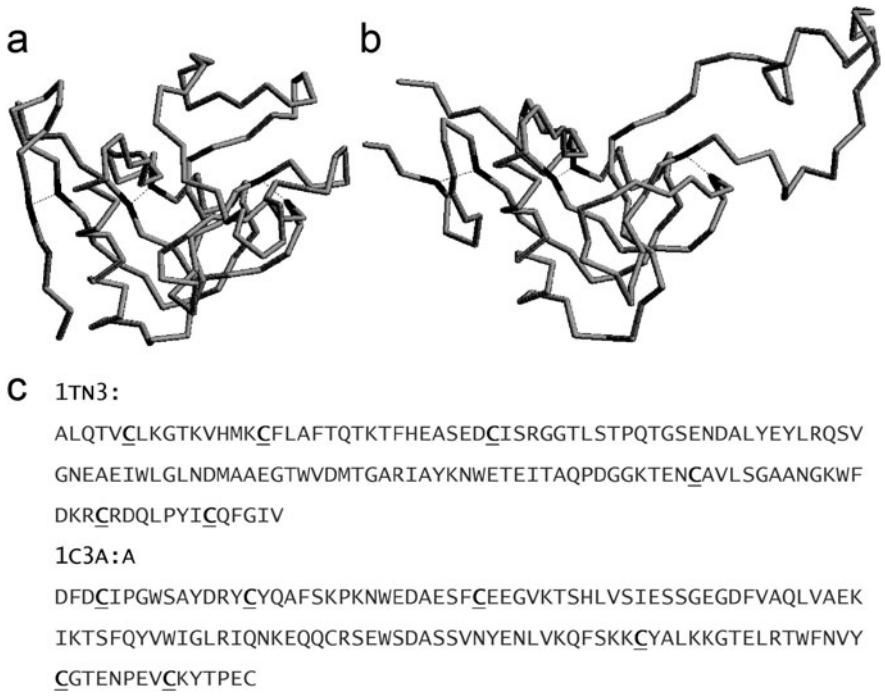

Fig. 3. Structures of (**a**) C-type lectin carbohydrate recognition domain of human tetranectin (PDB id 1TN3), (**b**) flavocetin-A from Habu snake venom (PDB id 1C3A:A), and (**c**) their sequences with cysteine residues highlighted. The divergence score D between these two protein sequences is 6. Both proteins have disulfide connectivity [1–2, 3–6, 4–5] and their sequence identity is 17.7%.

Table 4. Number of possible disulfide connectivity patterns (N_p) for protein chains with different disulfide bridge numbers

Number of disulfide bridges (B)	N_p^a	Observed N_p^b
$B = 2$	3	3
$B = 3$	15	15
$B = 4$	105	43
$B = 5$	945	45
$B = 6$	10 395	29
$B = 7$	135 135	14

[a]Number of possible disulfide connectivity patterns.
[b]Observed number of disulfide connectivity patterns. Statistics obtained from the SSDB database (http://www.e106.life.nctu.edu.tw/~ssbond/) (Chuang *et al.*, 2003).

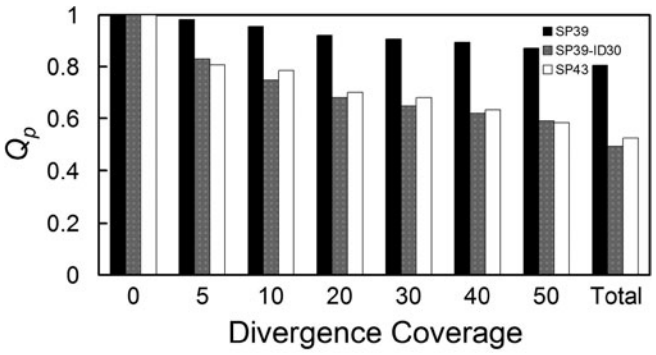

Fig. 4. Prediction accuracy of the datasets with various divergence coverage, which means that a profile matches with divergence score smaller than or equal to that specified. The prediction accuracy (Q_p) is higher with lower divergence coverage. With $D \leq 50$ the prediction accuracy is still slightly higher than the overall Q_p. See text for details.

connectivity patterns of the other sequences in the same dataset can be elucidated by neural networks (Vullo and Frasconi, 2004), support vector machines or other machine-learning approaches.

5 CONCLUSIONS

In this work, we have shown that cysteine separation profiles (CSPs) can be used in predicting disulfide connectivity patterns based on the hypothesis that proteins with similar cysteine separations in sequences may have similar disulfide bonding patterns. The prediction accuracy of CSP proposed in this study is higher than those obtained by other approaches. The handout prediction of new sequences in SP43 dataset can reach 53%. The method mentioned here is extremely simple; therefore the computation time is minimum compared to other methods. The rationale behind our method is completely different from previous studies using sequence and evolutionary information. Our method suggests that topology itself may be an important factor in disulfide connectivity, as it has been proposed by theoretical study (Abkevich and Shakhnovich, 2000) and observations in structure databases (Chuang *et al.*, 2003). Although many efforts have been made to predict the disulfide connectivity patterns, current prediction accuracy is limited around 50%. However, by combining CSP and other algorithms proposed previously

(Fariselli and Casadio, 2001; Vullo and Frasconi, 2004), it is possible to further improve the prediction accuracy. The use of predicted disulfide connectivity patterns in *ab initio* protein structure prediction and other applications would become more reliable in the foreseeable future.

ACKNOWLEDGEMENTS

The authors thank National Science Council of Taiwan for financial support (project number NSC-93-3112-B-002-022).

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Exhibit E

Current Pharmaceutical Design, 2004, *10*, 1235-1244

The Design and Development of Pegfilgrastim (PEG-rmetHuG-CSF, Neulasta®)

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Abstract: Recombinant protein technology produces drugs for human therapy in unprecedented quantity and quality. Research is now focusing on the relationship between pharmacokinetic and pharmacodynamic properties of molecules, with the aim of engineering proteins that possess enhanced therapeutic characteristics in contrast to being used as simple replacements for the natural equivalent.

The addition of a polyethylene glycol (PEG) moiety to filgrastim (rmetHu-G-CSF, Neupogen®) resulted in the development of pegfilgrastim. Pegfilgrastim is a long-acting form of filgrastim that requires only once-per-cycle administration for the management of chemotherapy-induced neutropenia. The covalent attachment of PEG to the N-terminal amine group of the parent molecule was attained using site-directed reductive alkylation. Pegylation increases the size of filgrastim so that it becomes too large for renal clearance. Consequently, neutrophil-mediated clearance predominates in elimination of the drug. This extends the median serum half-life of pegfilgrastim to 42 hours, compared with between 3.5 and 3.8 hours for Filgrastim, though in fact the half-life is variable, depending on the absolute neutrophil count, which in turn reflects of the ability of pegfilgrastim to sustain production of those same cells. The clearance of the molecule is thus dominated by a self-regulating mechanism. Pegfilgrastim retains the same biological activity as filgrastim, and binds to the same G-CSF receptor, stimulating the proliferation, differentiation and activation of neutrophils. Once-per-chemotherapy cycle administration of pegfilgrastim reduces the duration of severe neutropenia as effectively as daily treatment with filgrastim. In clinical trials, patients receiving pegfilgrastim also had a lower observed incidence of febrile neutropenia than patients receiving filgrastim.

INTRODUCTION

Since the early part of the last century it has been recognized that the administration of exogenous proteins can be an effective therapeutic strategy. In particular the replacement of a deficient protein with material from a source outside the body can ameliorate the disease symptoms associated with the original deficiency. Research in this area has made substantial progress [1] since the initial successes, which generally involved replacement of hormones with xenobiotic proteins, such as porcine or bovine insulin for diabetes. The advent of genetic engineering made available in unprecedented quantities recombinant equivalents of several human hormone-like proteins [2, 3]. These recombinant proteins were derived from prokaryotic or eukaryotic organisms into which had been inserted the human gene encoding the protein of interest and were typically of a purity, consistency and similarity to the endogenous equivalent that could not be approached by material derived from animal or human sources. Significant protein therapies obtained in this manner include erythropoietin (rHuEPO) for the treatment of the anaemia in chronic renal disease [4], insulin for diabetes [5], interferon alpha-2b for the management of hairy cell leukaemia [2] and viral infections and recombinant human granulocyte colony-stimulating factor (rHuG-CSF) for the treatment of chemotherapy-induced neutropenia [6]. It is with the latter that the current review is concerned.

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GRANULOCYTE COLONY-STIMULATING FACTOR (G-CSF)

Granulocyte colony-stimulating factor (G-CSF) is a major regulator of the development of antibacterial neutrophilic granulocytic leukocytes (neutrophils). In keeping with the functions of both G-CSF and neutrophils, the mouse molecule was first purified from medium which had bathed the explanted tissues of animals which had been previously injected with a bacterial cell wall extract [7], presumably in an experimental imitation of profound bacterial infection. Several years later the equivalent human molecule was purified, this time from a cancer cell line growing in culture that inadvertently expressed G-CSF in extraordinarily high concentrations [8-10]. The human material was found to be a glycoprotein of around 19kD, which was variably acidic depending upon the proportion of sialic acid in the carbohydrate component [10]. It was later found that the carbohydrate was optional for biological activity and the core protein comprised 174 amino acids [11, 12].

Natural human G-CSF is the product of a single locus on chromosome 17q21-22 [13] and regulation of the gene is by both transcriptional and post-transcriptional processes [14-16].

The cloning and characterization of recombinant human G-CSF (rHuG-CSF) took place between 1984 and 1986 [8, 12, 16] and led to it's expression in E Coli, trials in humans and eventually approval of Filgrastim (r-metHuG-CSF) for administration to patients in the US in 1991 [17-20]. A second, Chinese hamster ovary (CHO)-derived form (lacking

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the N-terminal methionine of the E Coli-derived version) was also approved for human use in Europe in 1993 (lenograstim). The administration of G-CSF to patients was initially for the treatment of cancer chemotherapy-induced neutropenia and the prevention of the associated infections. Since those initial approvals G-CSF has been recommended for use after bone marrow transplantation, for treatment of severe congenital neutropenia, for support of patients with AIDS, acute leukemia, aplastic anemia and myelodysplastic syndromes. It has also been used to mobilize transplantable stem cells to the blood of both cancer patients and normal donors – an unexpected benefit which was not imagined in the early days of development [6, 21, 22].

Various forms of rHuG-CSF have been developed and are available in different countries. Notably filgrastim (Neupogen®, Amgen Inc), lenograstim (Granocyte®, Chugai Pharmaceutical Co Ltd) and KW-2228 (Nartograstim® (Kyowa-Hakko Kogyo Co).

FILGRASTIM (NEUPOGEN®)

Recombinant methionyl G-CSF (r-metHuG-CSF) is a 175 amino acid protein produced in Escherichia coli. Natural G-CSF is a 204 amino acid glycoprotein including a 30 amino acid signal sequence that is removed from the secreted form. The bacterially synthesized version has an additional N-terminal methionine which aids stability in bacterial expression systems. Due to its origin it is also devoid of the O-linked carbohydrate on the threonine at position 133 of the natural protein [23], but retains all 5 cysteines typical of the human sequence (at positions 17, 36, 42, 64 and 74 – the murine version lacks the Cys-17). The latter 4 of these cysteines contribute to disulfide bonds, which stabilize the structure as 4 antiparallel helices [24].

As outlined above r-metHuG-CSF was the first G-CSF to be approved for clinical use and has been used in over 2 million patients [6, 25].

MECHANISM OF G-CSF ACTION

1) The Cellular Targets of G-CSF

One of the properties that allowed G-CSF to first be purified was its ability to act as a colony-stimulating factor or CSF. In this case the colonies that were stimulated to grow consisted entirely of neutrophilic granulocytes – hence the name granulocyte colony-stimulating factor [7].

G-CSF is normally present in the serum at levels of less than 10pg/mL [26]. In conditions such as aplastic anemia, neutropenia, infection, complicated pregnancy etc levels may be substantially higher – up to 100,000pg/mL [27, 28]. Though these studies suggested that G-CSF may be detectable in conditions like infection, especially when accompanied by neutropenia, it's role in normal hemostasis was not fully understood until the work of Lieshke *et al.* [29]. These investigators produced a G-CSF "knockout" (KO) animal by targeted disruption of the gene in embryonal stem (ES) cells. This produced an animal that was devoid of G-CSF for the whole of its life and which had only 20-30% of a normal neutrophil count. Though this may suggest that at least some neutrophils were produced by a G-CSF-independent pathway, the impaired ability of the KO animals to counter infection, despite having 20-30% of their normal complement of neutrophils, argued that not only was G-CSF involved in the majority (70-80%) of baseline neutropoiesis, but also played an essential "emergency" role in response to bacterial infection.

2) Pharmacokinetics of G-CSF

G-CSF has been administered to patients intravenously [18, 19], subcutaneously [30] and intramuscularly [31]. In each case the neutrophil response was similar. Serum G-CSF levels rise very quickly after intravenous infusion and peak within a matter of minutes at over 350pg/mL [18, 19]. Circulating levels attained after subcutaneous administration also suggest a very rapid absorption into the blood stream [30].

The clearance of G-CSF from the body in the presence of normal neutrophil counts has been studied in several species and in all cases the serum half-life has been found to be between 1 and 2 hours [32-34]. In humans the half-life has been reported to be 4.7 hours in the absence of neutrophils, but less than 2 hours at higher absolute neutrophil counts (ANC) [35]. The mode of clearance of the drug is of some interest not only because it is biologically appealing, but also because it led to the development of the pegfilgrastim derivative that is the subject of this review.

Clearance of hematopoietic cytokines by cells in the blood has been reported for several important regulators (thrombopoietin, the main regulator of platelet production [36-39], erythropoietin, which controls red blood cell production [40, 41], M (macrophage)-CSF [42, 43] and G-CSF [44]). This model suggests that the production of mature cell types in the blood would be promoted by a lineage-specific cytokine, the mature cells so formed would then negatively control their own number by being responsible for clearance of that very cytokine. To emphasize this point for G-CSF the reciprocal relationship between G-CSF levels and neutrophil counts has been reported repeatedly [45-48], as has the ability of neutrophils to destroy G-CSF *in vitro* [49]. Neutrophils have therefore been shown to be capable of removing a substantial amount of G-CSF. Since even relatively mature neutrophils express the G-CSF receptor on their surface it has been suggested that the process of neutrophil mediated removal of G-CSF may follow binding of the ligand to the receptor prior to internalisation and intracellular destruction. Extracellular processes may also contribute; the neutrophil is particularly adept at secreting proteolytic enzymes which *in vitro* at least are known to degrade several hematopoietic regulators [49]. In addition to this cellular clearance mechanism the role of the kidney in removal of G-CSF from the body has been reported in a manner consistent with proteins of this size [50-52]. It is suggested by these observations that two mechanisms predominate in the clearance of G-CSF from the body. One process, renal clearance, is of sufficient magnitude that in order to maintain effective serum levels even in conditions of absolute neutropenia, daily injections are required. The other mechanism, mediated by neutrophils, is dependent on the number of neutrophils and perhaps their immediate precursors. It would therefore be expected to be saturable (depending on the number of neutrophils) and would lead to a progressive shortening of the G-CSF half-life as neutrophils number increased – this is what was actually observed in early clinical studies [44].

The implications of these combined mechanisms of clearance would be that: 1) persistent stimulation of neutropoiesis would require continuous production of the endogenous cytokine or frequent injections of the recombinant equivalent (since even in the total absence of neutrophils substantial amounts of G-CSF would still be lost via the kidney) and 2) as neutrophil numbers increase, G-CSF would be more efficiently removed from the body.

CLINICAL BENEFITS OF G-CSF

The dangers of neutropenia are manifold whether it is iatrogenic or congenital in nature since neutrophils play a critical role in protecting the body against microorganisms. The most intuitively obvious application of rHuG-CSF would be in those circumstances where endogenous G-CSF levels are low – it would not, for instance, be predicted that an autoimmune neutropenia involving destruction of neutrophils might respond to rHuG-CSF. Unfortunately, as mentioned above, circulating levels of G-CSF cannot be used to make routine decisions due to the low levels in the circulation. Among iatrogenic neutropenias the chemotherapy-induced neutropenia in cancer patients is widely discussed and is the best documented. It is in this setting that the first study of rHuG-CSF was performed. In 1987 Bronchud *et al.* [17] completed a small trial in patients with lung cancer who received rHuG-CSF on alternating cycles of chemotherapy. They noted a reduction in the severity of neutropenia in those cycles where rHuG-CSF was administered. Since then a large number of studies in a wide variety of chemotherapy regimes and other diseases have been published. These include lung cancer, lymphoma, breast cancer, bone marrow transplantation, testicular cancer, AIDS-related malignancies, myleodysplastic disorders, acute leukemia, congential and cyclic neutropenias and aplastic anemia, all of which are reviewed by Mortsyn *et al.* [53].

Overall, the benefits of rHuG-CSF are that with its administration in chemotherapy- or radiotherapy-induced neutropenia, neutrophil recovery will begin sooner, proceed at a more rapid pace and reduce the period when a patient might be in danger of developing febrile episodes. The reason that rHuG-CSF works as well as it does in this circumstance is not well understood. For instance, the body will usually produce its own G-CSF in response to neutropenia so why would administration of equivalent material from an external source be beneficial ?. It is widely assumed that the advantage conferred by exogenous administration of G-CSF is due to it being administered before the body can mount its own endogenous response – though data to support this contention have yet to be published.

In addition to being used to directly increase neutrophil counts, G-CSF can also be used to mobilize progenitor cells from the bone marrow to the blood, where they can be more easily collected and processed. These progenitor cells, sometimes somewhat loosely called peripheral blood stem cells, have found extensive use in settings where the bone marrow (the normal source of all blood cells) is either damaged or diseased. Though the mechanism by which G-CSF moblizes these cells is largely unknown, by 1995 over 80% of all transplants reported to the European Group for Blood and Marrow Transplantation (EBMT) were performed with cells harvested from the blood rather than the bone marrow [54]. These peripheral blood progenitor cells (PBPC) have also found use beyond the oncology setting in for instance autoimmune disease [55].

Uses for G-CSF in addition to these have been suggested, some supported by preliminary clinical data. For instance closure of fistulas in Crohn's disease [56, 57] has been reported, as has quicker resolution of ulcers on the feet of diabetic patients [58].

G-CSF has few side effects with bone pain being the only adverse event of note and that would normally be managed with non-narcotic analgesics.

THE NEED FOR AN IMPROVED FORM OF FILGRASTIM

Cancer chemotherapy is an evolving science the intention of which is to selectively suppress growth of neoplastic cells while preserving normal tissues. Since the blood-forming tissues of the bone marrow are among the most sensitive in the body to poisoning by cancer chemotherapy they may suffer damage sufficient that treatment of the cancer must be delayed or halted. In cancer chemotherapy, especially intensive or dose-dense treatment regimens homeostatic mechanisms are responding to unnatural, often repeated iatrogenic insults, the endogenous or natural cytokine response often needs to be augmented if therapy is to be continued. rHuG-CSF [53] is administered as prophylaxis to patients receiving chemotherapy who are at risk of neutropenic complications. However, the limited half-life places constraints around its therapeutic use because daily injections are required. In some cases patients do not receive treatment or are left to self-inject over weekends. Others may reside some distance from their treatment center and logistics prevent them from receiving the required frequency of dosing, or place a significant burden on caregivers.

In considering how the requirement for repeated injections of Filgrastim might be overcome the two broad categories of modification considered were either sustained delivery (i.e. prolonged delivery or release from a reservoir of drug) or sustained duration (i.e. a form which persists longer in the circulation). Given the relative instability of G-CSF at physiological temperatures, salt concentration and pH the development of a slow release or depot formulation was considered feasible, but essentially second in line to manipulation of the duration of G-CSF residence in the circulation. It was also considered that a sustained duration form would likely require treatment with a relatively large amount of drug to sustain effective serum levels for up to 4 weeks, which was the initial target. This "front-loading" dosing strategy would expose the patient to high levels of drug immediately after injection with levels diminishing over time. This profile is likely to result in the highest drug levels when the need is greatest (assuming of course that the rationale for rHuG-CSF's effectiveness is it's early provision as discussed above), concentrations would then diminish as the need declined. It was hoped that this profile may promote faster recovery after chemotherapy through providing a very strong stimulus for recovery at the point when the bone marrow is ready to respond – a timepoint which is likely to be variable from patient to patient.

As illustrated by recent experience [59], hematopoietic cytokines can be subjected to molecular modifications that slow clearance from the body and enable less frequent dosing. The rational design of darbepoetin alfa, a novel analog of erythropoietin with extended dosing interval, was effected through first understanding and then manipulating of the nature of the carbohydrate component, which was known to be obligatory for *in vivo* activity. This was achieved by increasing the N-linked sialic acid-containing carbohydrate content of the molecule beyond that found in the natural or recombinant forms. The resulting molecule has a longer serum half-life and greater *in vivo* biological activity than rHuEPO [60]. In considering how G-CSF might be improved, modification of the carbohydrate content was considered unlikely to be fruitful since a comparison of the commercially available forms (which may be either glycosylated or not) revealed that the O-linked, sialic acid-containing carbohydrate found on the natural material and at least one commercially available recombinant form, is entirely optional for the activity of the drug.

As outlined above, detailed knowledge of the dual clearance mechanisms of G-CSF had existed for some time but the ability to manipulate only one or the other pathway had not been demonstrated. It was reasoned that clearance through the kidney could be controlled by attaching a carrier molecule to the parent drug; a carrier molecule which could control renal elimination yet would not affect neutrophil-mediated clearance. The reasoning was that filgrastim is typically administered to patients who are neutropenic. It was already known that high neutrophil numbers were associated with accelerated loss of G-CSF and that low neutrophil counts allowed filgrastim to remain in the circulation for as long as renal loss would allow – admittedly not long, but substantially longer than in conditions of neutrophilia. The aim of the conjugation process was therefore to create a molecule of sufficiently large hydrodynamic volume that losses through renal filtration would be minimized, yet it was important that the conjugated protein remain sensitive to neutrophil-mediated destruction. Should the process of conjugation provide a form of filgrastim which was also resistant to neutrophil mediated clearance, it was a possibility that neutrophil numbers could increase out of control – an outcome that was not considered desirable. Further constraints were placed on the design of a successor to filgrastim by its exemplary safety record. After being used in over 2 million patients the major reported side-effect remains bone pain – which itself may be considered an "on-target" activity of G-CSF upon the activity of osteoclasts or osteoclast precursors rather than a side-effect or "off-target" activity. Off-target activities are uncommon for G-CSF and it was important that a follow-up molecule had at least a similar safety profile. One other aspect of Filgrastim use was also considered at the time of design of its successor. Resulting from the regulatory approval process the dosing recommendations in the USA for filgrastim were that daily doses be administered at the rate of 5 micrograms per kilogram of body weight until a leukocyte count of 10, 000/L was attained. Practical issues dictate that the drug be supplied in standardized vial sizes – in this case containing 300 or 450μg of Filgrastim; yet practical issues also exist in the clinic and these mean that dosing is often rounded to the nearest vial – in effect compromising the precision of dosing calculations with the undesirable possibilities of under-dosing some patients so they do not obtain the full benefit, and in others running the risk of expensive and unnecessary overdosing.

PEGYLATION

Covalent adduction of proteins with poly(ethylene glycol) (PEG) has become a widely used drug delivery strategy, usually with the hope of improving the pharmaceutical and pharmacological characteristics of candidate therapeutics to include prolonged serum half-life [61-69] or reduced immunogenicity [65, 66, 68]. This has been demonstated in several drugs approved for clinical use e.g. PEGasparaginase [70], PEG interferon [71].

The beneficial effects of PEG-conjugation are conferred by the unique properties of PEG itself. Chief among these is the propensity of the polymer to occupy a large volume in an aqueous environment (due to chain flexibility and extensive hydration). PEG was thought to be uniquely suitable for the derivitization of filgrastim. A major goal of the project was to control elimination through the kidney and the volume occupied by a PEG molecule of modest molecular mass could be expected to exceed the threshold for renal loss far sooner than a protein conjugation partner of similar mass. Also, from the drug development perspective, PEG is relatively inert and has been shown to have an acceptable toxicological profile.

A wide range of chemical approaches has been reported for the generation of PEG-protein conjugates [72, 73]. In practice, however, the majority of synthetic procedures rely on the nucleophilic attack of functional groups (usually amino groups) from polypeptide amino acid side chains on the electrophilic center of an appropriately derivitized terminal residue of PEG. Where the chemical conjugation can be conducted in a reproducible fashion, the resulting conjugates can be shown to be consistent forms in terms of the number and location of the PEG chains attached to the protein molecule [71], though in essence the precise nature of the resulting conjugate is, if you will, at the behest of the chemistry; little can be done to control with precision the actual attachment site(s). Precisely this technique has proven itself in two cases of FDA approved and clinically used PEG-enzyme conjugates [74].

However, when PEG-conjugation of, for instance, a cytokine is considered it remains of primary importance that the interaction with the cognate receptor (a large macromolecule of complex 3-dimensional structure) not be affected by the derivitization. In this case these less selective PEG-conjugation strategies might not prove to be optimum. In systems such as cytokine/receptor pairings it is often considered that PEG-conjugation may result in two major and opposing effects on the biologically active component of the conjugate: reduced receptor-binding affinity (presumably due to stearic hindrance of the interaction) versus slowed clearance and a resultant prolonged systemic exposure in comparison to the unmodified protein [75]. The balance of each of these factors will determine the final properties of the drug and in reality it is very difficult to predict which way it will go - mostly the desired properties are attained in a semi-empirical manner.

In order to overcome the limitations of non-specific PEG conjugation, innovative site-specific conjugation strategies have been developed. Many of the problems discussed above can be managed by a well designed, site directed PEGylation scheme. Site-specific PEGylation has been successfully deployed for topographically mapping attachment sites that do not interfere with biological activity [67, 69], for disassociation of different aspects of the biological activity of a molecule [76, 77] or for probing structure/function relationships within proteins [78]. Rather than attempting to direct a PEGylation reaction to, say, specific lysines within a peptide structure, site-directed techniques can also be used to target general sites of attachment such as the N-terminus [64, 65, 79, 80] or C-terminus [61, 65] or the linker regions of chimeric molecules [81, 68]. Since current analytical methodology sets a high standard for molecular homogeneity, site-specific techniques for protein PEGylation offer the greatest potential for meeting criteria of product consistency routinely required in modern drug production. While each of the approaches to site-directed PEGylation employs a widely different conjugation strategy, the unifying objective is to preserve biological activity while incorporating the benefits of PEGylation in a molecule that is simple to make in high yield and is largely homogeneous.

Many different strategies have been developed to achieve site-directed PEG-protein conjugation. Some approaches, which target the primary α- and ε-amines that are found at the protein N-terminus or on lysine side-chains respectively, have sought to direct the coupling reaction to specific amines. By converting lysines at vulnerable sites in the target protein to less reactive arginines [81] or by inserting lysines in desirable positions [66] it has been possible to either create or remove potential reaction sites. Occasionally, the preferred amines are the most reactive and reasonably selective reactions can be optimized with the unaltered protein [77], by coupling through several different lysines [62]. Often this will result in heterogeneous poly-PEGylated molecules though some of these will retain good biological activity. In the case of G-CSF there exist 4 lysines, mostly within the first (A) and fifth (E) helices at positions in the amino acid sequence that, from 3 dimensional mapping data, would not appear to be desirable targets.

PEGylation can also be used to target the polysaccharide component of glycoproteins. Under relatively mild conditions the sugars can be oxidized and reacted specifically with a variety of activated PEG derivatives [82, 83]. Since carbohydrates on naturally occurring glycoproteins tend to be removed from the protein active sites and are readily accessible, they may often represent good attachment sites for PEG. Further, N-linked glycosylation sites have been successfully engineered into proteins for the express purpose of providing polysaccharide targets for PEGylation [81, 84].

A very common approach to site directed PEGylation is to target cysteine thiols using PEGs activated with maleimides, vinylsulfones, pyridyl disulphides or a variety of other chemistries. Many of these reagents are very specific for thiols and take advantage of the relative scarcity of cysteine in proteins [61]. Mutagenesis can also be applied to either remove or add cysteine residues as appropriate to optimize site-specific PEGylation and retention of biological activity [68, 85]. Targeting the cysteines in G-CSF was not considered a viable pathway. The 4 helix structure of G-CSF is stabilized by disulfide bonds which are known to be essential for activity. In addition, cytokines have proven very sensitive to changes in structure with respect to immunogenicity.

A variety of methods for PEGylating the N-termini of proteins have been developed. These include several approaches for selective chemical activation of the N-terminus [64, 80], enzymatic ligation [79] as well as recombinant methods [63]. However, in spite of the many approaches to site-specific PEGylation described in the literature, very few have been used to develop commercially viable products. Here, the development of a simple and scaleable chemical approach has allowed the preparation of a stoichiometrically defined, site-directed conjugate of PEG to the N-terminus of filgrastim via reductive alkylation with PEG aldehydes in an aqueous environment.

Several types of PEG aldehyde derivatives have been described and been used by a number of groups for conjugation with proteins [86-88]. Particularly prominent in the literature are the monofunctional PEG derivatives which can be prepared from mono-methoxyPEG (mPEG). In general, the modification of primary amines in proteins by reductive alkylation is a well understood [89].

The approach adopted for the development of pegfilgrastim was to take advantage of the different pKa values of the α-amino group of the N-terminal methionine (7.6-8.0 [90]) and the ε-amino group on the lysines (10-10.2 [90]) spread through the molecule as discussed above. It was anticipated that targeting specifically the N-terminal amino acid for PEGylation would 1) minimize any interference with receptor interaction, 2) not unduly upset the tertiary structure of the molecule, and 3) produce a consistent, defined product.

MONO-N-TERMINAL POLY(ETHYLENE GLYCOL) CONJUGATES OF FILGRASTIM

PEG-G-CSF conjugates were prepared by reductive alkylation of the protein with linear, mono-functional mPEG aldehydes of several molecular weights. These candidates were then screened in a battery of *in vivo* and *in vitro* assays to select the prime candidate. To obtain screening candidates typical conditions for N-terminal mono-PEG conjugate preparation were as follows: a stirred solution of r-metHuG-CSF (5mg/ml) was cooled in 100 mM sodium acetate, pH 5, 20 mM sodium cyanoborohydride, and a five-fold molar excess of mPEG-aldehyde was added to the solution. The reaction mixture continued to be stirred under the same conditions. The degree of the protein modification was monitored by size exclusion HPLC employing a Bio-Sil SEC 250-5 column (Bio-Rad) eluted with 100 mM sodium phosphate, 150 mM sodium chloride, 10mM sodium azide, pH 6.8, at 1ml/min. After 10 hrs, when the HPLC analysis indicated that approximately 92 % of the protein had been converted to the mono-PEG conjugate, the pH of the reaction mixture was adjusted to 4 (with 100 mM HCl) and diluted 5-fold with 1 mM HCl. The mono-mPEG-G-CSF conjugate was isolated by ion-exchange chromatography using a HiLoad 16/10 SP Sepharose HP column (Amersham Pharmacia Biotech) equilibrated with 20 mM sodium acetate buffer, pH

4 and eluted with a linear 0-1M NaCl gradient. Various mono-mPEG-G-CSF conjugates were obtained in a similar manner by selecting mPEG aldehydes of different molecular weights (between 12 and 30 kDa).

The location of the conjugated PEG moiety in the derivatives was determined by endoproteinase peptide mapping. Additional results obtained using a variety of physicochemical protein characterization methods (ultracentrifugation, MALDI TOF-MS, size exclusion HPLC with on-line multi-angle laser light scattering) confirmed the expected composition and structure of these conjugates as being that of a single linear PEG macromolecule conjugated to the N-terminus of the protein [91, 92] (see Fig. **1**).

PRECLINICAL AND CLINICAL DEVELOPMENT OF PEGFILGRASTIM

Normal clinical practice in cancer patients undergoing chemotherapy is to begin daily dosing with Filgrastim on the day after chemotherapy and to continue until neutrophils attain a safe level [53], around 10,000 neutrophils/mL. In animals it has been shown that a single dose of Filgrastim, no matter how large, cannot substitute for repeated daily administrations due to the short circulating half life of the protein [93]. The PEGylated derivatives of filgrastim were shown from *in vivo* screening in mice to be, almost without exception, to be sustained-duration forms, elevating ANC for substantially longer than the parent molecule. The final selection of a candidate comprising a 20 kd PEG molecule covalently conjugated to the N-terminus of filgrastim was based on prolonged *in vivo* activity, relatively low impact on *in vitro* activity and issues such as substrate availability, yield, and process robustness. Pegfilgrastim has similar pharmacodynamic and biological effects on neutrophils as filgrastim, stimulating the production and maturation of neutrophil precursors and enhancing the functions of mature neutrophils in the same manner as filgrastim [94]. Preclinical studies indicated that the desired properties of the novel form fulfilled the design parameters outlined above – similar safety and pharmacologic profile to the parent and predictable, extended and neutrophil-controlled pharmacokinetics. Phase 1 trials were uneventful in normal volunteers and indicated marked neutrophilia and unprecedented mobilization of PBPC [92]. In a phase 2 study in lung cancer patients comparing a single dose per chemotherapy cycle of pegfilgrastim with daily administration of filgrastim, both regimens caused a rapid increase in ANC, though as predicted from preclinical and Phase 1 data the duration of response was longer in patients receiving pegfilgrastim compared with filgrastim recipients [95]. In addition the residence time of pegfilgrastim was 1) longer than filgrastim and 2) longer in the absence of neutrophils.

Following a series of Phase 2 trials in which no surprise finding were uncovered, two randomised, double-blind, phase 3 trials of once-per-chemotherapy cycle pegfilgrastim versus daily filgrastim were conducted in patients with breast cancer receiving doxorubicin (60 mg/m^2) and docetaxel (75 mg/m^2) chemotherapy. The primary endpoint in both of these studies was the duration of severe neutropenia (days with ANC below 0.5 x 10^9/L). Interestingly, the trials employed slightly different dosing calculation methods. In one trial pegfilgrastim was dosed based on patient weight (100 µg/kg/

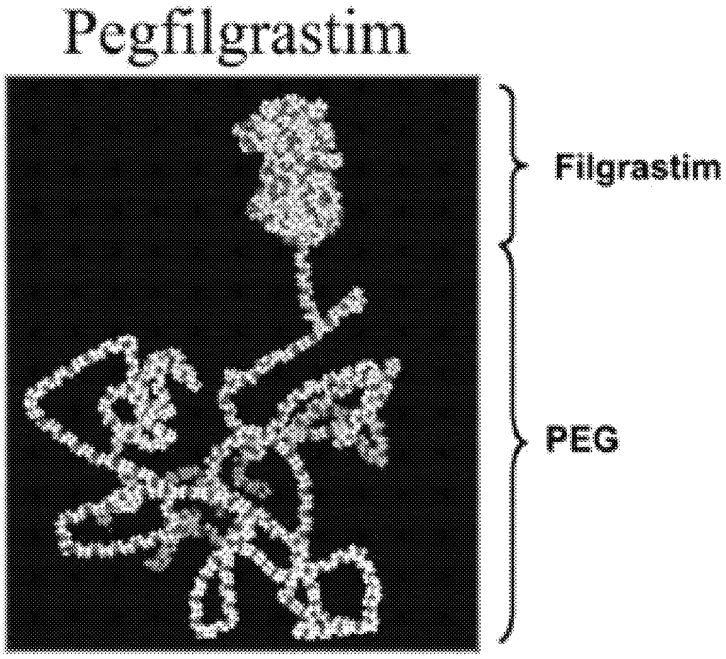

Fig. (1). Space filling model of pegfilgrastim. Note the disproportionate volume occupied by the poly(ethylene glycol) despite similar molecular mass to the protein component. PEG is an ideal conjugation partner to increase hydrodynamic size, and if attached by site-directed means may not niterfere with cytokine/receptor interaction.

cycle), while in the second patients received a fixed 6 mg dose of pegfilgrastim. Both trials used the standard 5 μg/kg/day of filgrastim as control, and it was dosed in the standard manner – starting 24 hours post-chemotherapy and continuing until ANC >10 x 10^9/L or for up to 14 days [96, 97].

In the trial that dosed by weight, the mean duration of severe neutropenia in cycle 1 was similar in both the pegfilgrastim and filgrastim groups (1.7 days vs. 1.8 $p > 0.5$) and comparable in subsequent cycles for each treatment group, although it tended to be shorter in the pegfilgrastim group. The fixed dose study also showed a similar effectiveness in patients treated with pegfilgrastim and Filgrastim [97]. Among the 77 patients in the pegfilgrastim group and the 75 patients in the filgrastim group who could be analyzed, the mean duration of severe neutropenia during cycle 1 was 1.8 and 1.6 days, respectively. Pegfilgrastim had a comparable efficacy to daily filgrastim across a range of body weights when administered as a 6 mg fixed dose. Fixed dosing was part of the desired profile and benefits patients by reducing the possibility of errors and simplifying greatly their supportive treatment. ANC recovery in patients in the pegfilgrastim group was similar to patients who received filgrastim, but without the fluctuations associated with daily filgrastim injections. Whether this fluctuation has any biological significance is unclear. It reflected the preclinical experience where more frequent blood sampling allowed a detailed demonstration of diurnal ANC fluctuation and the influence of daily filgrastim dosing on the cycling [92]. Though it is tempting to speculate that the absence of trough pegfilgrastim serum levels and ANC following treatment with the long-acting form may yield some prophylactic benefit, it remains to be determined whether consistent ANC is a good thing; for instance, where do the neutrophils go when not circulating ? Have they merely emigrated to the marginal pool or have they in fact been lost permanently – the former may be a good thing, the latter obviously less useful.

Febrile neutropenia (defined as a serious neutropenia accompanied by a temperature ≥38.2℃) is usually associated with serious infection, and often prompts hospitalisation and administration of anti-infectives [98, 99]. In addition to the threat to the patient and potential delay in chemotherapy dosing, treatment of febrile neutropenia itself can have a detrimental effect on the quality of life of patients and their carers through the need for hospitalisation and intravenous anti-infective therapy. Febrile neutropenia was prospectively defined as an end-point in both the phase 3 trials. In both the by-weight trial and the fixed dose trial the incidence of febrile neutropenia was reduced over all cycles compared with filgrastim (18% vs. 9%; $p = 0.029$ and 13% vs. 20%; p = NS respectively) [96, 97]. By combining data from the two trials (to include a greater number of treated and control patients) the incidence of febrile neutropenia was shown to be significantly reduced in pegfilgrastim-treated patients compared with those receiving filgrastim (11% vs. 19%; $p < 0.05$) [98]. Furthermore, the duration of the episodes was significantly shorter ($p < 0.05$) resulting in a [non-significant] trend towards reduced risk of hospitalisation and use of intravenous anti-infectives.

The tolerability of pegfilgrastim was similar in both phase 3 trials to that of filgrastim with mild to moderate bone pain the only reported significant treatment-related adverse event (25% vs. 26%) [96, 97]. Pegfilgrastim has also been shown in a by-weight phase 2 trial not to be inferior (a curious statement based on the unusual statistical study design required where placebo control is considered unethical) to filgrastim in patients with non-Hodgkin's lymphoma and Hodgkin's disease receiving ESHAP chemotherapy (etoposide, methylprednisolone, cisplatin and cytarabine) [100].

Pegylation of filgrastim slowed clearance of the parent molecule without affecting its clinical activity. A single injection per chemotherapy cycle is no less effective than daily administration of filgrastim in reducing the duration of severe neutropenia. The improved administration schedule for pegfilgrastim may have advantages over filgrastim in terms of compliance, simplicity and patient quality of life [101]. As a result of careful consideration of prospectively stated design parameters these advantages were gained despite both pegfilgrastim and its parent molecule having similar adverse event profiles [95].

CONCLUSIONS

The revolution in biotechnology heralded the development of recombinant protein therapeutics and the advent of treatment strategies centred on supplementation of natural hormones with synthetic versions. These strategies relied upon attempts to effectively reproduce the exposure profile of the natural hormone by manipulating recombinant protein administration schedules. Often this resulted in only partially optimised exposure, or the need for frequent injections of carefully titrated doses of the synthetic drugs. In attempts to relieve the burden of frequent injections and response monitoring on both patients and caregivers, several new generation protein drugs have been developed to offer superior performance as therapeutics. In the case of darbepoetin alfa an understanding of the role that carbohydrate played in erythropoietin activity suggested that a "more-of-the-same" approach would be optimum for this molecule. A similar approach was considered and rejected for G-CSF due to the non-essential nature of the glycosylation on natural G-CSF. In developing pegfilgrastim an understanding of the routes of clearance suggested PEGylation might offer a viable improvement pathway. Site-directed addition of the relatively inert polymer allowed retention of neutrophil-mediated clearance, yet eliminated renal loss as a significant factor by markedly increasing the hydrodynamic volume of the molecule. This led to the acquisition of many of the desired properties typical of PEGylation, but in addition added a unique "self-regulating" feature to the molecule. In this scenario, pegfilgrastim stimulates accelerated production of neutrophils, just like the parent drug, but then the neutrophils, the very cells produced in response to pegfilgrastim, remove the drug. In combination with a fixed dose strategy (in contrast to by-weight dosing) this self-regulating activity results in a one-size-fits-all, or at least a one-size-fits-most, drug which can be administered to all adult patients at significant risk of developing febrile neutropenia irrespective of their chemotherapy aggressiveness or cycle length and also independent of their (unknown and prospectively unknowable) idiosyncratic susceptibility to cancer treatment toxicity. Thus the development of pegfilgrastim has the potential to improve the management of chemotherapy-induced neutropenia beyond anything currently in the oncologists' armamentarium. Merely

increasing the duration of effective drug plasma concentrations has positive implications for ameliorating the impact of certain illnesses, but the front-loading paradigm attained somewhat serendipitously with pegfilgrastim may add another dimension to improving patient response. Less frequent dosing, increased patient compliance and improved therapeutic effects will decrease the burden of disease for both patients and medical services. Future advances in biomolecular engineering will accelerate the design and development of diverse and complex biomolecules, expanding the sophisticated range already available and providing further benefits in healthcare.

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Exhibit F

Protein refolding for industrial processes

Eliana De Bernardez Clark

Inclusion body refolding processes are poised to play a major role in the production of recombinant proteins. Improving renaturation yields by minimizing aggregation and reducing chemical costs are key to the industrial implementation of these processes. Recent developments include solubilization methods that do not rely on high denaturant concentrations and the use of high hydrostatic pressure for simultaneous solubilization and renaturation.

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Current Opinion in Biotechnology 2001, **12**:202–207

0958-1669/01/$ – see front matter

Abbreviations

CTAB	n-cetyl trimethylammonium bromide
DTE	dithioerythritol
DTT	dithiothreitol
GdmCl	guanidinium chloride
PDGF	platelet-derived growth factor
SDS	sodium dodecyl sulfate
SEC	size-exclusion chromatography

Introduction

The need for the efficient production of genetically engineered proteins has grown and will continue to grow as a consequence of the success of the human genome project. A variety of hosts may be used to produce these proteins, with expression in bacteria poised to play a major role, particularly when the biological activity of the protein product is not dependent on post-translational modifications. Expression of genetically engineered proteins in bacteria often results in the accumulation of the protein product in inactive insoluble deposits inside the cells, called inclusion bodies. Faced with the prospect of producing an insoluble and inactive protein, researchers usually attempt to improve solubility by a variety of means, such as growing the cells at lower temperatures, co-expressing the protein of interest with chaperones and foldases and using solubilizing fusion partners, among others [1]. However, expressing a protein in inclusion body form can be advantageous. Large amounts of highly enriched proteins can be expressed as inclusion bodies. Trapped in insoluble aggregates, these proteins are for the most part protected from proteolytic degradation. If the protein of interest is toxic or lethal to the host cell, then inclusion body expression may be the best available production method. The challenge is to take advantage of the high expression levels of inclusion body proteins by being able to convert inactive and misfolded inclusion body proteins into soluble bioactive products [2–5].

The recent literature includes many examples of the refolding of genetically engineered proteins. A significant number of these publications deal with the expression and purification of small amounts of proteins for structure/function relationship and biophysical characterization studies. Although valuable, the processes described in these publications are usually inefficient, include multiple unnecessary steps and have very low recovery yields. A second significant fraction of the refolding literature deals with understanding the folding pathway of a variety of proteins and, in particular, early folding events. These studies are performed with purified proteins that are subjected to unfolding under a variety of conditions, followed by carefully designed and monitored refolding experiments. A third fraction of the refolding literature, and the focus of this review, deals with the development of more efficient refolding methods that can be used for the commercial production of genetically engineered proteins

The general strategy used to recover active protein from inclusion bodies involves three steps: inclusion body isolation and washing; solubilization of the aggregated protein; and refolding of the solubilized protein (Figure 1a). Although the efficiency of the first two steps can be relatively high, renaturation yields may be limited by the accumulation of inactive misfolded species as well as aggregates. Because the majority of industrially relevant proteins contain one or more disulfide bonds, this review focuses on recent advances in oxidative protein refolding, that is, refolding with concomitant disulfide-bond formation.

Inclusion body isolation, purification and solubilization

Inclusion bodies are dense, amorphous protein deposits that can be found in both the cytoplasmic and periplasmic space of bacteria [1,6•]. Structural characterization studies using ATR-FTIR (attenuated total reflectance Fourier-transformed infrared spectroscopy) have shown that the insoluble nature of inclusion bodies may be due to their increased levels of non-native intermolecular β-sheet content compared with native and salt-precipitated protein [7,8]. Cells containing inclusion bodies are usually disrupted by high-pressure homogenization or a combination of mechanical, chemical and enzymatic methods [6•,9•]. The resulting suspension is treated by either low-speed centrifugation or filtration to remove soluble proteins from the particulate containing the inclusion bodies. The most difficult to remove contaminants of inclusion body protein preparations are membrane-associated proteins that are released upon cell breakage. Washing steps are performed to remove membrane proteins and other contaminants. Methods used to solubilize prokaryotic membrane proteins can be adapted to wash inclusion bodies. The most common washing steps utilize EDTA, and low concentrations of denaturants and/or weak detergents such as Triton X-100, deoxycholate and octylglucoside [6•,9•,10,11•,12,13,P1,P2].

Batas, Schiraldi and Chaudhuri [10] recently compared centrifugation and membrane filtration for the recovery and washing of inclusion bodies. Two membrane pore sizes (0.1 and 0.45 μm) were compared; the larger pore size membrane gave better solvent flux and protein purity. Centrifugation resulted in higher protein purity, probably because it takes advantage of the density differences between cell debris and inclusion bodies.

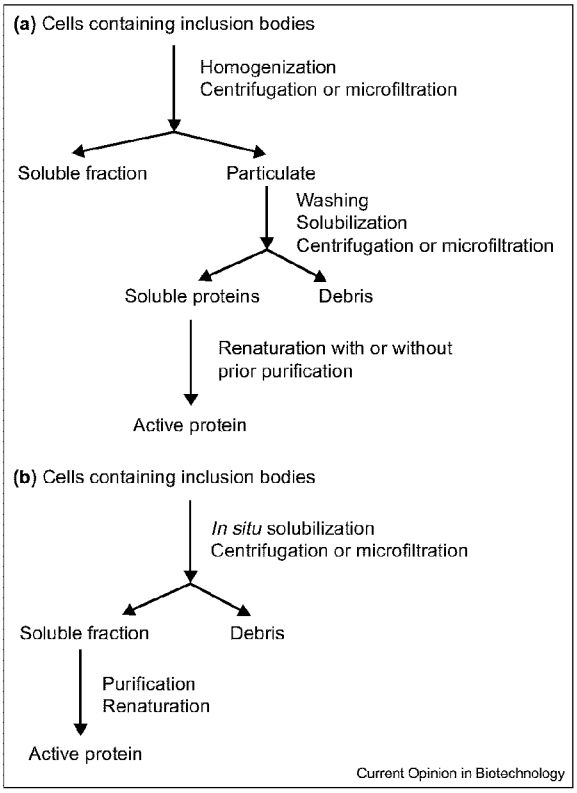
A variety of methods may be used to solubilize inclusion bodies; however, the choice of solubilizing agent can greatly impact the subsequent refolding step and the cost of the overall process. The most commonly used solubilizing agents are denaturants, such as guanidinium chloride (GdmCl) and urea. Using these denaturants, solubilization may be accomplished by the complete disruption of the protein structure (unfolding) or by the disruption of intermolecular interactions with partial unfolding of the protein. The latter approach has the advantage that it requires lower amounts of denaturant to succeed. Although proteins have been successfully refolded from the denatured state, it may prove to be difficult to fold proteins from a partially folded state. Key to the development of an efficient and economic denaturant-based solubilization step is the determination of the minimum amount of denaturant needed to solubilize the protein and to allow for full bioactivity recovery in the refolding step. The majority of the published work on inclusion body protein refolding has used relatively high denaturant (6–8 M) and protein (1–10 mg/ml) concentrations in the solubilization step [5,9•,10,11•,12–14].

Lower denaturant concentrations (1–2 M) have been used to solubilize cytokines from *Escherichia coli* inclusion bodies [P3]. The purity of the solubilized protein was much higher at GdmCl concentrations of 1.5–2 M compared with the more commonly used 4–6 M concentrations, because at the higher GdmCl concentrations contaminating proteins were also released from the particulate fraction. No information was provided about the efficiency of this solubilization process or the range of inclusion body protein concentrations for successful solubilization.

Extremes of pH have also been used to solubilize inclusion bodies. Gavit and Better [15] used a combination of low pH (≤ 2.6) and high temperature (85°C) to solubilize antifungal recombinant peptides from *E. coli*. Lower temperatures and higher pH values resulted in increased solubilization time. Reddy and coworkers [16] utilized 20% acetic acid to solubilize a maltose-binding protein fusion from inclusion bodies. These low pH solubilization processes may not be applicable to many proteins, particularly those that undergo irreversible chemical modifications at these conditions or those susceptible to acid cleavage.

High pH (≥ 12) has been used to solubilize growth hormones [17,18] and proinsulin [P4]. Exposure to elevated pH conditions for extended periods of time may also cause irreversible chemical modifications to the protein. Thus, this high pH solubilization method, although attractive for its simplicity and low cost, may not be applicable to most pharmaceutical proteins. More effective solubilization methods for growth hormones combine high pH with low denaturant concentrations [17,18], 20–40% isopropyl or n-propyl alcohol solutions [P1] or acyl glutamate detergents [P5].

Figure 1



Processes for the recovery of inclusion body proteins. **(a)** Inclusion body isolation followed by solubilization. **(b)** The *in situ* solubilization of inclusion bodies.

Detergents have also been used to solubilize inclusion bodies. Commonly used detergents are sodium dodecyl sulfate (SDS) and n-cetyl trimethylammonium bromide (CTAB) [3,18,19]. Detergents offer the advantage that the solubilized protein may already display biological activity, thus avoiding the need for a refolding step. If this is the case, it is important to remove contaminating membrane-associated proteases in the inclusion body washing step to avoid proteolytic degradation of the solubilized inclusion body protein [6•]. One acknowledged drawback of the use of detergents as solubilizing agents is that they may interfere with downstream chromatographic steps. Extensive washing may be needed to remove solubilizing detergents [P5]. Alternatively, detergents may be extracted from refolding mixtures by using cyclodextrins [20], linear dextrins [21] or cycloamylose [22].

Patra and coworkers [18] compared several solubilization methods for the recovery of human growth hormone from *E. coli* inclusion bodies. They observed similar solubilization efficiencies when using 8 M urea, 6 M GdmCl, 1% SDS or 1% CTAB (all at pH 8.5) or 2 M urea (at pH 12.5). Refolding for the first four solubilization conditions required a dilution step resulting in increased process volumes. Solubilization in 2 M urea at pH 12.5 was simple, economical and efficient, and refolding could be accomplished by a simple pH adjustment without dilution. However, this high pH solubilization method may not be applicable to proteins that might undergo irreversible chemical modifications under these conditions.

A key to the solubilization process is the addition of a reducing agent to maintain cysteine residues in the reduced state and thus prevent non-native intra- and inter-disulfide bond formation in highly concentrated protein solutions at alkaline pH. Typically used reducing agents are dithiothreitol (DTT), dithioerythritol (DTE), and 2-mercaptoethanol [2,3]. These reducing agents should be added in slight excess to ensure complete reduction of all cysteine residues. Chelating agents are added to the solubilization solution to prevent metal-catalyzed air oxidation of cysteines. Alternatively, reduced cysteines may be protected from oxidation by the formation of S-sulfonate derivatives [23,P6,P7] or mixed disulfides [9•,P7].

When expression levels are very high, a competitive alternative is to add the solubilizing agents directly to the broth at the end of the fermentation process. This *in situ* solubilization method has been used to recover insulin-like growth factor using urea under alkaline conditions [P8] and antifungal recombinant peptides using a combination of low pH (< 2.6) and high temperature (85°C) [15]. The main disadvantage of *in situ* solubilization concerns the release of both proteinaceous and nonproteinaceous contaminants that may have to be removed before renaturation is attempted. It has been shown that protein refolding in the presence of impurities may result in decreased yields [6•,24]. The main advantage of this method is the elimination of time-consuming and energy-consuming mechanical disruption methods and of one centrifugation and/or filtration step (Figure 1b).

Solubilization may also be accomplished by applying high hydrostatic pressures (1–2 kbar) in the presence of reducing agents and low concentrations of solubilizing agents [25•,P9].

Renaturation of the solubilized protein

When inclusion bodies have been solubilized using a combination of reducing agents and high concentrations of denaturants, renaturation is then accomplished by the removal of excess denaturants by either dilution or a buffer-exchange step, such as dialysis, diafiltration, gel-filtration chromatography or immobilization onto a solid support. Because of its simplicity, dilution of the solubilized protein directly into renaturation buffer is the most commonly used method in small-scale refolding studies. The main disadvantages of dilution refolding for commercial applications are the need for larger refolding vessels and additional concentration steps after renaturation. The key to successful dilution refolding is to control the rate of the addition of denatured protein to renaturation buffer and to provide good mixing in order to maintain low protein concentration during refolding and thus prevent aggregation. Dilution refolding can also be accomplished in multiple steps, also known as 'pulse renaturation', in which aliquots of denatured reduced protein are added to renaturation buffer at successive time intervals [2,9•], or semicontinuously via fed-batch addition of the denatured reduced protein to refolding buffer [26]. Recently, Katoh and Katoh [26] developed a continuous refolding method in which denatured reduced protein is gradually added from the annular space of a membrane tube to renaturation buffer flowing continuously through the inner space of the membrane tube. Refolding yields obtained using this continuous refolding method were similar to those obtained using fed-batch dilution and about 10% higher than those using batch dilution [26].

Buffer exchange to remove high denaturant concentrations can also be accomplished by diafiltration [27] and dialysis [28] using ultrafiltration membranes. Renaturation yields using these membrane-based methods can be significantly affected by protein binding to the membranes. Binding can be minimized by using highly hydrophilic materials, such as cellulose acetate, which are more compatible with unfolded protein molecules. With typical hydrophobic membrane materials, such as polyether sulfone, the majority of the denatured protein was found bound to the membrane [28]. Significant losses of unfolded protein occurred via transmission through the membrane. These losses could be reduced by dialysis against lower denaturant concentrations that lead to molten-globule or native configurations [28].

Size-exclusion chromatography (SEC) is an alternative buffer-exchange method to remove high denaturant concentrations and promote renaturation [11•,13,29•]. Fahey, Chaudhuri and Binding [13] examined the effect of gel type on renaturation yields and found that as the fractionation range of the gel matrix increases from Sephacryl (S)-100 to S-400, aggregation decreases but the resolution between protein and denaturant decreases. Thus, optimum renaturation yields were obtained with the S-300 gel. In a separate study, Fahey, Chaudhuri and Binding [29•] compared batch dilution and SEC refolding. Dilution also takes place during SEC but, for similar dilution factors, SEC resulted in higher refolding yields when compared with batch dilution as long as the dilution factor was below 40. Sample application conditions were found to have a strong effect on the efficiency of SEC refolding, because rapid structural collapse takes place during sample application that can lead to aggregation. Renaturation yields decreased with higher protein concentrations and sample

volumes and lower flow rates [29•]. Muller and Rinas [11•] circumvented the problem of aggregation during sample application by allowing the denatured protein to penetrate the column under denaturing conditions and then changing the buffer to renaturation conditions. They successfully refolded the complex heterodimeric protein platelet-derived growth factor (PDGF) using a combination of SEC for refolding of the monomeric species followed by prolonged incubation under renaturation conditions to promote dimerization.

Buffer exchange to remove high denaturant concentrations can also be achieved by transiently binding the denatured protein to a solid support. Intermolecular interactions leading to aggregation are minimized when the refolding molecules are isolated through binding to the support. Freedom for structure formation during renaturation is facilitated by binding through fusion partners, such as a His-tag [30] or the cellulose-binding domain [31], which retain their binding capabilities under the denaturing conditions required for loading the solubilized inclusion body protein onto the column. *In situ* purification is achieved by washing the bound protein before elution.

Disulfide-bond formation during folding

In the case of disulfide-bonded proteins, renaturation buffers must promote disulfide-bond formation (oxidation). The simplest and most inexpensive oxidation method uses air in the presence of a metal catalyst and a reducing agent to facilitate disulfide-bond reshuffling [P1,P8]. The rate of disulfide-bond formation through air oxidation may be limited by the slow mass transfer rate of oxygen in aqueous solutions. Increased agitation, which can be used to improve mass transfer rates, may also lead to aggregation due to increased shear and interfacial stresses [32].

Oxidation rates can be accelerated using an oxido-shuffling system, which consists of mixtures of reduced and oxidized low molecular weight thiol reagents. The most commonly used oxido-shuffling reagents are reduced and oxidized glutathione (GSH/GSSG) but the pairs cysteine/cystine, cysteamine/cystamine, DTT/GSSG and DTE/GSSG have also been utilized [2,3,9•]. Molar ratios of reduced to oxidized thiol of 3:1 to 1:1 and total thiol concentrations between 5–15 mM have been found to be optimal [14,33]. Disulfide-bond formation using the oxido-shuffling system can be accelerated by using a small-molecule mimic of protein disulfide isomerase [34,P10]. A disadvantage of the oxido-shuffling system is the high cost of some of the reagents, particularly glutathione.

A third oxidation method uses a two-step approach: the formation of mixed disulfides between glutathione and the denatured protein before renaturation, followed by refolding in the presence of catalytic amounts of a reducing agent to promote disulfide-bond formation and reshuffling [9•,P7]. The refolding yield of recombinant human tissue plasminogen activator could be increased sixfold when the oxido-shuffling refolding system was replaced with the mixed disulfide approach [9•]. Alternatively, cysteines in the denatured protein may be protected by sulfonation, followed by the addition of a reducing agent such as cysteine [P7] and 2-mercaptoethanol [P6] or a thiol/disulfide mixture such as cysteamine/cystamine [23].

Improving renaturation yields

The formation of incorrectly folded species, and in particular aggregates, is usually the cause of decreased renaturation yields. A very efficient strategy to suppress aggregation is the inhibition of the intermolecular interactions leading to aggregation by the use of low molecular weight additives. These small molecules are relatively easy to remove when refolding is complete. Numerous additives have been shown to prevent aggregation [3,9•]. The mechanism of action of additives is still unclear. They may influence both the solubility and the stability of the native, denatured and intermediate state(s), they may act by changing the ratio of the rates of proper folding and aggregate formation or they might simply act by solubilizing aggregates. The most commonly used low molecular weight additives are L-arginine (0.4–1 M), low concentrations of denaturants such as urea (1–2 M) and GdmCl (0.5–1.5 M) and detergents (Chaps, SDS, CTAB and Triton X-100). In a recent review, De Bernardez Clark, Schwarz and Rudolph [9•] discussed different approaches to inhibiting aggregation during refolding and provided a detailed list of low molecular weight additives and the concentration ranges needed to increase renaturation yields.

Low concentrations of urea and GdmCl, although widely used to inhibit aggregation [14,29•,P2,P3,P7], are not always effective folding enhancers. GdmCl concentrations as low as 0.25 M were found to inhibit the oxidative dimerization of PDGF [11•]. Similarly, bone morphogenetic protein-2 proved difficult to refold in the presence of low concentrations of denaturants [35].

Detergents such as Chaps [12,35], CTAB [20,21], Triton X-100 [20] and SDS [19] have been successfully used to improve renaturation yields. As noted earlier under solubilization methods, one drawback of the use of detergents is that they may be difficult to remove and may affect downstream chromatographic steps. Detergents have been extracted from refolding mixtures using cyclodextrins [20], linear dextrins [21], cycloamylose [22] and ion-exchange chromatography in the case of ionic detergents [19].

The *in vivo* competition between folding and aggregation is modulated by chaperones and foldases [1]. It is not surprising that these proteins can also affect the *in vitro* competition between folding and aggregation [36]. Because chaperones and foldases are proteins that need to be removed from the renaturation solution at the end of the refolding process and as they may be costly to produce, their commercial use will require a recovery–reuse scheme. Altamirano and coworkers [37] developed a

Table 1

Most frequently used oxidative renaturation methods.

Solubilization method	Renaturation method*	Comments	References
High denaturant concentrations: urea (≥ 8 M) or GdmCl (≥ 6 M) in the presence of a reducing agent (DTT, DTE, 2-mercaptoethanol)	Removal of the denaturant by dilution or buffer exchange (dialysis, diafiltration, size-exclusion chromatography or binding to a matrix) with concomitant disulfide-bond formation†	Residual denaturant concentrations may interfere with the assembly of oligomeric proteins	[9•,11•,12–14, 18,29•,P2]
Detergents: SDS, CTAB in the presence of a reducing agent (DTT, DTE, 2-mercaptoethanol)	Oxidation† followed by detergent removal by extensive washing or stripping	Residual detergent concentrations may interfere with downstream purification processes	[18–22]
Extremes of pH in the presence of low concentrations of denaturants	pH adjustment to 7.5–9.5 to promote disulfide-bond formation†	Extreme pH may cause irreversible chemical modifications	[15–18]

*Low molecular weight additives may be added to prevent aggregation. †Disulfide-bond formation may be accomplished by air oxidation in the presence of a metal catalyst and a reducing agent or by using an oxido-shuffling system.

reusable molecular chaperone system for oxidative refolding chromatography that utilizes a GroEL minichaperone, which can prevent aggregation, the oxido-shuffling catalyst DsbA, and peptidyl-prolyl isomerase, all immobilized on an agarose gel. Recently, Kohler, Preuss and Miller [38] developed a chaperone-assisted refolding bioreactor that uses a stirred-cell membrane system to immobilize the GroEL–GroES complex. In its current design, the bioreactor could only be used for three cycles of refolding. Further design improvements will be needed before this bioreactor can be considered as a commercially viable refolding alternative.

Interestingly, high hydrostatic pressures (1–2 kbar) in combination with low concentrations of denaturants have been used for the simultaneous solubilization and refolding of inclusion body proteins [25•,P9]. Similarly, high hydrostatic pressures can be used in the refolding process to prevent aggregation [39,40].

Conclusions

The recovery of bioactive proteins from inclusion bodies is a complex process. Despite its complexity, there are clear guidelines on how to proceed when faced with the task of refolding an inclusion body protein (Figure 1; Table 1). As with other protein recovery processes, however, optimum conditions have to be determined on a case by case basis. The key to a commercially viable renaturation process lies in minimizing the number of steps (to increase the overall yield) and the amounts and costs of chemicals needed. This can be accomplished by eliminating unnecessary buffer-exchange steps, by exploring the use of alternative solubilization methods that do not rely on high denaturant concentrations, and by developing efficient oxidation methods that do not require the use of expensive oxido-shuffling systems. Future developments in protein refolding will benefit from a more fundamental understanding of inclusion body solubilization methods, and on the role that additives play in the inhibition of aggregation.

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Exhibit G

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ProductInformation

L-Cystine

Product Number **C 8755**
Store at Room Temperature

Product Description

Molecular Formula: $C_6H_{12}N_2O_4S_2$
Molecular Weight: 240.3
CAS Number: 56-89-3
Synonyms: [*R*-(*R**,*R**)]-3,3'-dithiobis[2-aminopropanoic acid], dicysteine, β,β'-dithiodialanine[1]

Cystine is a derived amino acid that is formed from the oxidative linkage of two cysteine residues to give a disulfide covalent bond. Cystines form in many proteins after incorporation of free cysteines into the primary structure to stabilize their folded conformation. Cystine is the form in which cysteine exists in blood and urine.[2]

The two cystine-related clinical conditions are cystinuria, which involves the defective membrane transport of cystine, and cystinosis, the accumulation of cystine in lysosomes.[2,3,4] A review of cystine transport into rat brain cells has been published.[5] An investigation into cysteine and cystine levels in normal and malignant cells with a relationship to γ-cystathionase levels and tumor sensitivity to L-cysteine and cystine depletion has been reported.[6]

Mass spectrometry (GC-MS) methods for the analysis of cystine from granulocytes of cystinosis patients[7] and in the urine of homocystinuria patients[8] have been published.

Precautions and Disclaimer

For Laboratory Use Only. Not for drug, household or other uses.

Preparation Instructions

This product is soluble in 1 M HCl (50 mg/ml), with heat as needed, yielding a clear, colorless solution. The solubility of cystine in water is 0.112 mg/ml at 25 °C; cystine is more soluble in aqueous solutions with pH < 2 or pH > 8.[1]

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