IN THE UNITED STATES DISTRICT COURT
FOR THE WESTERN DISTRICT OF PENNSYLVANIA

AMGEN INC. and AMGEN MANUFACTURING LIMITED,	)	Civil Action
	)	No. ___________________
Plaintiffs,	)	
v.	)	***Electronically Filed***
MYLAN INC., MYLAN PHARMACEUTICALS INC., MYLAN GMBH and MYLAN N.V.,	)	**JURY TRIAL DEMANDED**
Defendants.	)	

COMPLAINT

Plaintiffs Amgen Inc. and Amgen Manufacturing Limited (collectively, "Plaintiffs"), by and through their undersigned attorneys, for their Complaint against Defendants Mylan Inc., Mylan Pharmaceuticals Inc., Mylan GmbH, and Mylan N.V. (collectively, "Defendants") hereby allege as follows:

THE PARTIES

1. Amgen Inc. ("Amgen") is a corporation existing under the laws of the State of Delaware, with its principal place of business at One Amgen Center Drive, Thousand Oaks, California 91320. Amgen discovers, develops, manufactures, and sells innovative therapeutic products based on advances in molecular biology, recombinant DNA technology, and chemistry. Founded in 1980, Amgen is a pioneer in the development of biological human therapeutics. Today, Amgen is the largest biotechnology company in the world, fueled in part by the success of NEULASTA® (pegfilgrastim).

2. Amgen Manufacturing Limited ("AML") is a corporation existing under the laws of the Territory of Bermuda with its principal place of business at Road 31 km 24.6, Juncos,

Puerto Rico 00777. AML manufactures and sells biologic medicines for treating particular diseases in humans. AML is a wholly owned subsidiary of Amgen.

3. Upon information and belief, Mylan Inc. is a corporation organized and existing under the laws of Pennsylvania, with its principal place of business in Canonsburg, Pennsylvania at 1000 Mylan Boulevard Canonsburg, Pennsylvania 15317. Upon information and belief, acting in concert with the other Defendants, Mylan Inc. is in the business of developing, manufacturing, and marketing biopharmaceutical products that are distributed and sold in the Commonwealth of Pennsylvania and throughout the United States.

4. Upon information and belief, Mylan Inc. is a United States agent for Mylan GmbH and Mylan N.V. for purposes including, but not limited to, corresponding with the Food and Drug Administration ("FDA").

5. Upon information and belief, Mylan Pharmaceuticals Inc. is a corporation organized and existing under the laws of West Virginia, with its principal place of business in Morgantown, West Virginia at 781 Chestnut Ridge Road, Morgantown, West Virginia 26505. Upon information and belief, acting in concert with the other Defendants, Mylan Pharmaceuticals Inc. is in the business of developing, manufacturing, and marketing biopharmaceutical products that are distributed and sold in the Commonwealth of Pennsylvania and throughout the United States.

6. Upon information and belief, Mylan Pharmaceuticals Inc. is a United States agent for Mylan GmbH and Mylan N.V. for purposes including, but not limited to, corresponding with FDA.

7. Upon information and belief, Mylan GmbH is a corporation existing under the laws of the Republic of Switzerland with its principal place of business at Thurgauerstrasse 40

Zurich, 8050 Switzerland. Upon information and belief, acting in concert with each of the other Defendants, Mylan GmbH is in the business of developing, manufacturing, and marketing biopharmaceutical products that are distributed and sold in the Commonwealth of Pennsylvania and throughout the United States.

8. Upon information and belief, Mylan N.V. is a corporation existing under the laws of the Republic of Netherlands with its global headquarters and principal offices located in Canonsburg, Pennsylvania, and its principal executive offices located Hatfield, Hertfordshire, England. Upon information and belief, acting in concert with each of the other Defendants, Mylan N.V. is in the business of developing, manufacturing, and marketing biopharmaceutical products that are distributed and sold in the Commonwealth of Pennsylvania and throughout the United States.

9. Upon information and belief, Mylan Inc., Mylan Pharmaceuticals Inc., and Mylan GmbH are wholly owned subsidiaries of Mylan N.V.

10. Upon information and belief, Mylan Pharmaceuticals Inc. is a wholly owned subsidiary of Mylan Inc.

11. Upon information and belief, Defendants collaborate to develop, manufacture, seek regulatory approval for, import, market, distribute, and sell biopharmaceutical products (including products intended to be sold as biosimilar versions of successful biopharmaceutical products developed by others) in the Commonwealth of Pennsylvania and throughout the United States.

NATURE OF THE ACTION

12. This is an action for patent infringement arising under the patent laws of the United States, Title 35, United States Code, including 35 U.S.C. § 271(e)(2)(C), which was enacted in 2010 as part of the Biologics Price Competition and Innovation Act of 2009 (“the

BPCIA”), Pub. L. No. 111-148, §§ 7001-7003, 124 Stat. 119, 804-21 (2010) (amending, inter alia, 35 U.S.C. § 271 and 42 U.S.C. § 262).

13. The asserted patents are U.S. Patent No. 8,273,707 (“the ’707 Patent”) and U.S. Patent No. 9,643,997 (“the ’997 Patent”). Amgen is the owner of all rights, title, and interest in the ’707 and ’997 Patents. The ’707 and ’997 Patents claim methods of purifying proteins used in the manufacture of a biological product.

14. The BPCIA created an abbreviated pathway for the approval of biosimilar versions of approved biologic drugs. 42 U.S.C. § 262(k). The abbreviated pathway (also known as “the subsection (k) pathway”) allows a biosimilar applicant (here, Mylan GmbH, acting in concert with the other Defendants) to rely on the prior licensure and approval status of the innovative biological product (here, NEULASTA®) that the biosimilar purports to copy. Amgen is the sponsor of the reference product (“reference product sponsor” or “RPS”), NEULASTA®, which is approved by FDA to decrease the incidence of infection in patients receiving myelosuppressive anti-cancer drugs. Under the subsection (k) pathway, the biosimilar applicant may rely on its reference product’s data rather than demonstrating that a biological product is safe, pure, and potent, as Amgen was required to do to obtain FDA licensure of its reference product under 42 U.S.C. § 262(a).

15. To avoid burdening the courts and parties with unnecessary disputes, the BPCIA also creates an intricate and carefully orchestrated set of procedures for the biosimilar applicant and the RPS to engage in a series of information exchanges and good-faith negotiations between parties prior to the filing of a patent infringement lawsuit. These exchanges are set forth in 42 U.S.C. § 262(*l*)(2)-(*l*)(5) and culminate in an “immediate patent infringement action” pursuant to 42 U.S.C. § 262(*l*)(6).

16. Seeking the benefits of the subsection (k) pathway, Mylan GmbH, acting in concert with the other Defendants, submitted Defendants' abbreviated Biologics License Application No. 761075 (the "Mylan aBLA") to FDA pursuant to the BPCIA, specifically 42 U.S.C. § 262(k), requesting that its biological product ("the Mylan Pegfilgrastim Product") be licensed by relying on Amgen's demonstration that NEULASTA® (pegfilgrastim) is "safe, pure, and potent."

17. Upon information and belief Mylan GmbH, acting in concert with the other Defendants, submitted the Mylan aBLA to FDA prior to February 2017, and thus before the expirations of the '707 Patent and the '997 Patent.

18. Upon information and belief, Defendants received FDA acceptance of the Mylan aBLA for review on or about February 7, 2017.

19. In March 2017, the parties began exchanging information as required by the BPCIA.

20. The '707 Patent was included on Amgen's May 1, 2017 disclosure pursuant to 42 U.S.C. § 262(*l*)(3)(A). Pursuant to 42 U.S.C. § 262(*l*)(7), the '997 Patent was included on Amgen's June 7, 2017 supplement to its 42 U.S.C. § 262(*l*)(3)(A) list.

21. Under 35 U.S.C. § 271(e)(2)(C)(i), it is an act of infringement to submit an application seeking approval of a biological product with respect to patents identified in the lists of patents described in 42 U.S.C. § 262(*l*)(3) if the purpose of such submission is to obtain approval to engage in the commercial manufacture, use, or sale of a biological product claimed in a patent or the use of which is claimed in a patent before the expiration of such patent. *See Sandoz Inc. v. Amgen Inc.*, 137 S. Ct. 1664, 1672 (2017).

22. Here, Defendants committed an act of infringement with respect to each of the ’707 and ’997 Patents under 35 U.S.C. § 271(e)(2)(C)(i) when they caused Mylan GmbH to submit the Mylan aBLA for the purpose of obtaining FDA approval to engage in the commercial manufacture, use, or sale of the Mylan Pegfilgrastim Product.

23. If FDA approves the Mylan aBLA and Defendants import the Mylan Pegfilgrastim Product into the United States, or offer to sell, sell, or use the Mylan Pegfilgrastim Product within the United States, Defendants will also infringe one or more claims of the ’707 and ’997 Patents under 35 U.S.C. § 271(g).

JURISDICTION AND VENUE

24. This action arises under the patent laws of the United States, Title 35 of the United States Code, Title 42 of the United States Code, and under the Declaratory Judgment Act of 1934 (28 U.S.C. §§ 2201-2202), Title 28 of the United States Code.

25. This Court has subject matter jurisdiction pursuant to 28 U.S.C. §§ 1331 and 1338(a).

26. Venue is proper in this District pursuant to 28 U.S.C. § 1391(b) and (c), and 28 U.S.C. § 1400(b).

27. Upon information and belief, Mylan Pharmaceuticals Inc. has a regular and established place of business in Pennsylvania. Upon information and belief, Mylan Pharmaceuticals Inc. is licensed to do business in Pennsylvania as a foreign business corporation.

28. This Court has personal jurisdiction over each of the Defendants for the reasons set forth below.

A. Mylan Inc.

29. Upon information and belief, Mylan Inc., Mylan Pharmaceuticals Inc., Mylan GmbH, and Mylan N.V. hold themselves out as a unitary entity and represent to the public that their activities are directed, controlled, and carried out as a single entity.

30. This Court has personal jurisdiction over Mylan Inc. by virtue of, among other things, Mylan Inc. being a Pennsylvania corporation; having its principal place of business in Canonsburg, Pennsylvania; having availed itself of the rights and benefits of Pennsylvania law; and having engaged in substantial and continuing contacts with Pennsylvania.

B. Mylan Pharmaceuticals Inc.

31. Upon information and belief, Mylan Inc., Mylan Pharmaceuticals Inc., Mylan GmbH, and Mylan N.V. hold themselves out as a unitary entity and represent to the public that their activities are directed, controlled, and carried out as a single entity.

32. Upon information and belief, Mylan Pharmaceuticals Inc. is a wholly owned subsidiary of Mylan Inc., which exercises considerable control over Mylan Pharmaceuticals Inc.

33. Upon information and belief, Mylan Pharmaceuticals Inc. develops, manufactures, seeks regulatory approval for, markets, distributes, and sells biopharmaceuticals for sale and use throughout the United States, including in Pennsylvania and this federal judicial District.

34. This Court has personal specific jurisdiction over Mylan Pharmaceuticals Inc. because, upon information and belief, following any FDA approval of the Mylan Pegfilgrastim Product, Mylan Pharmaceuticals Inc. will sell the Mylan Pegfilgrastim Product that is the subject of the patent infringement claims in this action in Pennsylvania and throughout the United States.

35. This Court has personal general jurisdiction over Mylan Pharmaceuticals Inc. by virtue of, inter alia, its having conducted business in this District, having availed itself of the rights and benefits of Pennsylvania law, and having engaged in substantial and continuing contacts with Pennsylvania. Upon information and belief, Mylan Pharmaceuticals Inc. has regular and continuous commercial business dealings with representatives, agents, distributors, and customers located in Pennsylvania and this District. In addition, Mylan Pharmaceuticals Inc. has availed itself of this Court by asserting claims in this District, *see, e.g.*, *Mylan Inc., Mylan Pharmaceuticals, Inc. v. Boehringer Ingelheim International GmbH, et al.*, Case No. 09-00990-GLL (W.D. Pa. complaint filed July 7, 2009), and by asserting counterclaims against plaintiffs in this judicial District and by consenting to this Court as a patent infringement defendant, *see, e.g.*, *Takeda Pharmaceutical Company Limited, et al. v. Mylan Inc., Mylan Pharmaceuticals Inc.*, Case No. 12-00026-AJS (W.D. Pa. answer and counterclaims filed Jan. 23, 2012).

C. Mylan GmbH

36. Upon information and belief, Mylan Inc., Mylan Pharmaceuticals Inc., Mylan GmbH, and Mylan N.V. hold themselves out as a unitary entity and represent to the public that their activities are directed, controlled, and carried out as a single entity.

37. Upon information and belief, Mylan GmbH collaborates with Mylan Inc., Mylan Pharmaceuticals Inc., and Mylan N.V. to develop, manufacture, seek approval for, and sell FDA-approved biopharmaceutical drugs, which are being marketed, distributed, and sold in Pennsylvania and in the United States.

38. Upon information and belief, Mylan GmbH operates as a subsidiary of Mylan N.V., which exercises considerable control over Mylan GmbH.

39. This Court has personal specific jurisdiction over Mylan GmbH because, upon information and belief, Mylan GmbH submitted the Mylan aBLA seeking approval from FDA to market and sell the Mylan Pegfilgrastim Product in the Commonwealth of Pennsylvania and throughout the United States, which directly gives rise to Plaintiffs' claims of patent infringement.

40. Further, upon information and belief, Mylan GmbH has or will directly or indirectly manufacture, import into the United States, and/or sell the Mylan Pegfilgrastim Product that is the subject of the infringement claim in this action in Pennsylvania and throughout the United States.

41. Additionally, upon information and belief, Mylan GmbH exercises considerable control over Mylan Inc. and Mylan Pharmaceuticals Inc. with respect to biosimilar products, and approves significant decisions of Mylan Inc. and Mylan Pharmaceuticals Inc. such as allowing Mylan Inc. and Mylan Pharmaceuticals Inc. to act as United States agents in connection with preparing and submitting the Mylan aBLA.

42. Additionally, and in the alternative, Plaintiffs allege that to the extent Mylan GmbH is not subject to the jurisdiction of the courts of general jurisdiction of the Commonwealth of Pennsylvania, Mylan GmbH likewise is not subject to the jurisdiction of the courts of general jurisdiction of any state, and accordingly is amenable to service of process based on its aggregate contacts with the United States, including but not limited to the above described contacts, as authorized by Rule 4(k)(2) of the Federal Rules of Civil Procedure.

D. Mylan N.V.

43. Upon information and belief, Mylan Inc., Mylan Pharmaceuticals Inc., Mylan GmbH, and Mylan N.V. hold themselves out as a unitary entity and represent to the public that their activities are directed, controlled, and carried out as a single entity.

44. Upon information and belief, Mylan N.V. collaborates with Mylan Inc., Mylan Pharmaceuticals Inc., and Mylan GmbH to develop, manufacture, seek approval for, and sell FDA-approved biopharmaceutical drugs, which are being marketed, distributed, and sold in Pennsylvania and in the United States.

45. Upon information and belief, Mylan GmbH operates as a subsidiary of Mylan N.V., which exercises considerable control over Mylan GmbH.

46. Mylan N.V. has issued at least one press release regarding the Mylan Pegfilgrastim Product and its regulatory status. *See* Press Release, Mylan N.V., "U.S. FDA Accepts Biologics License Application (BLA) for Mylan and Biocon's Proposed Biosimilar Pegfilgrastim for Review" (Feb. 16, 2017), http://newsroom.mylan.com/2017-02-16-U-S-FDA-Accepts-Biologics-License-Application-BLA-for-Mylan-and-Biocons-Proposed-Biosimilar-Pegfilgrastim-for-Review, attached hereto as Exhibit 1.

47. According to the Defendants' website (page attached hereto as Exhibit 2) "[t]he Chief Executive Officer and other executive officers of Mylan N.V. carry out the day-to-day conduct of Mylan N.V.'s worldwide businesses at the company's principal offices in Canonsburg, Pennsylvania."

48. This Court has personal jurisdiction over Mylan N.V. by virtue of, among other things, Mylan N.V. having its global headquarters and principal offices in Canonsburg, Pennsylvania; having availed itself of the rights and benefits of Pennsylvania law; and having engaged in substantial and continuing contacts with Pennsylvania.

49. Additionally, this Court has personal specific jurisdiction over Mylan N.V. because, upon information and belief, the acts of Mylan Inc., Mylan Pharmaceuticals Inc., and Mylan GmbH complained of herein were done, in part, for the benefit of Mylan N.V. Further,

upon information and belief, Mylan N.V. has or will directly or indirectly manufacture, import into the United States, and/or sell the Mylan Pegfilgrastim Product that is the subject of the infringement claim in this action in Pennsylvania and throughout the United States.

50. Additionally, upon information and belief, Mylan N.V. exercises considerable control over Mylan Inc. and Mylan Pharmaceuticals Inc. with respect to biosimilar products, and approves significant decisions of Mylan Inc. and Mylan Pharmaceuticals Inc. such as allowing Mylan Inc. and Mylan Pharmaceuticals Inc. to act as United States agents in connection with preparing and submitting the Mylan aBLA.

51. Additionally, and in the alternative, Plaintiffs allege that to the extent Mylan N.V. is not subject to the jurisdiction of the courts of general jurisdiction of the Commonwealth of Pennsylvania, Mylan N.V. likewise is not subject to the jurisdiction of the courts of general jurisdiction of any state, and accordingly is amenable to service of process based on its aggregate contacts with the United States, including but not limited to the above described contacts, as authorized by Rule 4(k)(2) of the Federal Rules of Civil Procedure.

BACKGROUND

A. Amgen's Innovative Biological Product: NEULASTA® (pegfilgrastim)

52. Amgen is one of the world's leading biopharmaceutical companies and is dedicated to using discoveries in human biology to invent, develop, manufacture, and sell new therapeutic products for the benefit of patients suffering from serious illnesses. Toward that end, Amgen has invested billions of dollars into its research and development efforts.

53. In 2002, Amgen introduced NEULASTA® (pegfilgrastim), an innovative biologic medicine which has benefited millions of cancer patients as a treatment of side effects of certain forms of cancer therapy. Amgen conducted extensive clinical trials and submitted the results of those trials to FDA in order to prove that NEULASTA® is safe, pure, and potent.

54. The active ingredient in Amgen's innovative NEULASTA® product is pegfilgrastim, a recombinantly expressed, 175-amino acid form of a protein known as human granulocyte-colony stimulating factor ("G-CSF") conjugated to a 20 kD monomethoxypolyethylene glycol (m-PEG) at the N-terminus of G-CSF.

55. NEULASTA® is indicated to decrease the incidence of infection in patients receiving myelosuppressive anti-cancer drugs. By binding to specific receptors on the surface of certain types of cells, NEULASTA® stimulates the production of a type of white blood cells known as neutrophils. Neutrophils are the most abundant type of white blood cells and form a vital part of the human immune system. A deficiency in neutrophils is known as neutropenia, a condition which makes the individual highly susceptible to infection. Neutropenia can result from a number of causes; it is a common side effect of chemotherapeutic drugs used to treat certain forms of cancer. NEULASTA® counteracts neutropenia.

56. NEULASTA® represented a major advance in cancer treatment by protecting chemotherapy patients from the harmful effects of neutropenia and by facilitating more effective chemotherapy regimens.

57. Prior to 2010, any other company wishing to sell its own version of NEULASTA® would have had to undertake the same extensive effort to conduct clinical trials to prove to FDA that its proposed version was also safe, pure, and potent.

58. Developing a new therapeutic product from scratch is extremely expensive: studies estimate the cost of obtaining FDA approval of a new biologic product at more than $2.5 billion. *See* DiMasi J.A. *et al.*, Innovation in the pharmaceutical industry: New estimates of R&D costs, 47 J. Health Econ. 20, 25-26 (2016), attached hereto as Exhibit 3.

B. Defendants Seek Approval To Market a Proposed Biosimilar Version of NEULASTA® (pegfilgrastim) by Taking Advantage of the Abbreviated Subsection (k) Pathway of the BPCIA

59. Upon information and belief, Mylan GmbH, acting in concert with the other Defendants, submitted the Mylan aBLA with FDA pursuant to Section 351(k) of the Public Health Service Act in order to obtain approval to commercially manufacture, use, offer to sell, and sell, and import into the United States the Mylan Pegfilgrastim Product, a biosimilar version of Plaintiffs' NEULASTA® (pegfilgrastim) product.

60. Upon information and belief, the Mylan aBLA references and relies on the approval and licensure of Plaintiffs' NEULASTA® (pegfilgrastim) product in support of Defendants' request for FDA approval.

61. Upon information and belief, the Mylan Pegfilgrastim Product is designed to copy and compete with Plaintiffs' NEULASTA® (pegfilgrastim).

62. Upon information and belief, Defendants did not seek to independently demonstrate to FDA that their biological product is "safe, pure, and potent" pursuant to 42 U.S.C. § 262(a), as Amgen did in its BLA for its innovative biological product NEULASTA® (pegfilgrastim). Rather, upon information and belief, Defendants requested that FDA evaluate the suitability of their biological product for licensure, expressly electing and seeking reliance on Amgen's FDA license for NEULASTA® (pegfilgrastim). Accordingly, Defendants submitted to FDA publicly available information regarding FDA's previous licensure determination that NEULASTA® (pegfilgrastim) is "safe, pure, and potent." 42 U.S.C. § 262(k)(2)(A)(iii)(I).

63. Defendants are piggybacking on the fruits of Plaintiffs' trailblazing efforts. Defendants have publicly announced that they submitted the Mylan aBLA under the subsection (k) pathway to obtain approval to commercially manufacture, use, offer to sell, and sell, and

import into the United States the Mylan Pegfilgrastim Product that they assert is a biosimilar version of Plaintiffs' NEULASTA®. *See* Press Release, Mylan N.V., "U.S. FDA Accepts Biologics License Application (BLA) for Mylan and Biocon's Proposed Biosimilar Pegfilgrastim for Review" (Feb. 16, 2017), http://newsroom.mylan.com/2017-02-16-U-S-FDA-Accepts-Biologics-License-Application-BLA-for-Mylan-and-Biocons-Proposed-Biosimilar-Pegfilgrastim-for-Review, attached hereto as Exhibit 1.

C. Information Exchange Under 42 U.S.C. § 262(*l*)

64. In March 2017, the exchange of information between Amgen and Mylan GbmH, as required by the BPCIA, began.

65. On March 2, 2017, pursuant to 42 U.S.C. § 262(*l*)(2)(A), Mylan GmbH provided Amgen's counsel with access to the Mylan aBLA.

66. Upon information and belief, the Mylan aBLA provided to Amgen was in a format different than and less complete than the format provided to FDA.

67. Upon information and belief, the Mylan aBLA was provided to FDA in Electronic Common Technical Document (eCTD) format with fully working hyperlinks and without restrictions on, inter alia, viewing, copying, and printing.

68. Mylan GmbH's failure to provide "a copy of the application submitted to the Secretary under subsection (k)" as required by 42 U.S.C. § 262(*l*)(2)(A) materially prejudiced and impeded Amgen's ability to review the Mylan aBLA. For example: Mylan GmbH uploaded the Mylan aBLA to a virtual data room (the "ShareVault data room") and provided Amgen's counsel with credentials to access the documents and data on the ShareVault data room. Mylan GmbH configured the ShareVault data room to prohibit Amgen from, inter alia, saving, copying, annotating, or printing any documents or data on the ShareVault data room. The ShareVault data room is also slow and cumbersome, and lacks fully working hyperlinks. In

addition, Amgen was and, in some cases, continues to be unable to view many of the documents and data on the ShareVault data room, including many of the xml, xsl, sas, xpt, jpeg, and txt files. Additionally, the ShareVault data room suffered periodic technological failures, preventing Amgen from accessing or viewing the documents and data on the ShareVault data room.

69. Mylan GmbH also failed to provide "other information that describes the process or processes used to manufacture the biological product that is the subject of" the Mylan aBLA, pursuant to 42 U.S.C. § 262(*l*)(2)(A). In April and May 2017, Amgen requested certain specific categories of documents that it believes exist and describe the Defendants' process for manufacturing the Mylan Pegfilgrastim Product. Mylan GmbH undertook to consider Amgen's request but, to date, has failed to provide such documents.

70. On May 1, 2017, Amgen provided Mylan GmbH with Amgen's list of patents under 42 U.S.C. § 262(*l*)(3)(A). That list included the '707 Patent and U.S. Patent No. 8,940,878 ("the '878 Patent"). On June 5, 2017, Mylan GmbH provided its detailed statement pursuant to 42 U.S.C. § 262(*l*)(3)(B) describing the factual and legal bases of Mylan GmbH's opinions that the '707 and '878 Patents are invalid, are unenforceable, or will not be infringed by the commercial marketing of the biological product that is the subject of the Mylan aBLA.

71. On June 7, 2017, pursuant to 42 U.S.C. § 262(*l*)(7) Amgen supplemented its 42 U.S.C. § 262(*l*)(3)(A) list to include the '997 Patent. On June 9, 2017, Mylan GmbH provided a detailed statement pursuant to 42 U.S.C. § 262(*l*)(7) describing the factual and legal bases of Mylan GmbH's opinions that the '997 Patent is invalid, is unenforceable, or will not be infringed by the commercial marketing of the biological product that is the subject of the Mylan aBLA.

72. On August 4, 2017, Amgen provided its detailed statement pursuant to 42 U.S.C. § 262(*l*)(3)(C) describing the factual and legal bases of Amgen's opinion that certain claims of the '707 and '878 Patents will be infringed by the commercial marketing of the biological product that is the subject of the Mylan aBLA, and Amgen's responses to the invalidity and unenforceability assertions against the '707 and '878 Patents in Mylan GmbH's statement under 42 U.S.C. § 262(*l*)(3)(B).

73. On August 8, 2017, Amgen provided Mylan GmbH with the factual and legal bases of Amgen's opinion that certain claims of the '997 will be infringed by the commercial marketing of the biological product that is the subject of the Mylan aBLA and responses to the invalidity and unenforceability assertions against the '997 Patent in Mylan GmbH's June 9, 2017 statement.

74. Amgen and Mylan GmbH then negotiated under 42 U.S.C. § 262(*l*)(4) as to "which, if any, patents listed under paragraph (3) by the subsection (k) applicant or the reference product sponsor shall be the subject of an action for patent infringement under paragraph (6)." Failing to reach agreement, Amgen and Mylan GmbH exchanged lists pursuant to the procedures of 42 U.S.C. § 262(*l*)(5) on August 25, 2017. Amgen asserted that there should be an immediate patent infringement action on the '707 and '997 Patents, but not on the '878 Patent.

75. Accordingly, Plaintiffs now file this immediate patent infringement action against Defendants pursuant to 42 U.S.C. § 262(*l*)(6)(B) on the '707 and '997 Patents. This action follows "not later than 30 days after the exchange of lists under paragraph (5)(B)."

THE PATENTS-IN-SUIT: U.S. PATENT NOS. 8,273,707 AND 9,643,997

76. Amgen is the owner of all rights, title, and interest in the '707 Patent.

77. AML is the exclusive licensee under the '707 Patent.

78. The ’707 Patent, titled “Process For Purifying Proteins,” was duly and legally issued on September 25, 2012 by the U.S. Patent and Trademark Office. A true and correct copy of the ’707 Patent is attached to this Complaint as Exhibit 4.

79. The ’707 Patent is directed to a process for purifying proteins.

80. Amgen is the owner of all rights, title, and interest in the ’997 Patent.

81. AML is the exclusive licensee under the ’997 Patent.

82. The ’997 Patent, titled “Capture Purification Processes for Proteins Expressed in a Non-Mammalian System,” was duly and legally issued on May 9, 2017 by the U.S. Patent and Trademark Office. A true and correct copy of the ’997 Patent is attached to this Complaint as Exhibit 5.

83. The ’997 Patent is directed to a process for purifying proteins.

CAUSES OF ACTION

FIRST COUNT:
INFRINGEMENT OF THE ’707 PATENT UNDER 35 U.S.C. § 271(e)(2)(C)(i)

84. Amgen incorporates by reference paragraphs 1-83 as if fully set forth herein.

85. Upon information and belief, Defendants seek FDA approval under Section 351(k) of the Public Health Service Act to engage in the commercial manufacture, use, or sale of the Mylan Pegfilgrastim Product, a proposed biosimilar version of Amgen’s NEULASTA® (pegfilgrastim) product.

86. Defendants committed an act of infringement with respect to the ’707 Patent under 35 U.S.C. § 271(e)(2)(C)(i) when they caused Mylan GmbH to submit the Mylan aBLA for the purpose of obtaining FDA approval to engage in the commercial manufacture, use, or sale of the Mylan Pegfilgrastim Product.

87. Upon information and belief, Defendants intend to manufacture, use, sell, and/or offer for sale within the United States, and/or import into the United States, the Mylan Pegfilgrastim Product before the expiration of the '707 Patent.

88. Upon information and belief, the manufacture, use, sale, and/or offer for sale within the United States, and/or the importation into the United States, of the Mylan Pegfilgrastim Product will infringe, literally or under the doctrine of equivalents, one or more claims of the '707 Patent.

89. Pursuant to 42 U.S.C. § 262(*l*)(3)(C), Amgen has provided Defendants with a detailed statement describing with respect to the '707 Patent, on a claim by claim basis, the factual and legal bases of Amgen's opinion that such patent will be infringed by the commercial marketing of the biological product that is the subject of the Mylan aBLA. Amgen's detailed statement includes, refers to, and relies on confidential information that Mylan GmbH provided to Amgen pursuant to 42 U.S.C. § 262(*l*)(2). Amgen does not repeat its detailed statement here because under 42 U.S.C. § 262(*l*)(1), Amgen is not permitted to include confidential information provided by Mylan GmbH "in any publicly-available complaint or other pleading." *See* 42 U.S.C. § 262(*l*)(1)(F).

90. Representative claim 1 of the '707 Patent recites:

> A process for purifying a protein on a hydrophobic interaction chromatography column such that the dynamic capacity of the column is increased for the protein comprising
>
> > mixing a preparation containing the protein with a combination of a first salt and a second salt,
> >
> > loading the mixture onto a hydrophobic interaction chromatography column, and
> >
> > eluting the protein,

> wherein the first and second salts are selected from the group consisting of citrate and sulfate, citrate and acetate, and sulfate and acetate, respectively, and wherein the concentration of each of the first salt and the second salt in the mixture is between about 0.1 M and about 1.0.

’707 Patent at col. 15:8-18. Upon information and belief, the process by which Defendants manufacture the Mylan Pegfilgrastim Product satisfies each limitation of at least claims 1, 2, 6, 8, 10, and 11, literally or equivalently. With respect to the requirement that the protein is purified on a hydrophobic interaction chromatography column, Defendants practice a process for purifying a protein on a hydrophobic interaction chromatography column as defined in the ’707 patent. With respect to the use of a combination of a first salt and a second salt, in the Defendants’ process, a preparation containing protein becomes mixed with a first salt and a second salt as recited in the claim. With respect to the salt concentration, the concentration of the salts in the Defendants’ process falls within the claimed range and/or is equivalent to a concentration within the claimed range. In the Defendants’ process, after the protein is loaded onto the hydrophobic interaction chromatography column in the presence of the combination of salts, the protein is eluted.

91. Plaintiffs will be irreparably harmed if Defendants are not enjoined from infringing the ’707 Patent. Amgen does not have an adequate remedy at law and is entitled to injunctive relief preventing Defendants from any further infringement under 35 U.S.C. § 271(e)(4)(B).

92. The manufacture, use, offer for sale, or sale within the United States, or importation into the United States, of the Mylan Pegfilgrastim Product before the expiration of the ’707 Patent will cause injury to Amgen, entitling it to damages or other monetary relief under 35 U.S.C. § 271(e)(4)(C).

SECOND COUNT:
DECLARATORY JUDGMENT OF INFRINGEMENT OF THE '707 PATENT UNDER 35 U.S.C. § 271(g)

93. Plaintiffs incorporate by reference paragraphs 1-92 as if fully set forth herein.

94. Upon information and belief, Defendants seek FDA approval under Section 351(k) of the Public Health Service Act to manufacture and sell the Mylan Pegfilgrastim Product, a biosimilar version of Amgen's NEULASTA® (pegfilgrastim) product.

95. Upon information and belief, FDA may act upon the Mylan aBLA as soon as October 2017. FDA has stated publicly that the agency's goal is to act upon 90% of aBLA applications within 10 months of the 60-day-filing-review period that begins on the date of FDA receipt of the original aBLA submission. *See* Biosimilar Biological Product Reauthorization Performance Goals and Procedures Fiscal Years 2018 Through 2022, *available at* https://www.fda.gov/downloads/forindustry/userfees/biosimilaruserfeeactbsufa/ucm521121.pdf.

96. Upon information and belief, Defendants intend to, and will upon FDA licensure of the Mylan aBLA, import into the United States or offer to sell, sell, or use within the United States the Mylan Pegfilgrastim Product, which will infringe one or more claims of the '707 Patent under 35 U.S.C. § 271(g).

97. An actual controversy has arisen and now exists between the parties concerning whether the Mylan Pegfilgrastim Product has or will infringe one or more claims of the '707 Patent.

98. Plaintiffs are entitled to a declaratory judgment that Defendants have infringed or will infringe one or more claims of the '707 Patent by making, using, offering to sell, or selling within the United States, or importing into the United States the Mylan Pegfilgrastim Product before the expiration of the '707 Patent.

99. Plaintiffs will be irreparably harmed if Defendants are not enjoined from infringing the ’707 Patent. Plaintiffs do not have an adequate remedy at law and are entitled to injunctive relief under 35 U.S.C. § 283 prohibiting Defendants from making, using, offering to sell, or selling within the United States, or importing into the United States the Mylan Pegfilgrastim Product before the expiration of the ’707 Patent.

100. Defendants’ manufacture, use, offer for sale, or sale within the United States, or importation into the United States, of the Mylan Pegfilgrastim Product before the expiration of the ’707 Patent will cause injury to Plaintiffs, entitling them to damages under 35 U.S.C. § 284.

THIRD COUNT: INFRINGEMENT OF THE ’997 PATENT UNDER 35 U.S.C. § 271(e)(2)(C)(i)

101. Amgen incorporates by reference paragraphs 1-100 as if fully set forth herein.

102. Upon information and belief, Defendants seek FDA approval under Section 351(k) of the Public Health Service Act to engage in the commercial manufacture, use, or sale of the Mylan Pegfilgrastim Product, a proposed biosimilar version of Amgen’s NEULASTA® (pegfilgrastim) product.

103. Defendants committed an act of infringement with respect to the ’997 Patent under 35 U.S.C. § 271(e)(2)(C)(i) when they caused Mylan GmbH to submit the Mylan aBLA for the purpose of obtaining FDA approval to engage in the commercial manufacture, use, or sale of the Mylan Pegfilgrastim Product.

104. Upon information and belief, Defendants intend to manufacture, use, sell, and/or offer for sale within the United States, and/or import into the United States, the Mylan Pegfilgrastim Product before the expiration of the ’997 Patent.

105. Upon information and belief, the manufacture, use, sale, and/or offer for sale within the United States, and/or the importation into the United States, of the Mylan

Pegfilgrastim Product will infringe, literally or under the doctrine of equivalents, one or more claims of the ’997 Patent.

106. Amgen has provided Defendants with a statement describing with respect to the ’997 Patent the factual and legal bases of Amgen’s opinion that such patent will be infringed by the commercial marketing of the biological product that is the subject of the Mylan aBLA. Amgen’s statement includes, refers to, and relies on confidential information that Mylan GmbH provided to Amgen pursuant to 42 U.S.C. § 262(*l*)(2). Amgen does not repeat its statement here because under 42 U.S.C. § 262(*l*)(1), Amgen is not permitted to include confidential information provided by Mylan GmbH “in any publicly-available complaint or other pleading.” *See* 42 U.S.C. § 262(*l*)(1)(F).

107. Representative claim 9 of the ’997 Patent recites:

> 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.

’997 Patent at col. 22:36-55. Upon information and belief, the process by which Defendants manufacture the Mylan Pegfilgrastim Product satisfies each limitation of at least independent claim 9 and also certain dependent claims, literally or equivalently. With respect to the

requirement that the protein is expressed in a non-native limited solubility form in a non-mammalian expression system, Defendants practice a process for purifying a protein expressed in a non-native limited solubility form in a non-mammalian expression system. With respect to the requirement of the "solubilizing" step, in the Defendants' process, protein is solubilized in a solubilization solution comprising one or more of a denaturant, reductant, and surfactant. With respect to the requirement of the "forming" step, in the Defendants' process, a refold solution is formed comprising the solubilization solution and a refold buffer, the refold buffer comprising one or more of a denaturant, aggregation suppressor, protein stabilizer, and redox component. With respect to the requirement of the "applying" step, the Defendants' refold solution is applied to a separation matrix under conditions suitable for the protein to associate with the matrix. With respect to the requirement of the "washing" step, the Defendants' separation matrix is washed. With respect to the requirement of the "eluting" step, Defendants' protein is eluted from the separation matrix.

108. Plaintiffs will be irreparably harmed if Defendants are not enjoined from infringing the '997 Patent. Amgen does not have an adequate remedy at law and is entitled to injunctive relief preventing Defendants from any further infringement under 35 U.S.C. § 271(e)(4)(B).

109. The manufacture, use, offer for sale, or sale within the United States, or importation into the United States, of the Mylan Pegfilgrastim Product before the expiration of the '997 Patent will cause injury to Amgen, entitling it to damages or other monetary relief under 35 U.S.C. § 271(e)(4)(C).

FOURTH COUNT: DECLARATORY JUDGMENT OF INFRINGEMENT OF THE '997 PATENT UNDER 35 U.S.C. § 271(g)

110. Plaintiffs incorporate by reference paragraphs 1-109 as if fully set forth herein.

111. Upon information and belief, Defendants seek FDA approval under Section 351(k) of the Public Health Service Act to manufacture and sell the Mylan Pegfilgrastim Product, a biosimilar version of Amgen's NEULASTA® (pegfilgrastim) product.

112. Upon information and belief, FDA may act upon the Mylan aBLA as soon as October 2017. FDA has stated publicly that the agency's goal is to act upon 90% of aBLA applications within 10 months of the 60-day-filing-review period that begins on the date of FDA receipt of the original aBLA submission. *See* Biosimilar Biological Product Reauthorization Performance Goals and Procedures Fiscal Years 2018 Through 2022, *available at* https://www.fda.gov/downloads/forindustry/userfees/biosimilaruserfeeactbsufa/ucm521121.pdf.

113. Upon information and belief, Defendants intend to, and will upon FDA licensure of the Mylan aBLA, import into the United States or offer to sell, sell, or use within the United States the Mylan Pegfilgrastim Product, which will infringe one or more claims of the '997 Patent under 35 U.S.C. § 271(g).

114. An actual controversy has arisen and now exists between the parties concerning whether the Mylan Pegfilgrastim Product has or will infringe one or more claims of the '997 Patent.

115. Plaintiffs are entitled to a declaratory judgment that Defendants have infringed or will infringe one or more claims of the '997 Patent by making, using, offering to sell, or selling within the United States, or importing into the United States the Mylan Pegfilgrastim Product before the expiration of the '997 Patent.

116. Plaintiffs will be irreparably harmed if Defendants are not enjoined from infringing the '997 Patent. Plaintiffs do not have an adequate remedy at law and are entitled to injunctive relief under 35 U.S.C. § 283 prohibiting Defendants from making, using, offering to

sell, or selling within the United States, or importing into the United States the Mylan Pegfilgrastim Product before the expiration of the ’997 Patent.

117. Defendants’ manufacture, use, offer for sale, or sale within the United States, or importation into the United States, of the Mylan Pegfilgrastim Product before the expiration of the ’997 Patent will cause injury to Plaintiffs, entitling them to damages under 35 U.S.C. § 284.

DEMAND FOR A JURY TRIAL

118. Plaintiffs hereby demand a jury trial on all issues so triable.

PRAYER FOR RELIEF

WHEREFORE, Plaintiffs respectfully request that this Court enter judgment in its favor against Defendants and grant the following relief:

A. a judgment that Defendants have infringed one or more claims of the ’707 Patent under 35 U.S.C. § 271(e)(2)(C)(i);

B. a judgment that Defendants have infringed or will infringe one or more claims of the ’707 Patent under 35 U.S.C. § 271(g);

C. a judgment that Defendants have infringed one or more claims of the ’997 Patent under 35 U.S.C. § 271(e)(2)(C)(i);

D. a judgment that Defendants have infringed or will infringe one or more claims of the ’997 Patent under 35 U.S.C. § 271(g);

E. a judgment compelling Defendants to pay to Plaintiffs damages or other monetary relief adequate to compensate for Defendants’ infringement, in accordance with 35 U.S.C. § 271(e)(4)(C) and 35 U.S.C. § 284;

F. an order enjoining Defendants, as well as all officers, employees, agents, representatives, affiliates, assignees, successors, and affiliates of Defendants, and all persons acting on behalf of or at the direction of, or in concert with Defendants, from infringing the ’707

Patent, or contributing to or inducing anyone to do the same, in accordance with 35 U.S.C. § 271(e)(4)(B) and 35 U.S.C. § 283;

G. an order enjoining Defendants, as well as all officers, employees, agents, representatives, affiliates, assignees, successors, and affiliates of Defendants, and all persons acting on behalf of or at the direction of, or in concert with Defendants, from infringing the '997 Patent, or contributing to or inducing anyone to do the same, in accordance with 35 U.S.C. § 271(e)(4)(B) and 35 U.S.C. § 283;

H. a declaration that this is an exceptional case and awarding to Plaintiffs their attorneys' fees and costs pursuant to 35 U.S.C. § 285, and expenses; andsuch other relief as this Court may deem just and proper.

Respectfully submitted,

THE WEBB LAW FIRM

Dated: September 22, 2017

s/ *Kent E. Baldauf, Jr.*

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Attorneys for Plaintiffs

CIVIL COVER SHEET

The JS 44 civil cover sheet and the information contained herein neither replace nor supplement the filing and service of pleadings or other papers as required by law, except as provided by local rules of court. This form, approved by the Judicial Conference of the United States in September 1974, is required for the use of the Clerk of Court for the purpose of initiating the civil docket sheet. *(SEE INSTRUCTIONS ON NEXT PAGE OF THIS FORM.)*

I. (a) PLAINTIFFS
Amgen Inc. and Amgen Manufacturing Limited

DEFENDANTS
Mylan Inc., Mylan Pharmaceuticals Inc., Mylan GmbH, and Mylan N.V.

(b) County of Residence of First Listed Plaintiff: Ventura County, CA
(EXCEPT IN U.S. PLAINTIFF CASES)

County of Residence of First Listed Defendant:
(IN U.S. PLAINTIFF CASES ONLY)
NOTE: IN LAND CONDEMNATION CASES, USE THE LOCATION OF THE TRACT OF LAND INVOLVED.

(c) Attorneys *(Firm Name, Address, and Telephone Number)*
Kent E. Baldauf, Jr., Thomas C. Wolski and Cecilia R. Dickson, The Webb Law Firm, One Gateway Center, 420 Ft. Duquesne Blvd., Suite 1200, Pittsburgh, PA 15222; 412.471.8815

Attorneys *(If Known)*

II. BASIS OF JURISDICTION *(Place an "X" in One Box Only)*

- ❒ 1 U.S. Government Plaintiff
- ☒ 3 Federal Question *(U.S. Government Not a Party)*
- ❒ 2 U.S. Government Defendant
- ❒ 4 Diversity *(Indicate Citizenship of Parties in Item III)*

III. CITIZENSHIP OF PRINCIPAL PARTIES *(Place an "X" in One Box for Plaintiff and One Box for Defendant)*
(For Diversity Cases Only)

	PTF	DEF		PTF	DEF
Citizen of This State	❒ 1	❒ 1	Incorporated *or* Principal Place of Business In This State	❒ 4	❒ 4
Citizen of Another State	❒ 2	❒ 2	Incorporated *and* Principal Place of Business In Another State	❒ 5	❒ 5
Citizen or Subject of a Foreign Country	❒ 3	❒ 3	Foreign Nation	❒ 6	❒ 6

IV. NATURE OF SUIT *(Place an "X" in One Box Only)*

Click here for: Nature of Suit Code Descriptions.

CONTRACT
- ❒ 110 Insurance
- ❒ 120 Marine
- ❒ 130 Miller Act
- ❒ 140 Negotiable Instrument
- ❒ 150 Recovery of Overpayment & Enforcement of Judgment
- ❒ 151 Medicare Act
- ❒ 152 Recovery of Defaulted Student Loans (Excludes Veterans)
- ❒ 153 Recovery of Overpayment of Veteran's Benefits
- ❒ 160 Stockholders' Suits
- ❒ 190 Other Contract
- ❒ 195 Contract Product Liability
- ❒ 196 Franchise

REAL PROPERTY
- ❒ 210 Land Condemnation
- ❒ 220 Foreclosure
- ❒ 230 Rent Lease & Ejectment
- ❒ 240 Torts to Land
- ❒ 245 Tort Product Liability
- ❒ 290 All Other Real Property

TORTS

PERSONAL INJURY
- ❒ 310 Airplane
- ❒ 315 Airplane Product Liability
- ❒ 320 Assault, Libel & Slander
- ❒ 330 Federal Employers' Liability
- ❒ 340 Marine
- ❒ 345 Marine Product Liability
- ❒ 350 Motor Vehicle
- ❒ 355 Motor Vehicle Product Liability
- ❒ 360 Other Personal Injury
- ❒ 362 Personal Injury - Medical Malpractice

PERSONAL INJURY
- ❒ 365 Personal Injury - Product Liability
- ❒ 367 Health Care/ Pharmaceutical Personal Injury Product Liability
- ❒ 368 Asbestos Personal Injury Product Liability

PERSONAL PROPERTY
- ❒ 370 Other Fraud
- ❒ 371 Truth in Lending
- ❒ 380 Other Personal Property Damage
- ❒ 385 Property Damage Product Liability

CIVIL RIGHTS
- ❒ 440 Other Civil Rights
- ❒ 441 Voting
- ❒ 442 Employment
- ❒ 443 Housing/ Accommodations
- ❒ 445 Amer. w/Disabilities - Employment
- ❒ 446 Amer. w/Disabilities - Other
- ❒ 448 Education

PRISONER PETITIONS

Habeas Corpus:
- ❒ 463 Alien Detainee
- ❒ 510 Motions to Vacate Sentence
- ❒ 530 General
- ❒ 535 Death Penalty

Other:
- ❒ 540 Mandamus & Other
- ❒ 550 Civil Rights
- ❒ 555 Prison Condition
- ❒ 560 Civil Detainee - Conditions of Confinement

FORFEITURE/PENALTY
- ❒ 625 Drug Related Seizure of Property 21 USC 881
- ❒ 690 Other

LABOR
- ❒ 710 Fair Labor Standards Act
- ❒ 720 Labor/Management Relations
- ❒ 740 Railway Labor Act
- ❒ 751 Family and Medical Leave Act
- ❒ 790 Other Labor Litigation
- ❒ 791 Employee Retirement Income Security Act

IMMIGRATION
- ❒ 462 Naturalization Application
- ❒ 465 Other Immigration Actions

BANKRUPTCY
- ❒ 422 Appeal 28 USC 158
- ❒ 423 Withdrawal 28 USC 157

PROPERTY RIGHTS
- ❒ 820 Copyrights
- ☒ 830 Patent
- ❒ 835 Patent - Abbreviated New Drug Application
- ❒ 840 Trademark

SOCIAL SECURITY
- ❒ 861 HIA (1395ff)
- ❒ 862 Black Lung (923)
- ❒ 863 DIWC/DIWW (405(g))
- ❒ 864 SSID Title XVI
- ❒ 865 RSI (405(g))

FEDERAL TAX SUITS
- ❒ 870 Taxes (U.S. Plaintiff or Defendant)
- ❒ 871 IRS—Third Party 26 USC 7609

OTHER STATUTES
- ❒ 375 False Claims Act
- ❒ 376 Qui Tam (31 USC 3729(a))
- ❒ 400 State Reapportionment
- ❒ 410 Antitrust
- ❒ 430 Banks and Banking
- ❒ 450 Commerce
- ❒ 460 Deportation
- ❒ 470 Racketeer Influenced and Corrupt Organizations
- ❒ 480 Consumer Credit
- ❒ 490 Cable/Sat TV
- ❒ 850 Securities/Commodities/ Exchange
- ❒ 890 Other Statutory Actions
- ❒ 891 Agricultural Acts
- ❒ 893 Environmental Matters
- ❒ 895 Freedom of Information Act
- ❒ 896 Arbitration
- ❒ 899 Administrative Procedure Act/Review or Appeal of Agency Decision
- ❒ 950 Constitutionality of State Statutes

V. ORIGIN *(Place an "X" in One Box Only)*

- ☒ 1 Original Proceeding
- ❒ 2 Removed from State Court
- ❒ 3 Remanded from Appellate Court
- ❒ 4 Reinstated or Reopened
- ❒ 5 Transferred from Another District *(specify)*
- ❒ 6 Multidistrict Litigation - Transfer
- ❒ 8 Multidistrict Litigation - Direct File

VI. CAUSE OF ACTION

Cite the U.S. Civil Statute under which you are filing ***(Do not cite jurisdictional statutes unless diversity)***:
35 U.S.C. § 271

Brief description of cause:
Patent Infringement

VII. REQUESTED IN COMPLAINT:

❒ CHECK IF THIS IS A **CLASS ACTION** UNDER RULE 23, F.R.Cv.P.

DEMAND $

CHECK YES only if demanded in complaint:
JURY DEMAND: ☒ Yes ❒ No

VIII. RELATED CASE(S) IF ANY

(See instructions): JUDGE Chief Judge Leonard P. Stark DOCKET NUMBER No. 17-546 (LPS)(CJB)

DATE: 09/22/2017

SIGNATURE OF ATTORNEY OF RECORD: s/ Kent E. Baldauf, Jr.

FOR OFFICE USE ONLY

RECEIPT # ______ AMOUNT ______ APPLYING IFP ______ JUDGE ______ MAG. JUDGE ______

JS 44A REVISED June, **2009**
IN THE UNITED STATES DISTRICT COURT FOR THE WESTERN DISTRICT OF PENNSYLVANIA
THIS CASE DESIGNATION SHEET MUST BE COMPLETED

PART A

This case belongs on the (○ Erie ○ Johnstown ◉ Pittsburgh) calendar.

1. **ERIE CALENDAR** - If cause of action arose in the counties of Crawford, Elk, Erie, Forest, McKean. Venang or Warren, OR any plaintiff or defendant resides in one of said counties.
2. **JOHNSTOWN CALENDAR** - If cause of action arose in the counties of Bedford, Blair, Cambria, Clearfield or Somerset OR any plaintiff or defendant resides in one of said counties.
3. Complete if on **ERIE CALENDAR**: I certify that the cause of action arose in__________ County and that the ____________________resides in_________________County.
4. Complete if on **JOHNSTOWN CALENDAR**: I certify that the cause of action arose in ________________County and that the____________resides in ________________County.

PART B (You are to check ONE of the following)

1. ◉ This case is related to Number 17-546 (LPS)(CJB) . Short Caption Amgen v. Coherus, No. 17-546 (D. Del.)
2. ○ This case is not related to a pending or terminated case.

DEFINITIONS OF RELATED CASES:
CIVIL: Civil cases are deemed related when a case filed relates to property included in another suit or involves the same issues of fact or it grows out of the same transactions as another suit or involves the validity or infringement of a patent involved in another suit EMINENT DOMAIN: Cases in contiguous closely located groups and in common ownership groups which will lend themselves to consolidation for trial shall be deemed related.
HABEAS CORPUS & CIVIL RIGHTS: All habeas corpus petitions filed by the same individual shall be deemed related. All pro se Civil Rights actions by the same individual shall be deemed related.

PARTC

I. CIVIL CATEGORY (Select the applicable category).

1. ○ Antitrust and Securities Act Cases
2. ○ Labor-Management Relations
3. ○ Habeas corpus
4. ○ Civil Rights
5. ◉ Patent, Copyright, and Trademark
6. ○ Eminent Domain
7. ○ All other federal question cases
8. ○ All personal and property damage tort cases, including maritime, FELA, Jones Act, Motor vehicle, products liability, assault, defamation, malicious prosecution, and false arrest
9. ○ Insurance indemnity, contract and other diversity cases.
10. ○ Government Collection Cases (shall include HEW Student Loans (Education), V A 0verpayment, Overpayment of Social Security, Enlistment Overpayment (Army, Navy, etc.), HUD Loans, GAO Loans (Misc. Types), Mortgage Foreclosures, SBA Loans, Civil Penalties and Coal Mine Penalty and Reclamation Fees.)

I certify that to the best of my knowledge the entries on this Case Designation Sheet are true and correct

s/ Kent E. Baldauf, Jr.

Date: 09/22/2017

ATTORNEY AT LAW

NOTE: ALL SECTIONS OF BOTH FORMS MUST BE COMPLETED BEFORE CASE CAN BE PROCESSED.

Exhibit 1

Case 2:17-cv-01238-DSC Document 1-2 Filed 09/22/17 Page 2 of 4

News

Select ▼

Print

U.S. FDA Accepts Biologics License Application (BLA) for Mylan and Biocon's Proposed Biosimilar Pegfilgrastim for Review

Second Successful BLA Filing of the Partnership in the U.S.

HERTFORDSHIRE, England, PITTSBURGH and BENGALURU, India, Feb. 16, 2017 /PRNewswire/ -- Mylan N.V. (NASDAQ, TASE: MYL) and Biocon Ltd. (BSE code: 532523, NSE: BIOCON) today announced that the U.S. Food and Drug Administration (FDA) has accepted Mylan's Biologics License Application (BLA) for MYL-1401H, a proposed biosimilar to Neulasta® (pegfilgrastim), for filing through the 351(k) pathway.

The proposed biosimilar to Neulasta is used to reduce the duration of neutropenia (low count of neutrophils, a type of white blood cells) and the incidence of fever associated with neutropenia in adult patients treated with chemotherapy in certain types of cancer.

The FDA goal date set under the Biosimilar User Fee Act (BsUFA) is Oct. 9, 2017.

Mylan President Rajiv Malik commented: "We're proud of the FDA acceptance of our BLA for proposed biosimilar pegfilgrastim. This is the second BLA accepted for review by FDA as part of the Mylan and Biocon partnership within the past two months. The milestone builds upon the acceptance of regulatory filings for proposed biosimilar pegfilgrastim in Europe, Australia, and Canada and reinforces our dedication and commitment to establishing a global platform for this product. Once approved, proposed biosimilar pegfilgrastim will complement Mylan's broad oncology portfolio focused on expanding access to more affordable treatments for multiple types of cancer."

Dr. Arun Chandavarkar, CEO and Joint Managing Director, Biocon, said: "We are extremely pleased with the regulatory progress of our biosimilars in the U.S. The FDA's acceptance for review of our second BLA for a proposed biosimilar developed by Biocon and Mylan is an outcome of our strong R&D and manufacturing capabilities. Once approved, our proposed biosimilar pegfilgrastim will provide a high quality alternative to branded pegfilgrastim (Neulasta®) for cancer patients during cytotoxic chemotherapy. It will expand our oncology portfolio and further enable us to fulfil our promise of making cancer-care affordable and accessible for patients across the globe."

About the Biocon and Mylan Partnership

Biocon and Mylan are exclusive partners on a broad portfolio of biosimilars and generic insulin analogs. The proposed biosimilar pegfilgrastim is one of the six biologic products co-developed by Mylan and

Case 2:17-cv-01235-DSC Document 1-2 Filed 09/22/17 Page 3 of 4

Biocon for the global marketplace. Mylan has exclusive commercialization rights for the proposed biosimilar pegfilgrastim in the U.S., Canada, Japan, Australia, New Zealand and in the European Union and European Free Trade Association countries. Biocon has co-exclusive commercialization rights with Mylan for the product in the rest of the world.

About Mylan

Mylan is a global pharmaceutical company committed to setting new standards in healthcare. Working together around the world to provide 7 billion people access to high quality medicine, we innovate to satisfy unmet needs; make reliability and service excellence a habit; do what's right, not what's easy; and impact the future through passionate global leadership. We offer a growing portfolio of more than 2,700 generic and branded pharmaceuticals, including antiretroviral therapies on which approximately 50% of people being treated for HIV/AIDS worldwide depend. We market our products in more than 165 countries and territories. Our global R&D and manufacturing platform includes more than 50 facilities, and we are one of the world's largest producers of active pharmaceutical ingredients. Every member of our more than 35,000-strong workforce is dedicated to creating better health for a better world, one person at a time. Learn more at mylan.com

About Biocon

Biocon Limited, publicly listed in 2004, (BSE code: 532523, NSE Id: BIOCON, ISIN Id: INE376G01013) is India's largest and fully-integrated, innovation-led biopharmaceutical company. As an emerging global biopharmaceutical enterprise serving customers in over 100 countries, it is committed to reduce therapy costs of chronic diseases like diabetes, cancer and autoimmune. Through innovative products and research services it is enabling access to affordable healthcare for patients, partners and healthcare systems across the globe. It has successfully developed and taken a range of Novel Biologics, Biosimilars, differentiated Small Molecules and affordable Recombinant Human Insulin and Analogs from 'Lab to Market'. Some of its key brands are INSUGEN® (rh-insulin), BASALOG® (Glargine), CANMAb™ (Trastuzumab), BIOMAb-EGFR™ (Nimotuzumab) and ALZUMAb™ (Itolizumab), a 'first in class' anti-CD6 monoclonal antibody. It has a rich pipeline of Biosimilars and Novel Biologics at various stages of development including Insulin Tregopil, a high potential oral insulin.

Forward-Looking Statements: Mylan

This press release includes statements that constitute "forward-looking statements," including with regard to the FDA goal date; that the FDA acceptance builds upon the acceptance of regulatory filings for proposed biosimilar pegfilgrastim in Canada, Europe and Australia and reinforces Mylan's dedication and commitment to establishing a global platform for the product; that once approved, proposed biosimilar pegfilgrastim will complement Mylan's broad oncology portfolio focused on expanding access to more affordable treatments for all stages of cancer; and that proposed biosimilar pegfilgrastim, once approved, will provide a high quality alternative to branded pegfilgrastim for cancer patients during cytotoxic chemotherapy. These statements are made pursuant to the safe harbor provisions of the Private Securities Litigation Reform Act of 1995. Because such statements inherently involve risks and uncertainties, actual future results may differ materially from those expressed or implied by such forward-looking statements. Factors that could cause or contribute to such differences include, but are not limited to: any changes in or difficulties with Mylan's or its partners' ability to develop, manufacture, and commercialize products; any regulatory, legal, or other impediments to Mylan's or its partners' ability to bring products to market; Mylan's and its partners' ability to protect intellectual property and preserve intellectual property rights; the effect of any changes in Mylan's or its partners' customer and supplier relationships and customer purchasing patterns; other changes in third-party relationships; the impact of competition; changes in the economic and financial conditions of the businesses of Mylan or its partners; the scope, timing, and outcome of any ongoing legal proceedings and the impact of any such proceedings on Mylan's or its partners' business; actions and decisions of healthcare and pharmaceutical regulators, and changes in healthcare and pharmaceutical laws and regulations, in the United States and abroad; risks associated with international operations; other uncertainties and matters beyond the control of management; and the other risks detailed in Mylan's filings with the Securities and Exchange Commission.

Mylan undertakes no obligation to update these statements for revisions or changes after the date of this release.

Forward-Looking Statements: Biocon

Certain statements in this release concerning our future growth prospects are forward-looking statements, which are subject to a number of risks, uncertainties and assumptions that could cause actual results to differ materially from those contemplated in such forward-looking statements. Important factors that could cause actual results to differ materially from our expectations include, amongst others general economic and business conditions in India, our ability to successfully implement our strategy, our research and development efforts, our growth and expansion plans and technological changes, changes in the value of the Rupee and other currency changes, changes in the Indian and international interest rates, change in laws and regulations that apply to the Indian and global biotechnology and pharmaceuticals industries, increasing competition in and the conditions of the Indian biotechnology and pharmaceuticals industries, changes in political conditions in India and changes in the foreign exchange control regulations in India. Neither our company, our directors, nor any of our affiliates, have any obligation to update or otherwise revise any statements reflecting circumstances arising after this date or to reflect the occurrence of underlying events, even if the underlying assumptions do not come to fruition.

SOURCE Mylan N.V.

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Exhibit 2

Company

Corporate Governance ▼

Corporate Governance

Mylan N.V. is a publicly traded company on the NASDAQ Global Select Market and incorporated under the laws of the Netherlands, as set forth in its Articles of Association.

The Chief Executive Officer and other executive officers of Mylan N.V. carry out the day-to-day conduct of Mylan N.V.'s worldwide businesses at the company's principal offices in Canonsburg, Pennsylvania. Duties and standards of conduct for Mylan employees, officers and directors are set forth in the company's Code of Business Conduct and Ethics, as well as in the Code of Ethics for the Chief Executive Officer, Chief Financial Officer and Corporate Controller.

Mylan N.V. is managed and controlled under the oversight of the company's board of directors in the United Kingdom, where the board generally meets. The board has established seven committees, each of which operates pursuant to a written charter. Each director is elected annually by the company's shareholders. Certain of the directors' duties, rights and responsibilities are detailed in the company's Articles of Association, Board Rules, and Corporate Governance Principles.

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Committee Charters

Other Governance Documents

Political Contribution & Trade Association Memberships Report

Code of Ethics for the Chief Executive Officer, Chief Financial Officer and Corporate Controller

Code of Business Conduct and Ethics

CA Supply Chains & UK Modern Slavery Acts Statement and Mylan's Supplier Code of Conduct

Terms and Conditions for Purchase Orders

U.S. Supplier Diversity

California Declaration

Exhibit 3

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Innovation in the pharmaceutical industry: New estimates of R&D costs☆

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ABSTRACT

The research and development costs of 106 randomly selected new drugs were obtained from a survey of 10 pharmaceutical firms. These data were used to estimate the average pre-tax cost of new drug and biologics development. The costs of compounds abandoned during testing were linked to the costs of compounds that obtained marketing approval. The estimated average out-of-pocket cost per approved new compound is $1395 million (2013 dollars). Capitalizing out-of-pocket costs to the point of marketing approval at a real discount rate of 10.5% yields a total pre-approval cost estimate of $2558 million (2013 dollars). When compared to the results of the previous study in this series, total capitalized costs were shown to have increased at an annual rate of 8.5% above general price inflation. Adding an estimate of post-approval R&D costs increases the cost estimate to $2870 million (2013 dollars).

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1. Introduction

We provide an updated assessment of the value of the resources expended by industry to discover and develop new drugs and biologics, and the extent to which these private sector costs have changed over time. The costs required to develop these new products clearly play a role in the incentives to invest in the innovative activities that can generate medical innovation. Our prior studies also have been used by other researchers, including government agencies, to analyze various policy questions (US Congressional Budget Office, 1998, 2006).

The full social costs of discovering and developing new compounds will include these private sector costs, but will also include government-funded and non-profit expenditures on basic and clinical research that can result in leads and targets which drug developers can explore. These additional costs can be substantial.[1] However, it is difficult to identify and measure non-private expenditures that can be linked to specific new therapies. Thus, we focus here on the private sector costs.

The methodological approach used in this paper follows that used for our previous studies, although we apply additional statistical tests to the data (Hansen, 1979; DiMasi et al., 1991, 1995a,b, 2003, 2004; DiMasi and Grabowski, 2007). Because the methodologies are consistent, we can confidently make comparisons of the results in this study to the estimates we found for the earlier studies, which covered earlier periods, to examine and illustrate trends

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[1] For example, for fiscal year 2013, the United States National Institutes of Health (NIH) spent nearly $30 billion on the activities that it funds (http://officeofbudget.od.nih.gov/pdfs/FY15/Approp%20%20History%20by%20IC%20through%20FY%202013.pdf).

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in development costs. These studies used compound-level data on the cost and timing of development for a random sample of new drugs first investigated in humans and annual company pharmaceutical R&D expenditures obtained through surveys of a number pharmaceutical firms.

We analyze private sector R&D activities as long-term investments. The industrial R&D process is marked by substantial financial risks, with expenditures incurred for many development projects that fail to result in a marketed product. Thus, our approach explicitly links the costs of unsuccessful projects to those that are successful in obtaining marketing approval from regulatory authorities. In addition, the pharmaceutical R&D process is very lengthy, often lasting a decade or more (DiMasi et al., 2003). This makes it essential to model accurately how development expenses are spread over time.

Given our focus on resource costs and how they have changed over time, we develop estimates of the average pre-tax cost of new drug development and compare them to estimates covering prior periods. We corroborated the basic R&D cost results in this study by examining the representativeness of our sample firms and our study data, and by incorporating a number of independently derived results and data relating to the industry and the drug development process into analyses that provide rough comparators for at least components of our cost results. The details of those analyses are provided in our online supplement.

The remainder of this paper is organized as follows. We briefly discuss the literature on pharmaceutical industry R&D costs since our 2003 study in Section 2. Section 3 briefly outlines the standard paradigm for the drug development process. In Section 4 we describe the survey sample data and the population from which they were drawn, and briefly outline the methodology used to derive full R&D cost estimates from data on various elements of the drug development process. We present base case pre- and post-marketing approval R&D cost estimates in Section 5. Sensitivity analyses are presented in Section 6. We describe the representativeness of our data, various approaches to validating our results, and responses to various critiques in Section 7. Finally, we summarize our findings in Section 8.

2. Previous studies of the cost of pharmaceutical innovation

Much of the literature on the cost of pharmaceutical innovation dating back decades has already been described by the authors in their previous two studies (DiMasi et al., 1991, 2003). The interested reader can find references and discussions about the prior research in those studies. The earliest studies often involved a case study of a single drug (typically without accounting for the cost of failed projects) or they analyzed aggregate data. We will focus here on studies and reports that have emerged since DiMasi et al. (2003) that involve the use of new data for at least some parts of the R&D process. The basic elements of these analyses are shown in Table 1.

Adams and Brantner (2006, 2010) sought to assess the validity of the results in DiMasi et al. (2003) with some alternative data. Specifically, in their 2006 article, they used a commercial pipeline database to separately estimate clinical approval and phase attrition rates, as well as phase development times.[2] They found a similar overall cost estimate ($868 million versus $802 million in year 2000 dollars).[3] The authors followed that study with another study that featured clinical phase out-of-pocket cost estimates derived from regressions based on publicly available data on company R&D expenditures (Adams and Brantner, 2010). They found a somewhat higher overall cost estimate ($1.2 billion in year 2000 dollars).[4]

In a paper authored by two of the authors of this study (DiMasi and Grabowski, 2007), we provided a first look at the costs of developing biotech products (specifically, recombinant proteins and monoclonal antibodies). The methodological approach was the same as that used for our studies of traditional drug development. We used some data from DiMasi et al. (2003) combined with new data on the costs of a set of biotech compounds from a single large biopharmaceutical company. Biotech drugs were observed to have a higher average clinical success rate than small molecule drugs, but this was largely offset by other cost components. We found that the full capitalized cost per approved new compound was similar for traditional and biotech development ($1.3 billion for biotech and $1.2 billion for traditional development in year 2005 dollars), after adjustments to compare similar periods for R&D expenditures.

The other studies shown in Table 1 are discussed in detail in the online supplement. One important finding emerging from the survey of cost studies in Table 1 is that clinical success rates are substantially lower for the studies focused on more recent periods. This observed trend is consistent with other analyses of success probabilities (DiMasi et al., 2010; DiMasi et al., 2013; Hay et al., 2014; Paul et al., 2010) and our analysis below. Average R&D (inflation-adjusted) cost estimates are also higher for studies focused on more recent periods, suggesting a growth in real R&D costs. While suggestive, these studies are not strictly comparable to our earlier analyses of R&D costs given methodological differences and data omissions that are discussed in the online supplement (Appendix A).

3. The new drug development process

The new drug development process need not follow a fixed pattern, but a standard paradigm has evolved that fits the process well in general. We have described the process in some detail in previous studies, and the FDA's website contains a schematic explaining the usual set of steps along the way from test tube to new compound approval (http://www.fda.gov/Drugs/DevelopmentApprovalProcess/SmallBusinessAssistance/ucm053131.htm). Marketing approval applications for investigational compounds submitted to the FDA for review by manufacturers are referred to as new drug applications (NDAs) or biologic license applications (BLAs), depending on the type of product.

In basic form, the paradigm portrays new drug discovery and development as proceeding along a sequence of phases and activities (some of which often overlap). Basic and applied research initiate the process with discovery programs that result in the synthesis or isolation of compounds that are tested in assays and animal models in preclinical development. We do not have the level

[2] For mean out-of-pocket phase costs, they used the estimates in DiMasi et al. (2003).

[3] The Adams and Brantner (2006) study used records in the pipeline database that were reported to have entered some clinical testing phase from 1989 to 2002. Thus, they did not follow the same set of drugs through time. The data for the commercial pipeline databases are also thin prior to the mid-1990s. The DiMasi et al. (2003) study covered new drugs that had first entered clinical testing anywhere in the world from 1983 to 1994 and followed the same set of drugs through time.

[4] However, the authors interpreted their estimate as a marginal, as opposed to an average, drug cost. The concept, though, of marginal cost has an unclear meaning here. With high fixed costs and a development process that varies by drug, it is difficult to understand what marginal pharmaceutical R&D cost means in this context. It seems that the relevant marginal concept here is marginal profitability. The marginally profitable drug could have a very high or a very low cost. What's more, marginal profitability may only have meaning at the firm, not the industry, level. The cost of a marginally profitable drug in the pipeline of a firm may be high for one firm and low for another firm.

Table 1
Prior studies and analyses of pharmaceutical R&D costs (2003–2012).

Study	Study period	Clinical success rate	Real cost of capital	Inflation adjustment	Cost estimate
DiMasi et al. (2003)	First-in-humans, 1983–1994	21.5%	11.0%	2000 dollars	$802 million
Adams and Brantner (2006)	First-in-humans, 1989–2002	24.0%	11.0%	2000 dollars	$868 million
Adams and Brantner (2010)	Company R&D expenditures, 1985–2001	24.0%	11.0%	2000 dollars	$1.2 billion
DiMasi and Grabowski (2007)	First-in-humans, 1990–2003 (large molecule)	30.2% (large molecule)	11.5%	2005 dollars	$1.2 billion
Gilbert et al. (2003)	2000–2002 (launch)	8.0%	NA	2003 dollars	$1.7 billion
O'Hagan and Farkas (2009)	2009 (launch)	NA	NA	2009 dollars	$2.2 billion
Paul et al. (2010)	≈2007	11.7%	11.0%	2008 dollars	$1.8 billion
Mestre-Ferrandiz et al. (2012)	In clinical development, 1997–1999	10.7%	11.0%	2011 dollars	$1.5 billion

of granularity to disaggregate R&D expenditure data into discovery and preclinical development testing costs, so for the purposes of this study, as in prior studies, discovery and preclinical development costs are grouped and referred to as pre-human costs.[5]

Clinical (human) testing typically proceeds through three successive, sometimes overlapping phases. Historically, human testing has often been initiated first outside the United States (DiMasi, 2001). For any of these clinical phases, pharmaceutical companies may pursue development of their investigational compounds in multiple indications prior to and/or after the initial indication approval.

4. Data and methods

Ten multinational pharmaceutical firms of varying sizes provided data through a confidential survey of their new drug and biologics R&D costs.[6] Data were collected on clinical phase expenditures and development phase times for a randomly selected sample of the investigational drugs and biologics of the firms participating in the survey.[7] The sample was taken from a Tufts Center for the Study of Drug Development (CSDD) database of the investigational compounds of top 50 firms. Tufts CSDD gathered information on the investigational compounds in development and their development status from commercial pipeline intelligence databases (*IMS R&D Focus* and *Thomson Reuters Cortellis* database [formerly the *IDdb3* database]), published company pipelines, clinicaltrials.gov, and web searches. Cost and time data were also collected for expenditures on the kind of animal testing that often occurs concurrently with clinical trials.[8] The compounds chosen were self-originated in the following sense. Their development from synthesis up to initial regulatory marketing approval was conducted under the auspices of the surveyed firm. This inclusion criterion is broader than it might at first seem since it includes compounds of firms that were acquired or merged with the survey firm during development and drugs that originated with the survey firm and were co-developed (and for which full cost data were available).[9] Licensed-in and co-developed compounds without partner clinical cost data were excluded because non-survey firms would have conducted significant portions of the R&D.[10]

We also collected data from the cost survey participants on their aggregate annual pharmaceutical R&D expenditures for the period 1990–2010. The firms reported on total annual R&D expenditures broken down by expenditures on self-originated new drugs, biologics, diagnostics, and vaccines. Data were also provided on annual R&D expenditures for licensed-in or otherwise acquired new drugs, and on already-approved drugs. Annual expenditures on self-originated new drugs were further decomposed into expenditures during the pre-human and clinical periods.

The survey firms accounted for 35% of both top 50 firm pharmaceutical sales and pharmaceutical R&D expenditures. Of the 106 investigational compounds included in the project dataset, 87 are small molecule chemical entities (including three synthetic peptides), and 19 are large molecule biologics (10 monoclonal antibodies and nine recombinant proteins). For ease of exposition, we will refer to all compounds below as new drugs, unless otherwise indicated. Initial human testing anywhere in the world for these compounds occurred during the period 1995–2007. Development costs were obtained through 2013.

We selected a stratified random sample of investigational compounds.[11] Stratification was based on the status of testing as of the end of 2013. Reported costs were weighted to reflect the development status of compounds in the population relative to those in the cost survey sample, so that knowledge of the distribution of development status in the population from which the sample was drawn was needed. The population is composed of all investigational compounds in the Tufts CSDD investigational drug database that met study criteria: the compounds were self-originated and first tested in humans anywhere in the world from 1995 to 2007. We found 1442 investigational drugs that met these criteria. Of these compounds, 103 (7.1%) have been approved for marketing, 13 (0.9%) had NDAs or BLAs that were submitted and are still active, 11 (0.8%) had NDAs or BLAs submitted but abandoned, 576 (39.9%) were abandoned in phase I, 19 (1.3%) were still active in phase I, 492 (34.1%) were abandoned in phase II, 84 (5.8%) were still active in phase II, 78 (5.4%) were abandoned in phase III, and 66 (4.6%) were still active in phase III. For both the population and the cost survey sample, we estimated approval and discontinuation shares for the active compounds by phase so that the population and sample distributions consisted of shares of compounds that were approved or discontinued in phase I, phase II, phase III, or regulatory review. The

[5] We capture out-of-pocket discovery costs with our data, but the pre-synthesis discovery period is highly variable with no clear starting point. For our analyses we began our representative discovery and development timeline at the point of compound synthesis or isolation. Thus, our estimates of time costs are somewhat conservative.

[6] Using pharmaceutical sales in 2006 to measure firm size, 5 of the survey firms are top 10 companies, 7 are top 25 firms, and 3 are outside the top 25 (*Pharmaceutical Executive*, May 2007).

[7] A copy of the survey instrument can be found in our online supplement (Appendix G).

[8] Long-term teratogenicity and carcinogenicity testing may be conducted after the initiation of clinical trials, and is often concurrent with phase I and phase II testing.

[9] The criterion also does not preclude situations in which the firm sponsors trials that are conducted by or in collaboration with a government agency, an individual or group in academia, a non-profit institute, or another firm.

[10] Large and mid-sized pharmaceutical firms much more often license-in than license-out new drug candidates. Firms that license-in compounds for further development pay for the perceived value of the prior R&D typically through up-front fees, development and regulatory milestone payments, and royalty fees if the compound should be approved for marketing. For a breakdown of new drugs and biologics approved in the United States in the 2000s by business arrangements among firms initiated during clinical development, see DiMasi et al. (2014).

[11] To ease the burden of reporting and increase the likelihood that firms would respond, we limited the number of compounds to be reported on to a maximum of 15 for any firm (with fewer compounds for smaller firms).

cost survey sample was purposely weighted toward compounds that lasted longer in development to increase the amount of information on drugs that reached late-stage clinical testing. Weights, determined as described above, were then applied to the compounds in the cost dataset so that the results would reflect the development status distribution for the population from which the sample was drawn.

Some firms were not able to provide full phase cost data for every new drug sampled. For example, phase I cost data were available for 97 of the 106 new drugs in the dataset (92%). Of the 82 compounds in the dataset that had entered phase II, cost data were available for 78 (95%). For phase III, cost data were available for 42 of the 43 compounds that entered the phase (98%). However, we had cost data for at least one phase for each of the 106 drugs in the sample. In aggregate, we had cost data for all phases entered for 94 of the 106 compounds (89%).[12] In addition, five compounds were still active in a phase at the time that data were reported. For these drugs it is likely that there will be some additional future costs for the drug's most recent phase. Thus, for this reason our cost estimates are likely to be somewhat conservative. However, given the small number of drugs in this category and the fact that the impact would be on only one phase for each of these drugs, our overall cost estimates are not likely to be substantially affected.

The methodology that we use to estimate development costs is the same as the approach used in our earlier studies (Hansen, 1979; DiMasi et al., 1991, 2003). We refer the reader to the earlier studies and to our online supplement (Appendix A) for details. The methodology results in a full risk-adjusted cost per approved new compound that also takes into account time costs. That is, we link the cost of compound failures to the cost of the successes (investigational compounds that attain regulatory marketing approval), and we utilize a representative time profile along with an industry cost of capital to monetize the cost of the delay between when R&D expenditures are incurred and when returns to the successes can first be realized (date of marketing approval). We refer to the sum of out-of-pocket cost (actual cash outlays) and time cost per approved new compound as the capitalized cost per approved new compound. The full capitalized cost estimate is built through a number of estimates of various components of the drug development process. These individual component estimates are interesting as objects of analysis in their own right, and we provide estimates for those components.

5. Base case R&D cost estimates

5.1. Out-of-pocket clinical cost per investigational drug

To determine expected costs, we need estimates of the clinical development risk profile. We examined the dataset of 1442 self-originated compounds of top 50 pharmaceutical firms described above and estimated the phase transition probabilities shown in Fig. 1. The overall probability of clinical success (i.e., the likelihood that a drug that enters clinical testing will eventually be approved) was estimated to be 11.83%. This success rate is substantially lower than the rate of 21.50% estimated for the previous study, but consistent with several recent studies of clinical success rates.[13] Such an increase in overall risk will contribute greatly to an increase in costs per approved new drug, other things equal.

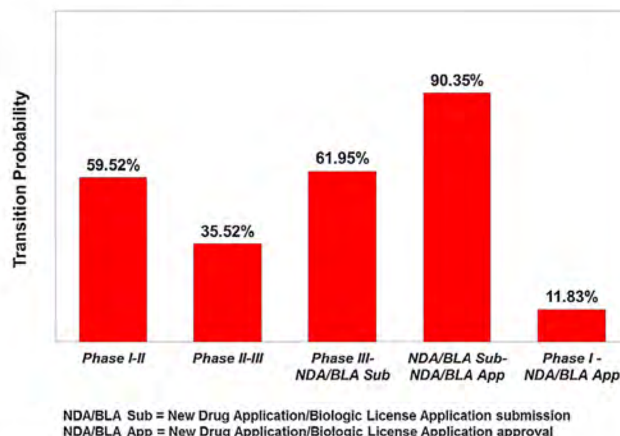

Fig. 1. Estimated phase transition probability and overall clinical approval success rates for self-originated new molecular entity (NME) and new therapeutically significant biologic entity (NBE) investigational compounds first tested in humans anywhere from 1995 to 2007.

As described above, we calculated weighted means, medians, standard deviations, and standard errors for clinical phase costs. Some of the firms could not separate out long-term animal testing costs during clinical development, and instead, included these costs in their phase cost estimates by year. To be consistent, therefore, for those compounds where animal costs were separately reported, we allocated those costs to the clinical phases according to when the animal testing costs were incurred. Thus, the clinical phase costs presented in Table 2 are inclusive of long-term animal testing costs.[14]

Weighted mean and median costs per investigational drug entering a phase[15] increase for later clinical phases, particularly for phase III (which typically includes a number of large-scale trials). In comparison to our previous study (DiMasi et al., 2003), both mean and median phase III cost are notably higher relative to the earlier phases. While the ratio of mean phase III cost to mean phase I cost was 5.7 for the previous study, it was 10.1 here. Similarly, the ratio of mean phase III to phase II cost was 3.7 for the earlier study, but was 4.4 for this study. Mean phase II cost was also higher relative to phase I cost in the current study compared to the previous one (2.3 times as high compared to 1.5 times as high).[16] Thus, while mean cost in real dollars for phase I increased 28% relative to the previous study,[17] phase I costs were notably lower relative to both phase II and phase III for the current study.

As we will see below, the differential in cost per approved new drug between the two studies will be much greater than cost per investigational drug because of the much lower overall clinical approval success rate. However, our results do show that the impact is mitigated to some degree by firms failing the drugs that they do abandon faster for the current study period. The distribution of clinical period failures for this study were 45.9% for phase I, 43.5% for phase II, and 10.6% for phase III/regulatory review. The

[12] Phase cost correlation results presented in the online supplement, together with an examination of relative phase costs for drugs that had some missing phase cost data, suggest that our phase cost averages (exclusive of missing data) are conservative.

[13] See, for example, Paul et al. (2010), DiMasi et al. (2013), and Hay et al. (2014).

[14] When animal testing costs occurred in a year during which costs were incurred for two clinical phases, the animal costs were allocated to the two phases according to their relative costs for the year.

[15] Averages for unweighted costs did not differ greatly from the weighted cost figures. On an unweighted basis, mean phase I, phase II, and phase III costs were $29.7 million, $64.7 million, and $253.5 million, respectively.

[16] The ratios for median costs for the current study are 11.6 for phase III relative to phase I, 4.5 for phase III relative to phase II, and 2.6 for phase II relative to phase I. The corresponding ratios for the previous study are 4.5, 3.6, and 1.2, respectively.

[17] In real terms, median phase I cost was actually 4% lower for the current study compared to the previous study.

Table 2
Average out-of-pocket clinical period costs for investigational compounds (in millions of 2013 dollars).[a]

Testing phase	Mean cost	Median cost	Standard deviation	Standard error	N^b	Probability of entering phase (%)	Expected cost
Phase I	25.3	17.3	29.6	3.0	97	100.0	25.3
Phase II	58.6	44.8	50.8	6.6	78	59.5	34.9
Phase III	255.4	200.0	153.3	34.1	42	21.1	54.0
Total							114.2

[a] All costs were deflated using the GDP implicit price deflator. Weighted values were used in calculating means, medians, and standard deviations.
[b] N = number of compounds with cost data for the phase.

Table 3
Nominal and real cost of capital (COC) for the pharmaceutical industry, 1994–2010.

	1994	2000	2005	2010
Nominal COC (%)	14.2	14.9	13.3	11.4
Inflation rate (%)	3.1	3.1	2.5	2.0
Real COC (%)	11.1	11.8	10.8	9.4

corresponding figures for the previous study were 36.9% for phase I, 50.4% for phase II, and 12.6% for phase III/regulatory review.

5.2. Cost of capital estimates

To account for the time value of money in our previous paper (DiMasi et al., 2003), we utilized an 11% real after-tax weighted average cost of capital (WACC). In particular, we employed the capital asset pricing model (CAPM) to estimate the cost of equity capital. This was combined with the cost of debt, appropriately weighted with the cost of equity, to yield a representative, pharmaceutical industry weighted after-tax cost of capital. The resultant parameters were estimated at regular intervals from the mid-1980s to the year 2000, given the time period spanned by our sample of R&D projects.

In the present paper, we follow the same methodology to compute WACC. In the current R&D cost analysis, we have a sample of new drugs that began clinical trials in 1995 through 2007 and which have an average introduction period in the latter part of the 2000 decade. Hence, a relevant time period for our cost of capital is the mid-1990s through 2010. Our analysis yielded an after-tax weighted cost of capital of 10.5%, moderately lower than in our last paper. This reflects the fact that the cost of equity capital has declined in pharmaceuticals since 2000 (as well as for other industrial sectors). Research intensive industries, including the pharmaceutical industry, generally finance most of their investments through equity, rather than through debt. This is the case even when the cost of debt is significantly below the cost of equity (Hall, 2002; Vernon, 2004). One of the primary reasons is that servicing debt requires a stable source of cash flows, while the returns to R&D activities are skewed and highly variable (Scherer and Harhoff, 2000; Berndt et al., 2015). Given the low debt-to-equity ratios that exist for pharmaceutical firms, the cost of equity component dominates the computed WACC values in Table 3.

To obtain a real cost of capital, we first compute the nominal values and then subtract the expected rate of inflation. The nominal cost of capital in 1994 is from a CAPM study by Myers and Howe (1997). The estimates for 2000, 2005, and 2010 are based on our own analysis, utilizing a comparable approach, with a large sample of pharmaceutical firms.[18] As this table shows, the estimated nominal cost of capital for pharmaceuticals was fairly stable during the period 1994–2000 (14.2–14.9%). However, it decreased during the decade of 2000s, particularly after the global recession occurred (with a value of 11.4% observed in 2010).

As discussed in DiMasi et al. (2003), the rate of inflation was above historical values during the first part of the 1980s, but then receded back to or below historical levels throughout most of the 1990s. Hence, we utilized the long run historical value for inflation for the expected inflation level in 1994 and 2000 (3.1%), as in our prior work. For the 2000s decade, inflation was significantly below historical values. In this case, we employed a 5-year lagged moving average to compute the expected rate of inflation in 2005 and 2010 (calculated as 2.5% and 2.0%, respectively).

As shown in Table 3, our estimates for the real cost of capital varied between 9.4% and 11.8% for pharmaceutical firms over the 1994–2010 period. We elected to use the midpoint of this range, or approximately 10.5%, as the representative COC to capitalize our R&D cost estimates.

The focus of our analysis is R&D investment expenditures and privately financed resources for new drugs undertaken by the biopharmaceutical industry. Accordingly we capitalized these expenditures utilizing a cost of capital estimate based on financial data from publicly listed firms. Drug development is also sponsored and funded by government and non-profit agencies (e.g., public–private partnerships devoted to developing medicines for neglected diseases). To the extent that our cost estimates are applicable to these ventures, a social rate of discount would be appropriate to capitalize R&D outlays. We provide a sensitivity analysis in Section 6 with respect to a wide spectrum of alternative cost of capital values.

5.3. Capitalized clinical cost per investigational drug

Opportunity cost calculations for clinical period expenditures require estimates of average phase lengths and average gaps or overlaps between successive clinical phases to generate an average clinical development and regulatory review timeline. Mean phase lengths and the mean lengths of time between successive phases are shown in Table 4, along with the associated capitalized mean phase costs and capitalized expected phase costs by phase for investigational compounds. The time between the start of clinical testing and submission of an NDA or BLA with the FDA was estimated to be 80.8 months, which is 12% longer (8.7 months) than the same period estimated for the previous study. The average time from the start of clinical testing to marketing approval for our timeline was 96.8 months for the current study, 7% (6.5 months) longer than for the earlier study. The difference is accounted for by shorter FDA approval times. The period for the previous study included, in part, a period prior to the implementation of the *Prescription Drug Use Fee Act of 1992* (PDUFA), and, in part, the early user fee era for which approval times were somewhat higher than for later user fee periods (Berndt et al., 2005).[19] While the approval

[18] The sample is composed of all publically traded drug firms in the *Value Line Survey* which also provides beta values and the other pharma-specific parameters used in the CAPM calculations for the relevant years. The long-term horizon equity risk premium, and the yield on long-term government bonds employed in the CAPM analysis, are from Ibbotson Valuation yearbooks for 2000, 2005, and 2010.

[19] The user fee legislation sunsets every 5 years. It has been renewed every 5 years since its original enactment. Performance goals for FDA review of marketing

Table 4
Average phase times and clinical period capitalized costs for investigational compounds (in millions of 2013 dollars).[a]

Testing phase	Mean phase length	Mean time to next phase	Capitalized mean phase cost[b,c]	Capitalized expected phase cost[b,c]
Phase I	33.1	19.8	49.6	49.6
Phase II	37.9	30.3	95.3	56.7
Phase III	45.1	30.7	314.0	66.4
Total				172.7

[a] All costs were deflated using the GDP implicit price deflator. Weighted values were used in calculating means for costs and phase times. Phase times are given in months.
[b] The NDA/BLA approval phase was estimated to be 16.0 months on average (2000–2012).
[c] Costs were capitalized at an 10.5% real discount rate.

phase averaged 18.2 months for the earlier paper's study period, that phase averaged 16.0 months for drugs covered by the current study.Other things being equal, the observed longer times from clinical testing to approval yielded higher capitalized costs relative to out-of-pocket costs. However, the discount rate that we used for the current study is also lower than for the previous study (10.5% versus 11.0%). The two effects work in offsetting ways. In addition, capitalized clinical cost per investigational compound will also depend on the gaps and overlaps between phases. On net, the ratio of mean capitalized to out-of-pocket cost per investigational compound was slightly lower for the current study compared to the previous one (1.5 versus 1.7).[20]

5.4. Clinical cost per approved new drug

Average cost estimates for investigational drugs are useful, but we are primarily interested in estimates of cost per approved new drug. As noted above, our analysis of drugs in development for the relevant period yielded a predicted overall clinical success rate of 11.83%. Applying this success rate to our estimates of out-of-pocket and capitalized costs per investigational drug results in estimates of cost per approved new drug that link the cost of drug failures to the successes.

Aggregating across phases, we found an out-of-pocket clinical period cost per approved new drug estimate of $965 million and a capitalized clinical period cost per approved new drug estimate of $1460 million. In constant dollars, these costs are 2.6 and 2.4 times higher than those we found in our previous study, respectively.

5.5. Pre-human out-of-pocket and capitalized costs per approved drug

The pre-human period, as defined here, includes discovery research as well as preclinical development. Some costs incurred during this period cannot be associated with specific compounds. To deal with this issue, we analyzed reported aggregate annual firm expenditures on self-originated new drugs by the pre-human and clinical periods. We gathered data on aggregate expenditures for these periods from survey firms for 1990–2010. Both times series tended to increase over time in real terms. Given this outcome, and the fact that the clinical expenditures in 1 year will be associated with pre-human expenditures that occurred years earlier, the ratio of total pre-human expenditures to total R&D (pre-human plus clinical) expenditures over the entire study period would yield an overestimate of the share of total cost per new drug that is accounted for by the pre-human period. To accurately estimate

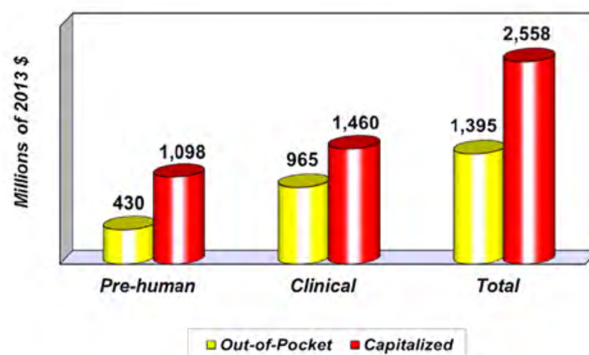

Fig. 2. Pre-human phase, clinical phase, and total out of-pocket and capitalized costs per approved new compound.

this share we built in a lag structure that associates pre-human expenditures with clinical expenditures incurred some time later.

The survey firms reported on dates of synthesis or isolation for compounds for which we sought cost data, as well as dates of first human testing. We had data for the period from synthesis to first human testing for 78 of the compounds. The average time from synthesis to initial human testing for these compounds was 31.2 months, down considerably from 52.0 months for the previous study.[21] Our analyses of clinical phase lengths and phase gaps and overlaps indicated a period of 95.2 months over which clinical period development costs are incurred. We approximated the lag between pre-human and clinical expenditures for a representative new drug as the time between the midpoints of each period. This yields a lag of 63.2 months, or approximately 5 years. Thus, we used a 5-year lag in analyzing the aggregate expenditure data, although we also examined 4-year and 6-year lags. A 5-year lag applied to the aggregate expenditure data resulted in a pre-human to total R&D expenditure ratio of 30.8%, which was only slightly different from the corresponding ratio used in our previous study (30.0%). The share was applied to our clinical cost estimates to determine associated pre-human cost estimates.

Given the estimates of out-of-pocket and capitalized clinical cost per approved new drug noted in Section 5.4 and the pre-human expenditure to total R&D expenditure ratio, we can infer pre-human out-of-pocket and capitalized costs per approved new drug of $430 million and $1098 million, respectively (Fig. 2). The results are very robust to different values for the length of the lag structure. For example, if we assume a lag of 4 years instead of 5 years, then out-of-pocket pre-human costs would be 6.8% higher. Alternatively, if we assume a 6-year lag, then out-of-pocket pre-human costs would be 8.5% lower.[22]

applications under PDUFA were tightened somewhat for some applications after the initial 5-year period.

[20] The differences in the ratios of capitalized to out-of-pocket cost for the individual phases were also small. For the current study they were 2.0, 1.6, and 1.2 for phase I, phase II, and phase III, respectively. For the earlier study, we found the ratios to be 2.0, 1.8, and 1.3 for phase I, phase II, and phase III, respectively.

[21] The results for the current study are consistent with data for a small number of compounds reported in a recently published study (Stergiopoulas and Getz, 2012). The mean time from synthesis to human testing there was 37.9 months for 17 compounds.

[22] The pre-human to total R&D expenditure ratios for four- and six-year lags were 32.2% and 28.9%, respectively.

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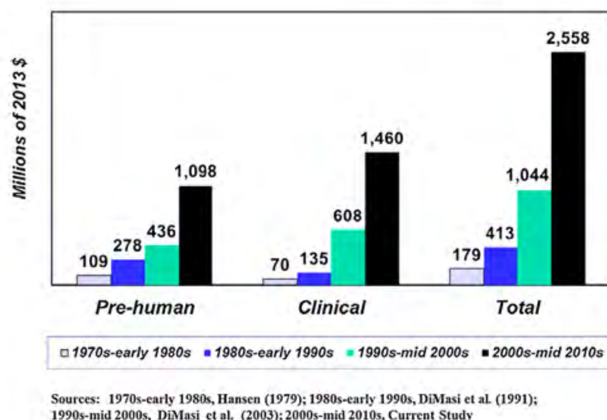

Fig. 3. Trends in capitalized pre-human, clinical and total cost per approved new drug.

5.6. Total capitalized cost per approved drug

Total cost estimates are the sum of pre-human and clinical period cost estimates. Our base case total out-of-pocket cost per approved new drug is $1395 million, while our fully capitalized total cost estimate is $2558 million (Fig. 2). Time costs (differences between capitalized cost and out-of-pocket cost) account for 45% of total cost. This share is down from the share in our previous study (50%) and that for the study that preceded it (51%). This is due in part to a shorter pre-human period and a lower discount rate.

5.7. Trends in R&D costs

Fig. 3 presents capitalized pre-human, clinical, and total cost per approved new drug for the previous three studies in this series and for our current study. In constant dollars, total capitalized cost increased 2.31 times for the second study in comparison to the first, 2.53 times for the third study in comparison to the second study, and 2.45 times for the current study in comparison to the third study. However, the samples for these studies include drugs that entered clinical testing over periods that are not uniformly distributed. In addition, while the samples were chosen on the basis of when drugs entered clinical testing, changes over time in the average length of the development process make ascribing differences in the study periods according to the year of first human testing problematic. An alternative is to determine an average approval date for drugs in each study's sample and use the differences in these dates to define the time differences between the studies. Our previous study described this approach and presented the corresponding annual growth rates between successive studies for the first three studies.

Drugs in the current study sample obtained FDA marketing approval from 2005 to 2013. The mean and median approval dates for drugs in the current study's sample were both in 2008. For the previous study, we reported that the average approval date was in 1997. Thus, we used 11 years as the relevant time span between the studies and calculated compound annual rates of growth between the two studies accordingly.

Using the period differences described here and in our previous study, we determined the compound annual growth rates between the studies for out-of-pocket and capitalized cost per approved drug for pre-human, clinical, and total costs (Table 5). Compared to the growth rate for the results in the previous study, the growth rates for total out-of-pocket and capitalized costs for the current study are somewhat higher (9.3% and 8.5% per year). The results for the current study in comparison to those for the previous study are also noteworthy in that, after a substantial decline in the growth rate for real pre-human costs described in the previous study and presented in Table 5, pre-human costs for the current study resumed a much higher rate of growth. Conversely, the growth rates for clinical period expenditures declined from the very high rates for the previous study, although they are still substantial.

5.8. Cost of post-approval R&D

As we did for our most recent study, we develop indirect estimates of post-approval R&D costs. Post-approval R&D consists of efforts subsequent to original marketing approval to develop the active ingredient for new indications and patient populations, new dosage forms and strengths, and to conduct post-approval (phase IV) research required by regulatory authorities as a condition of original approval. We follow the methodology that we used in previous study.[23] We utilize our pre-approval estimates together with aggregate pharmaceutical industry data regarding the drug development process to construct an estimate of the cost of post-approval R&D, which together with our pre-approval estimates, provide estimates of average total R&D cost per new drug covering the entire development and product life-cycle. The data that we collected from the survey firms on company annual aggregate expenditures on biopharmaceutical R&D show that over the study period these firms spent 73.1% of their prescription biopharmaceutical R&D expenditures on investigational self-originated new compounds,[24] 10.2% on investigational compounds that were licensed-in or otherwise acquired, and 16.5% on improvements to drugs that have already been approved.[25]

We cannot, however, use the percentage of aggregate R&D expenditures spent on post-approval R&D on a current basis and apply it to a pre-approval cost estimate to obtain an appropriate estimate of the cost of post-approval R&D per approved compound. The reason is that pre-approval costs occur years before post-approval costs. We used our aggregate annual firm R&D data to obtain an appropriate ratio by building in a reasonable lag structure between pre-approval and post-approval costs.

For our base results we used, as we did for the previous study, a 10-year lag for the aggregate data (which is the approximate time between median pre-approval development costs and median post-approval costs, given an 8-year post-approval expenditure period), we assumed that post-approval R&D cost per approval is the same, on average, for licensed-in and self-originated compounds, and we determined the percentage of approvals for the cost survey firms that are self-originated to estimate the ratio of post-approval R&D cost per approved compound to pre-approval cost per approved compound. The data indicated that this share was 33.4%. Applying this ratio, we estimated the out-of-pocket cost per approved compound for post-approval R&D to be $466 million (Fig. 4). Since these costs occur after approval and we are capitalizing all costs to the point of marketing approval, our discounted cost estimate is lower ($312 million). Thus, out-of-pocket cost per approved compound for post-approval R&D is 25.0% of

[23] We refer to the discussion in DiMasi et al. (2003) and an accompanying Appendix A for more detail on the method.

[24] This figure includes expenditures on biologics, vaccines, and diagnostics. The self-originated share for therapeutic investigational drugs and biologics was 71.2%.

[25] These expenditure shares are similar to those found for the previous study for the 1980 to 1999 period. The results here are also similar to figures that the trade association Pharmaceutical Research and Manufacturers of America (PhRMA) has published for its member firms for the years 2003 and 2005 to 2010. Those data do not separate out expenditures on existing products, but they do distinguish between self-originated and licensed products. Aggregating across those years, the shares for self-originated, licensed, and uncategorized were 74.3%, 17.6%, and 8.1%, respectively.

Table 5
Compound annual growth rates in out-of-pocket and capitalized inflation-adjusted costs per approved new drug.[a]

Approval periods	Out-of-pocket			Capitalized		
	Pre-human	Clinical	Total	Pre-human	Clinical	Total
1970s to 1980s	7.8%	6.1%	7.0%	10.6%	7.3%	9.4%
1980s to 1990s	2.3%	11.8%	7.6%	3.5%	12.2%	7.4%
1990s to early 2010s	9.6%	9.2%	9.3%	8.8%	8.3%	8.5%

[a] Costs for 1970s approvals are from Hansen (1979), costs for 1980s approvals are from DiMasi et al. (1991), costs for the 1990s to the early 2000s are from DiMasi et al. (2003), and costs for the 2000s to early 2010s are from the current study.

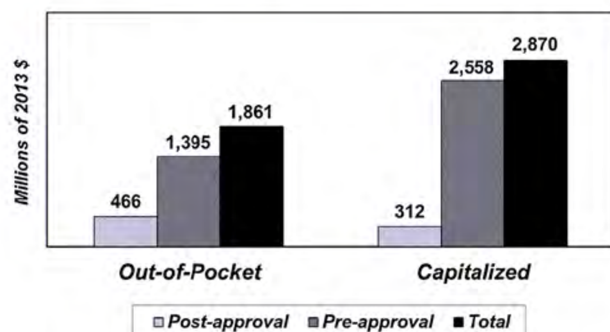

Fig. 4. Out-of-pocket and capitalized total cost per approved new drug for new drugs and for improvements to existing drugs.

Table 6
Capitalized pre-human, clinical, and total costs per approved new drug (in millions of 2013 dollars) by discount rate.

Discount rate	Pre-human	Clinical	Total
1.0%	472	1012	1476
2.0%	517	1044	1561
3.0%	567	1086	1653
4.0%	621	1129	1750
5.0%	679	1175	1854
6.0%	742	1222	1964
7.0%	811	1271	2082
8.0%	885	1322	2207
9.0%	965	1376	2341
10.0%	1052	1431	2483
11.0%	1145	1489	2634
12.0%	1246	1549	2795
13.0%	1355	1612	2967
14.0%	1473	1677	3150
15.0%	1600	1744	3344

total R&D cost (pre- and post-approval), while capitalized cost for post-approval R&D is 10.9% of total cost.

5.9. Extensions to the base case

We can extend the base case results on drug development costs prior to original approval in a number of interesting ways. The sample dataset includes information on compound-level costs for both chemical compounds (small molecules) and biologics (large molecules). As reported in the online supplement (Appendix B), we examined investigational compounds by molecule size for differences in individual clinical phase costs. Since the distributions of compounds across therapeutic classes differ for large and small molecules, we conducted a regression analysis of phase costs for investigational compounds for each of the three clinical phases, while controlling for molecules size and therapeutic class. Sample sizes were somewhat limited when cut by both sample size and therapeutic class, but we found statistically significant higher phase II costs for large molecules. However, we found that clinical approval success rates for large molecules are substantially higher than for small molecules. As a result, clinical period cost per approved compound was appreciably higher for small molecules, with the ratio of costs nearly the same as we had estimated in a previous paper for an earlier period (DiMasi and Grabowski, 2007). Compete results are given and discussed in the online supplement (Appendix B).

The base case results on full R&D costs link expenditures on drug failures to the costs of drugs that attain regulatory success. We can also estimate the clinical period cost of taking a successful drug all the way to approval by examining the data for just the approved drugs in the sample. Focusing on that subsample also allowed us to examine evidence on the costs for the more therapeutically significant drugs (according to what is known at the time of approval) by using an FDA prioritization system for reviewing drugs submitted to the agency for marketing approval. We found that clinical period costs were substantially higher for the approved compounds in the sample relative to our results for the sample as a whole, and that costs were lower (although not at a statistically significant level) for compounds that the FDA had designated for a priority review (compounds thought to represent a significant gain over existing therapy). These results are presented in full and discussed in the online supplement (Appendix B).

6. Sensitivity analysis

We examined how sensitive the results were to extreme values in the data and to changes in certain critical parameters. In particular, we focus in detail in this section on variation in the discount rate used to calculate capitalized costs. We also determine the extent to which key cost drivers (cash outlays, risks, time, and the cost of capital) explain the increase in total cost per approved drug found for this study relative to our previous study.

In addition, since all of the parameters are subject to sampling error, we conducted Monte Carlo simulations, reported on in detail in the online supplement (Appendix C), allowing all parameters to vary according to their sampling distributions (using Crystal Ball™ software). For the full capitalized pre-approval cost estimate, 80% of the simulation forecasts (set of 1000) varied between $2.3 billion and $2.8 billion. All of the forecasts varied between $1.9 billion and $3.2 billion.

Finally, we also conducted an outlier analysis to determine the impact of the most extreme values in the dataset. The results show that drugs with high and low costs have a fairly small impact on cost estimates. For example, if all cost data for the drugs with the highest and lowest aggregate clinical costs are dropped from the analysis, then the full capitalized cost estimate falls by only 3.0% (3.5% if only the drug with the highest aggregate cost is dropped). The online supplement (Appendix D) further describes in detail various outlier analyses, including those that examine results when a number of high and/or low values for each clinical phase are excluded even though no one drug has uniformly high or low values across all clinical phases.

6.1. Effects of variation in the discount rate

Table 6 shows how pre-human, clinical, and total capitalized costs would vary by discount rate at one percentage point intervals. The values for a zero percent discount rate are out-of-pocket costs. In the neighborhood of our base case discount rate (10.5%), clinical cost changed by approximately $30 million, pre-human cost changed by approximately $45 million, and total cost changed by approximately $75 million for every half of one percent shift in the discount rate. In our previous study, the base case discount rate was 11.0%. At an 11.0% discount rate, total capitalized cost here was $2634 million or 3% higher than our base case result. At more extreme values for the discount rate, Table 6 indicates that total capitalized cost with a 15% discount rate was $3334 million, or 30% higher than our base case result. Similarly, a 3% discount rate (a figure often used as a social discount rate) yielded a total capitalized cost per approved new drug of $1561 million, or 39% lower than the base case result.[26]

6.2. Impact of cost drivers

As noted in the previous section, the full cost estimate is a function of numerous parameters that interact in a non-linear (often multiplicative) manner. That makes it difficult to isolate the extent to which changes in individual parameters alone drive changes in total costs. However, we can get a sense for which parameters had the greatest impacts, in either direction, on the change in total R&D cost between the previous study and the current one by calculating what R&D costs would have been if only a single parameter (or a set of related parameters) had changed from what it was for the previous study to what we found it to be for the current study period.

Table 7 shows our results for these thought experiments for the major parameters categorized into four groupings (direct pre-human and clinical average phase cash outlays, technical risks, average development and approval times, and the cost of capital). The base result is total cost per approved new compound for the DiMasi et al. (2003) study in year 2013 dollars ($1044 million). The current study full cost estimate is 145% higher than the base result. That change reflects the cumulative effect of all parameter changes. For the table, we examined parameter-by-parameter changes from the parameter values for the DiMasi et al. (2003) study to those values found for the current study.

The largest impact on the change in costs between the studies was driven by changes in average out-of-pocket clinical phase costs, which resulted in an 82.5% increase in full cost.[27] Considering also the small difference between the studies in the estimated ratio of pre-human to clinical costs, the impact of the change in direct out-of-pocket phase costs was an increase in total cost of 85.5%. The increase in total cost was also driven to a substantial extent by much higher development risks. The overall clinical approval success rate declined from approximately one-in-five to approximately one-in-eight. That change alone accounts for a 57.3% increase in total cost. However, the impact of a lower clinical approval success rate was mitigated to a small extent by a shift in the distribution of failures to earlier in development. Taking both effects into account resulted in an increase in total cost of 47.3%. Changes in the development and approval timeline had a relatively small depressing effect on total cost. This impact was driven by a shorter pre-human testing phase and a shorter average approval phase. Average clinical development time increased modestly, and this had a relatively small impact on total cost. Overall, the effect of changes in the development and approval timeline was a 5.6% decrease in total cost. Finally, the small change in the cost of capital had a 3.1% depressing effect on total cost. The aggregation of the direct impacts across the four cost factor groupings accounted for a 124% increase in costs between the two studies. We attribute the residual increase (21%) to interaction effects.

Table 7
Impact on total capitalized cost per approved new drug due to changes in individual cost drivers (current study factor effect relative to prior study[a] cost).

Factor category	Factor (change to current study values)	Capitalized cost (millions of 2013 $)	Percentage change in cost
Direct cash outlays			
	Out-of-pocket clinical phase costs	1905	82.5%
	Pre-human/clinical cost ratio	1061	1.6%
	Overall out-of-pocket costs	1937	85.5%
Risk			
	Clinical approval success rate with prior study distribution of failures	1643	57.3%
	Distribution of failures with prior study clinical approval success rate	981	−6.0%
	Overall risk profile: clinical approval success rate plus distribution of failures	1538	47.3%
Time			
	Pre-human phase	993	−4.9%
	Clinical phase	1046	0.2%
	Regulatory review	1013	−3.0%
	Overall development timeline	985	−5.6%
Cost of capital			
	Discount rate	1012	−3.1%

[a] DiMasi et al. (2003). In 2013 dollars the capitalized cost per approved new drug for the prior study is $1044 million.

7. Critiques, sample representativeness, and validation

Our prior study results have been questioned on a number of methodological and data grounds (Angell, 2005; Goozner, 2004; Light and Warburton, 2005a,b; Love, 2003; Young and Surrusco, 2001). We have rebutted each of these criticisms in detail in a number of venues (e.g., DiMasi et al., 2004, 2005a,b). We review the critics' main arguments only briefly here.

Goozner (2004) and Angell (2005) reject opportunity cost calculations because they, in essence, deny that industrial pharmaceutical R&D expenditures can be viewed as investments at risk.[28] These points are addressed more fully in DiMasi et al. (2004). Clearly, industrial pharmaceutical R&D meets the criteria for being considered investments that have opportunity costs. In any event, an estimate with no opportunity costs is simply the out-of-pocket cost estimate.

[26] The appropriate social rate of discount for government backed expenditures has been analyzed and debated extensively in the economics literature. See for example, Moore et al., 2013 and Burgess and Zerbe, 2013. A standard reference in the cost-effectiveness literature (Gold et al., 1996) recommends 3% as the base case rate in comparing alternative medical therapies ("Therefore, we recommend that the base rate of 3% and an alternate rate of 5% be retained for a period of at least 10 years.", p.233).

[27] Given the methodology, higher out-of-pocket clinical phase costs also get associated with higher out-of-pocket pre-human phase costs.

[28] In the case of Goozner (2004), the claim is made that R&D expenditures are expenses rather than investments, because accountants have traditionally treated them as such for tax purposes (failing to recognize practical measurement problems underlying why this has been the practice, such as great uncertainty regarding future regulatory and commercial success). The basis offered for rejecting opportunity costs in Angell (2005, p.45) is simply the claim that pharmaceutical firms "have no choice but to spend money on R&D if they wish to be in the pharmaceutical business".

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A number of the critiques question how representative the data were for prior studies, whether tax deductions and credits must be included, and whether any FDA application for product marketing approval (as opposed to the active ingredient that is at the core of all such applications) should be taken as the unit of observation. As noted, we have addressed all of these issues in earlier publications as they relate to our prior studies. In this section we examine the representativeness of the survey firms and data used for this study, what the level of tax credits has been in relation to R&D expenditures in recent years, an analysis of molecules that have been approved for orphan drug indications recently, and we outline a variety of methods using independent data that can be used to validate our results (full details of the methods and analysis can be found in our online supplement).

7.1. Representativeness of the survey firm data

Questions about data representativeness should be framed in terms of the population from which the sample was selected. In particular, it is relevant to compare characteristics of the investigational drugs in our cost survey sample and for our cost survey firms generally to those of all drugs in our database of top 50 pharmaceutical firms, which is the relevant population.[29] This is the main focus of the analysis in this section.

Smaller research-oriented firms may have a comparative advantage in the discovery and pre-human stages because they often have scientific researchers with close ties to the basic research underlying new classes of therapies and technology platforms. Even if this is the case, the literature indicates that smaller firms also tend to have significantly higher costs of capital, especially when they are start-ups financed by venture firms. The literature also indicates that firms with larger R&D pipelines and greater R&D experience have a higher probability of success during the costly clinical stages of drug R&D. It is not evident, therefore, that the R&D costs for compounds originating in smaller firms, whether developed internally or in alliances would be systematically lower than those originating in mid-sized and large firms. We discuss what is known about R&D metrics for small firms in Appendix E of the online supplement.

As noted, the appropriate comparator dataset for our cost survey sample is the population of investigational compounds of the top 50 pharmaceutical firms over the relevant period. There are 1442 compounds in the top 50 firm database that met our study inclusion criteria. Of these, 510, or 35.4% belonged to nine of our 10 cost survey firms.[30] Thus, the cost survey sample ($n = 106$) constitutes 20.8% of the survey firm compounds and 7.4% of the population compounds.

We determined the therapeutic class distribution for the drugs in the larger dataset for the four largest therapeutic classes and one miscellaneous class (with a wide variety of drug types) for drugs in the dataset that met our study inclusion criteria and compared it to the therapeutic class distribution for our cost sample. The population shares for antineoplastic, cardiovascular, central nervous system (CNS), and systemic anti-infective drugs were 21.5%, 8.7%, 19.0%, and 8.5%, respectively. The corresponding shares for the cost survey sample were 19.8%, 9.4%, 24.5%, and 8.5%, respectively. We used a chi-squared goodness-of-fit test to compare the therapeutic class distributions for cost survey firm drugs and for the drugs of the entire set of 50 firms in the database, and found no statistically significant differences in the class shares ($\chi^2 = 2.4257$, df = 4).

We also examined the degree to which the top 50 firms in aggregate and the sample of cost survey firms agreed in terms of how molecule type (biologic versus small molecule) and the sourcing of compounds are distributed. For the set of top 50 firms, 14.6% of their self-originated investigational compounds over the study period are large molecules, compared to 13.7% for the survey firms ($p = 0.3933$). In terms of the share of investigational compounds for the study period that are self-originated (as broadly defined here), we found the share to be 74.1% for the cost survey firms and 71.1% for all top 50 firms ($p = 0.1039$).

Finally, we also examined the phase transition and overall approval success rates for the cost survey firms and compared them to the corresponding estimates for the larger dataset. The phase transition rates for just the cost survey firms were 58.0% for phase I to phase II, 36.0% for phase II to phase III, 58.2% for phase III to regulatory review, and 89.5% for regulatory review to approval. The corresponding figures for the population, as shown in Fig. 1, are 59.5%, 35.5%, 62.0%, and 90.4%. The overall clinical approval success rate for just the cost survey firms implied by the phase transition rates is 10.9%, which compares to 11.8% for the entire dataset.

7.2. Orphan drug development

Some past critiques have focused to some extent on orphan tax credits, which can provide incentives to develop some drugs for a class of indications. We examine the extent to which these tax credits and other tax issues are empirically significant in the context of drug development as a whole in the next section. Here we briefly discuss the nature of development of molecules that are approved for orphan indications and the distinction between costs for orphan drug indications and the full development costs for molecules with orphan drug indication approvals.

Compounds developed for orphan indications may well have lower clinical development costs for those indications, as trial sizes tend to be lower.[31] The share of U.S. original new drug approvals from 2000 to 2014 for drugs with an orphan indication was 27%, and has increased in relative terms over the last 3 years of that period.[32] The most recent approval experience aside, the share of approvals sponsored by the set of population firms (top 50) matches closely the historical average for all approvals from 1987 to 2010 (22% for top 50 firms versus 23% of all approvals).[33] The survey firms were nearly indistinguishable from the population non-survey firms by this metric (21% versus 23%).

[29] The data included in the top 50 firm dataset were curated primarily from information contained in two commercial investigational drug pipeline databases that are available after payment of subscription fees. Additional information was obtained from freely available web sites. See Section 4 above for a description of data sources.

[30] One of the participating firms was outside of the top 50.

[31] Drugs for these indications, with some notable exceptions, tend to garner lower sales given limited patient populations. This contention is supported by recent data analysis conducted by IMS Health (Divino et al., 2014). They found that sales in the United States for orphan indications varied from only 4.8% to 8.9% of total pharmaceutical sales over 2007–2013. The analysts also projected that growth in orphan drug expenditures would slow over 2014–2018.

[32] The result was calculated from information provided by the FDA on its website and included in a Tufts CSDD database of NME and therapeutically significant biologic approvals. The share of new drug approvals with orphan indications has increased very recently. The *Orphan Drug Act* was enacted in 1983, but it took several years for an appreciable number of such approvals to appear. From 1987 to 1999 the orphan drug share of all new drug approvals was 23%; the same share as for the 2000–2010 period. The orphan drug share was, however, unusually high for 2014 (41%), and above-average for 2011–2013 (approximately one-third of approvals).

[33] An FDA analysis of Center for Drug Evaluation and Research (CDER) marketing applications for NMEs and new biologics for 2006 to 2010 found that approximately one-third of the applications were sponsored by small firms, and that 75% of the applications for first-in-disease therapies for orphan indications came from small firms (Lesko, 2011). Such firms may find a low R&D cost orphan disease oriented strategy attractive, given that typical sales and operating profit levels may still be sufficient to increase their market valuations.

Table 8
Number of indications tested clinically prior to initial U.S. regulatory marketing approval for therapeutic compounds approved[a] in 2014 by orphan drug status.

	Mean	Median	Range	% multiple indications
Orphan (n = 17)	8.5	7.0	1–24	88%
Orphan cancer (n = 9)	10.9	9.0	1–24	89%
Non-orphan (n = 22)	2.7	2.0	1–7	73%
All approvals (n = 39)	5.3	3.0	1–24	79%

[a] Therapeutic new molecular entities (NMEs) and new biologic entities (NBEs) approved by the Center for Drug Evaluation and Research (CDER) of the United States Food and Drug Administration (FDA).

The cost survey sample contained two compounds that were approved originally for orphan indications.[34] The average clinical period cost for these two compounds was nearly the same as the average for all sample approved compounds (94% of the overall average). One of the compounds, though, was relatively low cost, while the other was relatively high cost. This may reflect the experience of molecules approved for orphan indications generally, as total molecule cost depends not only on the approved indication, but, critically, on the total number of indications (orphan and non-orphan) pursued.

To investigate this point further, we examined the development histories of all new therapeutic drugs and biologics approved in the United States in 2014. We studied the records for these compounds in two commercial pipeline database (*IMS R&D Focus* and *Cortellis*), as well as the clinicaltrials.gov website. Table 8 demonstrates that, even with a conservative notion of what constitutes different indications,[35] molecules approved for orphan indications were investigated in a substantial number of indications prior to original marketing approval. This was particularly true for compounds approved for treating orphan cancer indications, and, in general, the orphan drugs tended to be investigated in many more indications prior to approval than was the case for non-orphan compounds.

7.3. Taxes and R&D expenditures

As in our previous studies, the cost estimates presented here are pre-tax. Our objective was to measure the level of and trends in the private sector real resource costs of developing new drugs and biologics. As discussed in DiMasi et al. (2003), if one is calculating after-tax rates of return for R&D one would need to include the effect of taxes. Under current U.S. corporate income tax accounting practices, firms are able to deduct R&D expenses at the time they incur the costs. This is in contrast to many other investments, such as plants and equipment, which must be amortized and depreciated over a longer time period. This treatment reflects the difficulty of appropriately depreciating an intangible asset such as R&D. Later, when the company earns profits from the sales of approved pharmaceuticals it cannot depreciate the R&D investment for income tax purposes. The advantage for R&D investment over investment in plant and equipment is the timing of tax payments on net income. If one were calculating the rate of return on R&D investments one would need to take into account the tax implications. Making these adjustments is complicated by the fact that major firms operate in multiple tax jurisdictions.

In DiMasi et al. (2003) we also discussed several tax credits available in the United States to firms in the biopharmaceutical industry. In particular, we examined the Research & Experimentation tax credit for increasing qualified research expenditures, which we concluded had little impact on large multinational pharmaceutical firms.[36] Since then, the Qualifying Therapeutic Discovery Project tax credit was created as part of the Patient Protection and Affordable Care Act of 2010 (http://grants.nih.gov/grants/funding/QTDP_PIM/; accessed 14.08.14). However, it is quite restrictive in that it applies to discovery projects for small firms with a limit of $5 million per taxpayer. Recently, the U.S. Congress Joint Committee on Taxation (2013) estimated tax expenditures for fiscal years 2012–2017 for the credit for increasing research activities, the Qualifying Therapeutic Discovery Project tax credit, and the advantage from expensing, as opposed to amortizing, research and experimental expenditures to be, in aggregate, in the range of $10 billion to $12 billion per year for fiscal years 2012–2017 across all U.S. corporations engaged in research activities. It is not clear how much of this is accounted for by the biopharmaceutical industry.

We also examined in DiMasi et al. (2003) the impact of tax credits for orphan drug research, and found them to be quite small in relation to total R&D expenditures for large pharmaceutical firms. The reporting requirements for orphan drug credits are such that many companies do not take the credit. The major financial incentive of the orphan drug program appears to be the intellectual property protection that is created from the granting of 7 years of marketing exclusivity. With respect to the magnitude of orphan drug tax credits utilized in the United States, the U.S. Congress Joint Committee on Taxation (2013) estimated that expected tax credits for orphan drug research are fairly small at between $700 million and $1 billion per year from fiscal years 2012–2017.

To put these tax credits and tax advantages in perspective, Battelle and R&D Magazine's 2014 Global R&D Funding Forecast (http://www.battelle.org/docs/tpp/2014_global_rd_funding_forecast.pdf?sfvrsn=4; accessed 14.08.14) estimates that approximately $79 billion will be spent in the United States on R&D by the biopharmaceutical industry.[37] Some other countries also have a number of tax credit incentives in place for R&D. However, it seems unlikely that, in aggregate, their value in relation to R&D expenditures for the biopharmaceutical industry is disproportionately higher than is the case for the United States. The Battelle and R&D Magazine's prediction of global R&D spending by the biopharmaceutical industry is approximately $171 billion. In sum, in aggregate the value of R&D tax credits and the tax advantage of expensing versus amortizing R&D expenditures for the biopharmaceutical industry appear to be no more than one-sixth of total industry R&D expenditures (and perhaps significantly less than that).

7.4. Validation

We gathered publicly available data and performed a number of independent analyses on those data to corroborate our results. Details on methodology and data are provided in Appendix F of our online supplement. The validation efforts can be grouped into those

[34] Analyzing orphan drug status for investigational compounds is problematic because the designation may be granted at any point during the development process. Thus, some compounds that might have been granted orphan drug status can be abandoned before that would occur.

[35] Indications may be defined quite narrowly. We chose a broad definition that would limit the number of different indications pursued. Specifically, we considered all trials for the same disease and that applied to the same organ system as testing on the same indication. For example, oncology compounds may be tested as first-line treatment, second-line treatment, for refractory patients, as a monotherapy, in combination with other compounds, or for special patient populations. These cases were considered to be the same indication if they applied to the same organ (e.g., breast cancer or prostate cancer).

[36] The impact may be greater for small firms if their R&D expenditures are growing more rapidly.

[37] The report estimates that the industrial life sciences sector will spend $92.6 billion on R&D in the United States in 2014. However, the report also indicates that approximately 85% of all life sciences industrial expenditures are accounted for by the biopharmaceutical industry.

that utilize micro data on elements of the development process that are then used to develop growth rate estimates for portions of the process, and those that use publicly available aggregate financial time series data and compound approval statistics for biopharmaceutical firms as a check on our estimate of overall cost.

On a micro level, we examined survey data from the National Science Foundation (NSF), published estimates of trends in clinical trial complexity and clinical trial costs per subject, and published trade association times series data on R&D employment levels. Utilizing external data on costs per subject, along with clinical trial sizes and estimated clinical approval success rates from our analyses over time, we found a compound annual growth rate in real clinical trial costs between the study periods for our previous study and the current study of 9.9%, which is close to our clinical period cost growth rate of 9.2% for out-of-pocket costs shown in Table 5. We also examined measures of clinical trial complexity (number of procedures per trial) in the published literature (Getz et al., 2008; PAREXEL, 2005) and found a compound annual growth rate of 10.0% over our study period. Finally, we utilized trade association and 10-K information on R&D scientific and professional staff employment levels and NSF data on salary levels to estimate that labor costs increased at a rate of 8–9% per year across our study periods.

We examined PhRMA time series data on the R&D expenditures of its member firms. The reported growth rate for cost survey firms was 4.9%, compared to 4.2% for the PhRMA time series data for the portion of the survey period that could be compared.[38] We also used the industry time series data, as we had in the previous study, in two ways to get a sense for the magnitude of overall costs per approved new molecule. In one approach, we estimated the portion of the reported time series expenditure levels that could be attributed to self-originated compound development. Next we determined the annual number of approvals of PhRMA-member firms that were self-originated. Finally, we used our study estimated time-expenditure profile to link aggregate R&D expenditures to approvals. For reasons expounded upon in the supplement, this will likely yield an upper bound estimate. Using this approach we found our out-of-pocket cost per approved molecule estimate to be 56% of the estimate derived from aggregate published industry data. The second approach focuses on the published industry self-originated R&D expenditure level for a single year, assumes that every self-originated member-firm approval (inclusive of failures) costs what we found to be our average out out-of-pocket cost estimate, and uses our estimated time-expenditure profile to spread costs out over time to explain reported total R&D expenditures for the year considered. As with the previous method, the outcome would be problematic if using our average out-of-pocket cost estimate explained more than the reported aggregate R&D expenditure level. We found that this approach explained 57% of the reported expenditures.

Company total biopharmaceutical R&D expenditures reported for the cost survey are consistent with the audited financial statements of the firms in that the annual values are equal to or lower than company R&D expenses found in the financial statements.[39] As another check on our overall results, we examined what survey company total biopharmaceutical R&D expenditures would be given our estimate of out-of-pocket cost per approved molecule and assuming that entry rates to survey company pipelines are in a steady state. That figure can then be compared to R&D expenditure levels reported for these firms for our cost survey (which, as noted, match audited financial statements). Full details of these calculations are in Appendix F of the supplement. Depending on assumptions, we found that we could account for between 51% and 94% of the reported total annual biopharmaceutical R&D expenditures in this way. Thus, all three approaches using aggregate R&D expenditure data suggest that our estimate of out-of-pocket cost per approved molecule is, if anything, conservative.

8. Conclusions

Studies of the cost of developing new drugs have long been of substantial interest to drug developers, drug regulators, policy makers, and scholars interested in the structure and productivity of the pharmaceutical industry and its contributions to social welfare. The interest has been strong and growing over the last few decades during which cost containment pressures for drugs approved for marketing have expanded and concerns have been raised about industry productivity in an environment in which industry structure has been evolving (Munos, 2009; Pammolli et al., 2011). The changing industrial landscape has featured consolidation among large firms, growing alliances among firms of all sizes, and the growth of a small firm sector.

We have conducted the fourth in a series of comprehensive compound-based analyses of the costs of new drug development. In the last study we reported average out-of-pocket and capitalized R&D costs of $403 million and $802 million in 2000 dollars ($524 million and $1044 million in 2013 dollars), respectively. For our updated analysis, we estimated total out-of-pocket and capitalized R&D cost per new drug to be $1395 million and $2558 million in 2013 dollars, respectively. To examine R&D costs over the entire product and development lifecycle, we also estimated R&D costs incurred after initial approval. This increased out-of-pocket cost per approved drug to $1861 million and capitalized cost to $2870 million. We validated our results in a variety of ways through analyses of independently derived published data on the pharmaceutical industry.

Our pre-approval out-of-pocket cost estimate is a 166% increase in real dollars over what we found in our previous study, and our capitalized cost estimate is 145% higher. Roughly speaking, the current study covers R&D costs that yielded approvals, for the most part, during the 2000s and early 2010s. Our previous study (DiMasi et al., 2003) generally involved R&D that resulted in 1990s approvals. The compound annual rates of growth in total real out-of-pocket and capitalized costs between the studies are 9.3% and 8.5%, respectively. These growth rates are both somewhat higher than those we found for the two previous studies (7.6% and 7.4%, respectively). Growth in out-of-pocket clinical period costs have moderated some from the 1990s, but the growth rate is still high at 9.2%. While the compound annual growth rate for out-of-pocket pre-human costs declined substantially for the previous study (from 7.8% to 2.3%), this study showed a substantially higher growth rate for pre-human costs in the new century (9.6%).

The success rate found for this study is nearly 10 percentage points lower than for the previous study. The overall change in the risk profile for new drug development by itself still accounted directly for a 47% increase in costs. It is difficult to know definitively why failure rates have increased, but a number of hypotheses worthy of testing come to mind. One possibility is that regulators have become more risk averse over time, especially in the wake of high profile safety failures for drugs that have reached the marketplace (most notably, Vioxx™, but there have been others as well). It may also be the case that the industry has generally focused more in areas where the science is difficult and failure risks are high as a result (Pammolli et al., 2011). Finally, the substantial growth in identified drug targets, many of which may be poorly validated, may have encouraged firms to pursue clinical development of more

[38] As explained in the Supplement, the growth rate for the PhRMA time series may somewhat underestimate the true growth rate.

[39] Biopharmaceutical R&D expenditures may be less than total company R&D expenditures if the firm engages in non-biopharmaceutical R&D.

compounds with an unclear likelihood of success than they otherwise would.

As can be seen from results cited in the supplement developed external to this study, as well as our own data, out-of-pocket clinical cost increases can be driven by a number of factors, including increasing clinical trial complexity (Getz et al., 2008), larger clinical trial sizes, inflation in the cost of inputs taken from the medical sector that are used for development, and possibly changes in protocol design to include efforts to gather health technology assessment information and, relatedly, testing on comparator drugs to accommodate payer demands for comparative effectiveness data. The expansion of the scope of the clinical trial enterprise during our study period is illustrated by the finding in Getz and Kaitin (2015) that for a typical phase III trial information had been gathered by sponsors on nearly 500,000 data points in 2002, but more than 900,000 data points in 2012.

Finally, it is difficult to assess whether and how regulatory burdens may have impacted changes in industry R&D costs over time. However, occasionally, an exogenous shift in the types and amount of information perceived as necessary for regulatory approval for particular classes of drugs can be instructive. For example, during our study period the FDA issued guidance (Food and Drug Administration, 2008) for the development of drugs to treat diabetes in late 2008 that highlighted a need to better assess and characterize cardiovascular risks for this class of compounds, after a number of cardiovascular concerns emerged regarding a previously approved drug (Avandia®). A number of development metrics positively related to R&D costs can be examined pre- and post-guidance. DiMasi (2015), for example, found that average U.S. clinical development times increased from 4.7 to 6.7 years for diabetes drugs approved in the United States from 2000–2008 to 2009–2014, respectively. In addition, Viereck and Boudes (2011) found that the number of randomized patients and patient-years in NDAs for diabetes drugs approved from 2005 to 2010 increased more than 2.5 and 4.0 times, respectively, before and after the guidelines were issued. Our sample data show that diabetes drugs were among the most costly (particularly for phase III [92% higher than the overall average]).

Our analysis of cost drivers indicates that the rate of increase observed in the current study was driven mainly by increases in the real out-of-pocket costs of development for individual drugs and by much higher failure rates for drugs that are tested in human subjects, but not particularly by changes in development times or the cost-of-capital. Continued analysis of the productivity of biopharmaceutical R&D should remain an important research objective.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhealeco.2016.01.012.

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Exhibit 4

US008273707B2

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

(10) **Patent No.: US 8,273,707 B2**
(45) **Date of Patent: Sep. 25, 2012**

(54) **PROCESS FOR PURIFYING PROTEINS**

(75) Inventors: **Anna Senczuk**, Shoreline, WA (US); **Ralph Klinke**, Sammamish, WA (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 55 days.

(21) Appl. No.: **12/822,072**

(22) Filed: **Jun. 23, 2010**

(65) **Prior Publication Data**

US 2010/0311953 A1 Dec. 9, 2010

Related U.S. Application Data

(62) Division of application No. 10/895,581, filed on Jul. 21, 2004, now Pat. No. 7,781,395.

(60) Provisional application No. 60/540,587, filed on Jan. 30, 2004.

(51) **Int. Cl.**
C07K 1/16 (2006.01)

(52) **U.S. Cl.** **514/1.1**; 530/387.1; 530/417

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

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(57) **ABSTRACT**

The invention relates to a process for purifying a protein by mixing a protein preparation with a solution having a first salt and a second salt, wherein each salt has a different lyotropic value, and loading the mixture onto a hydrophobic interaction chromatography column. The dynamic capacity of the column for a protein using the two salt combination will be increased compared with the dynamic capacity of the column for either single salt alone.

13 Claims, 5 Drawing Sheets

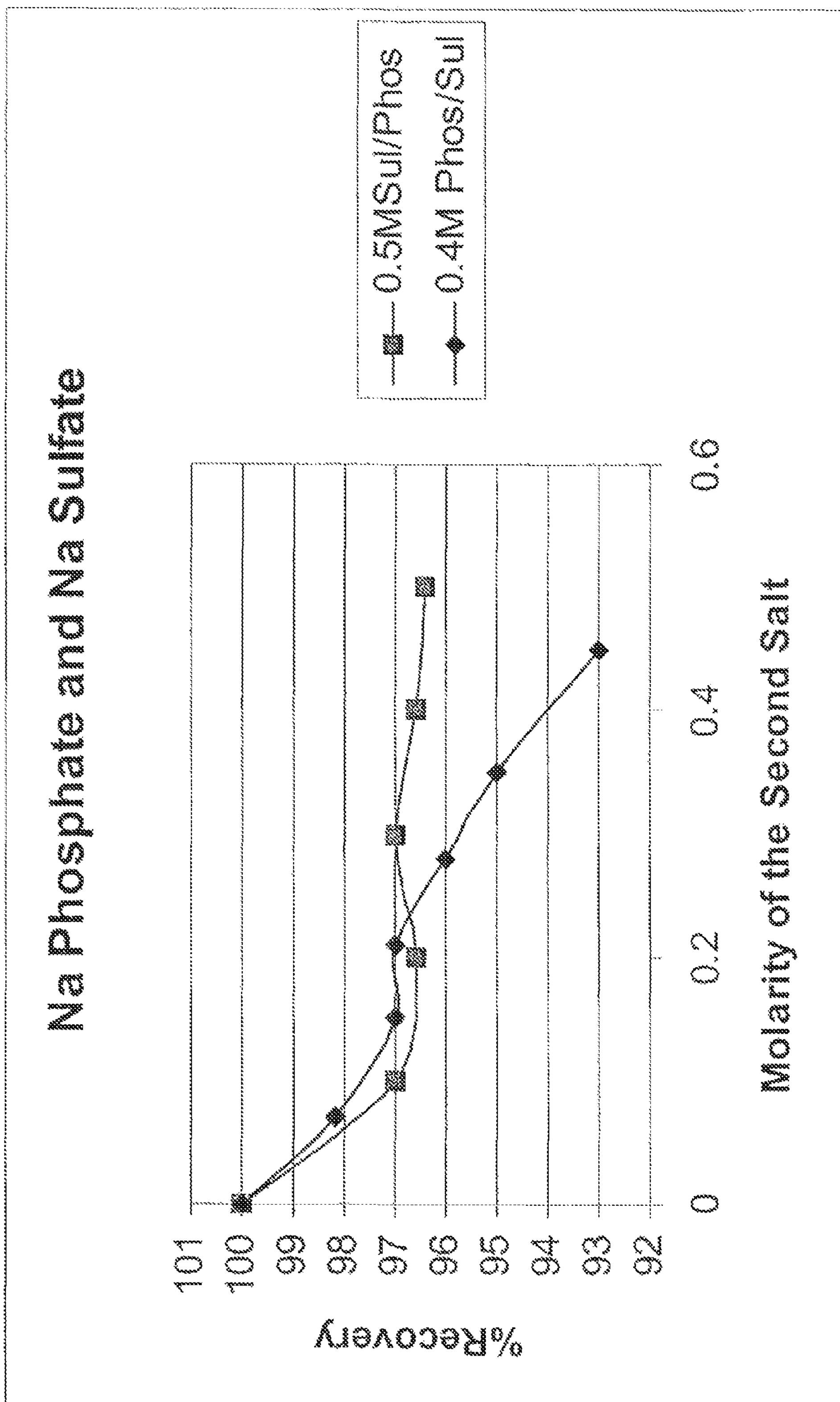

Figure 1A

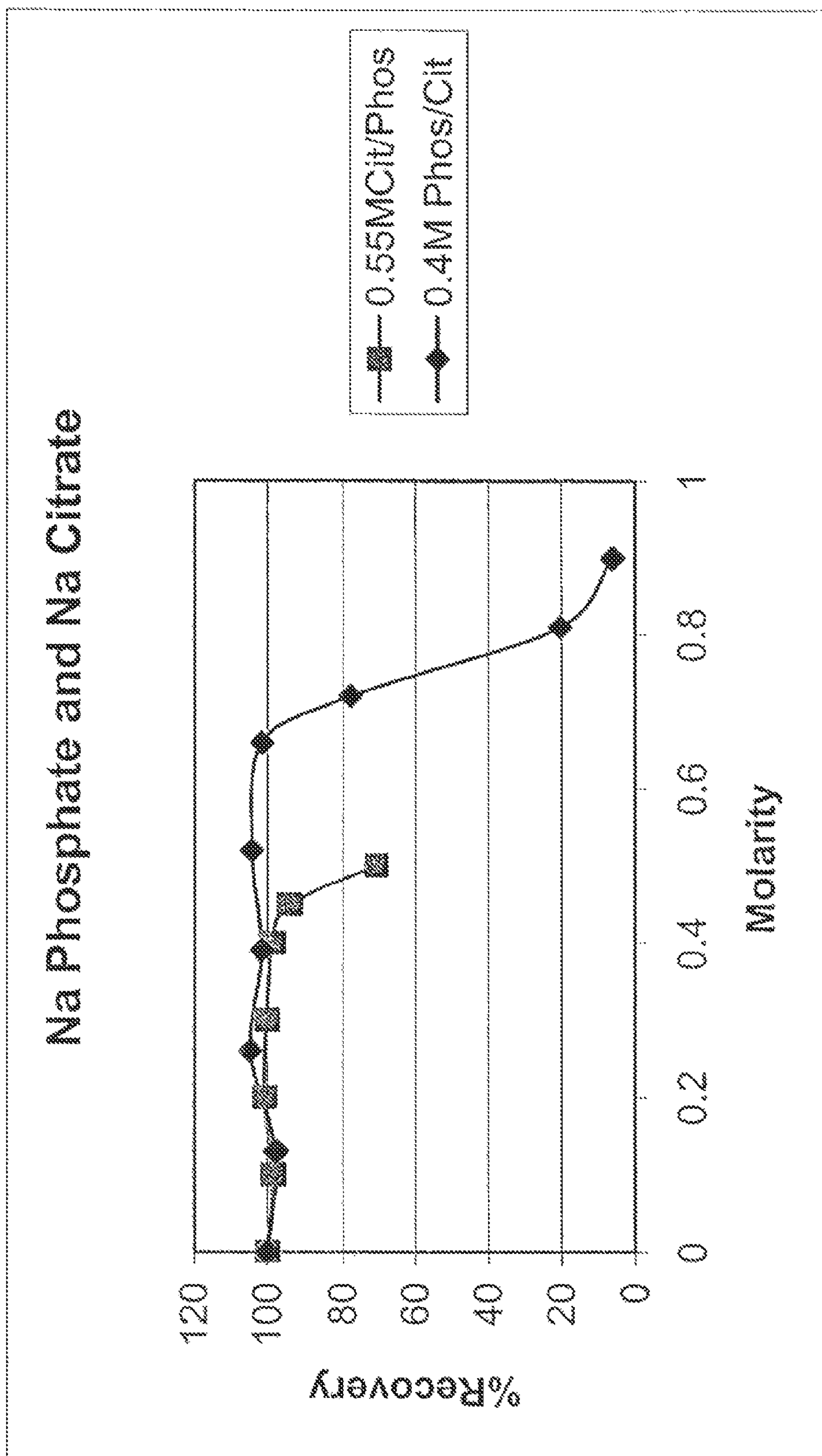

Figure 1B

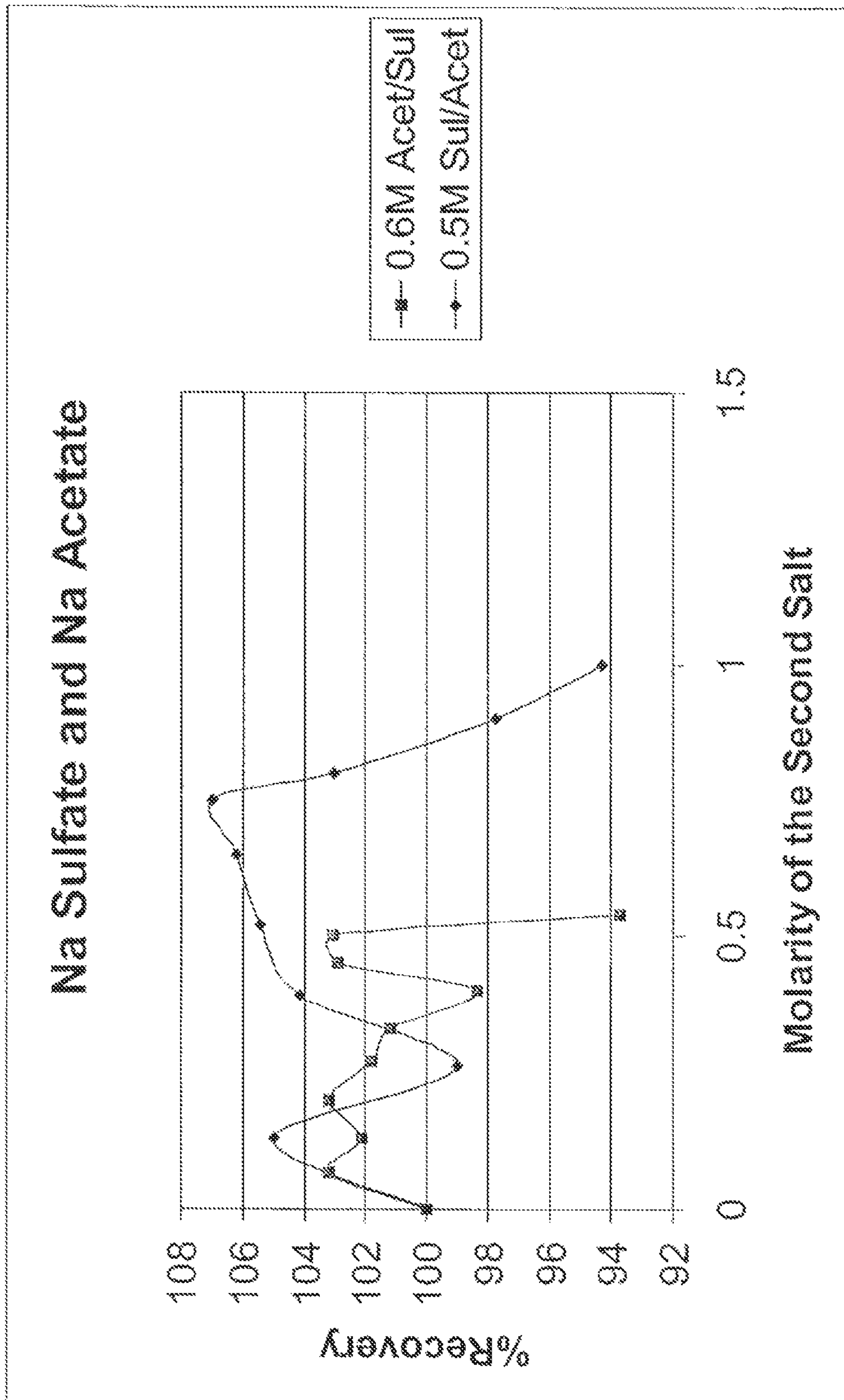

Figure 1C

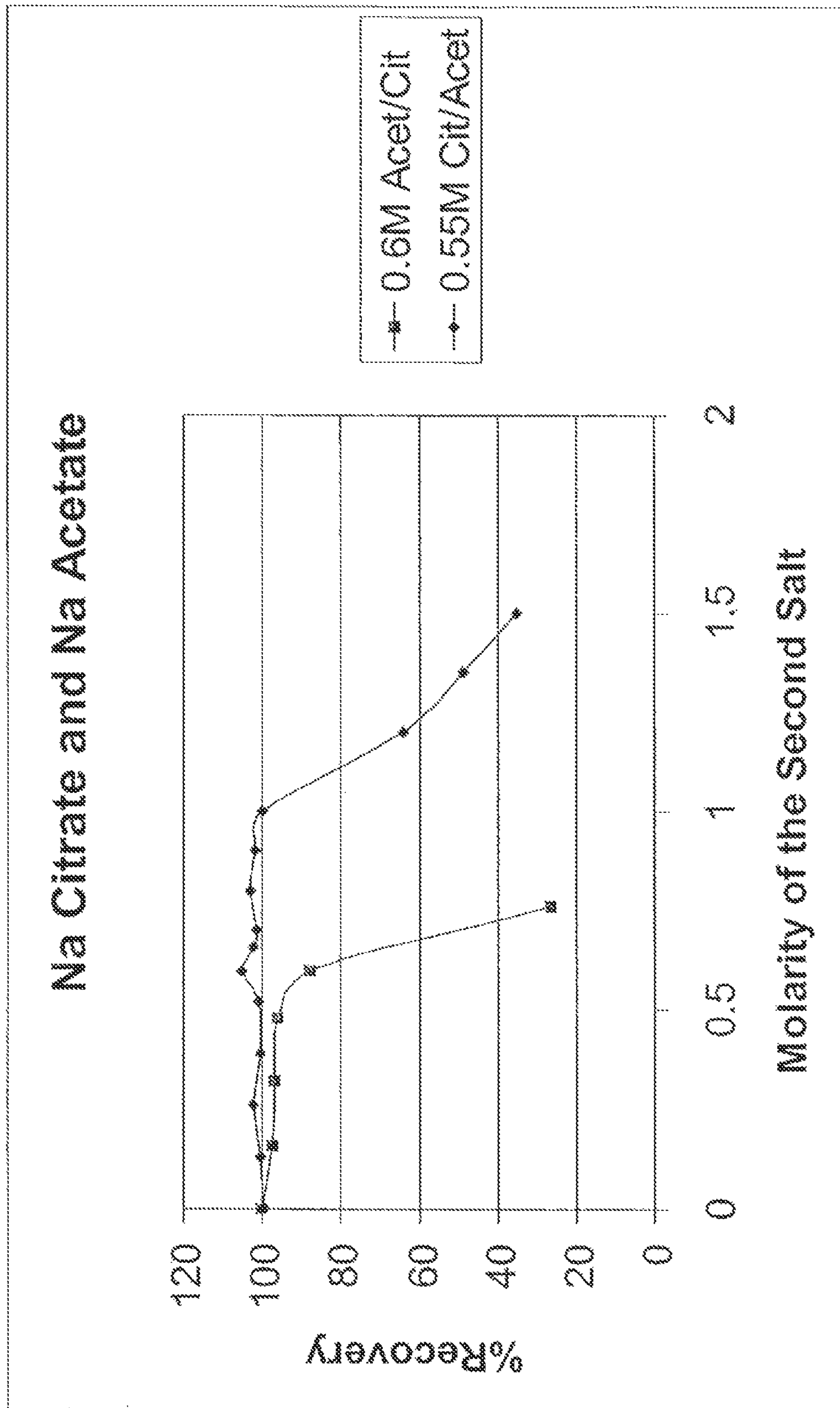

Figure 1D

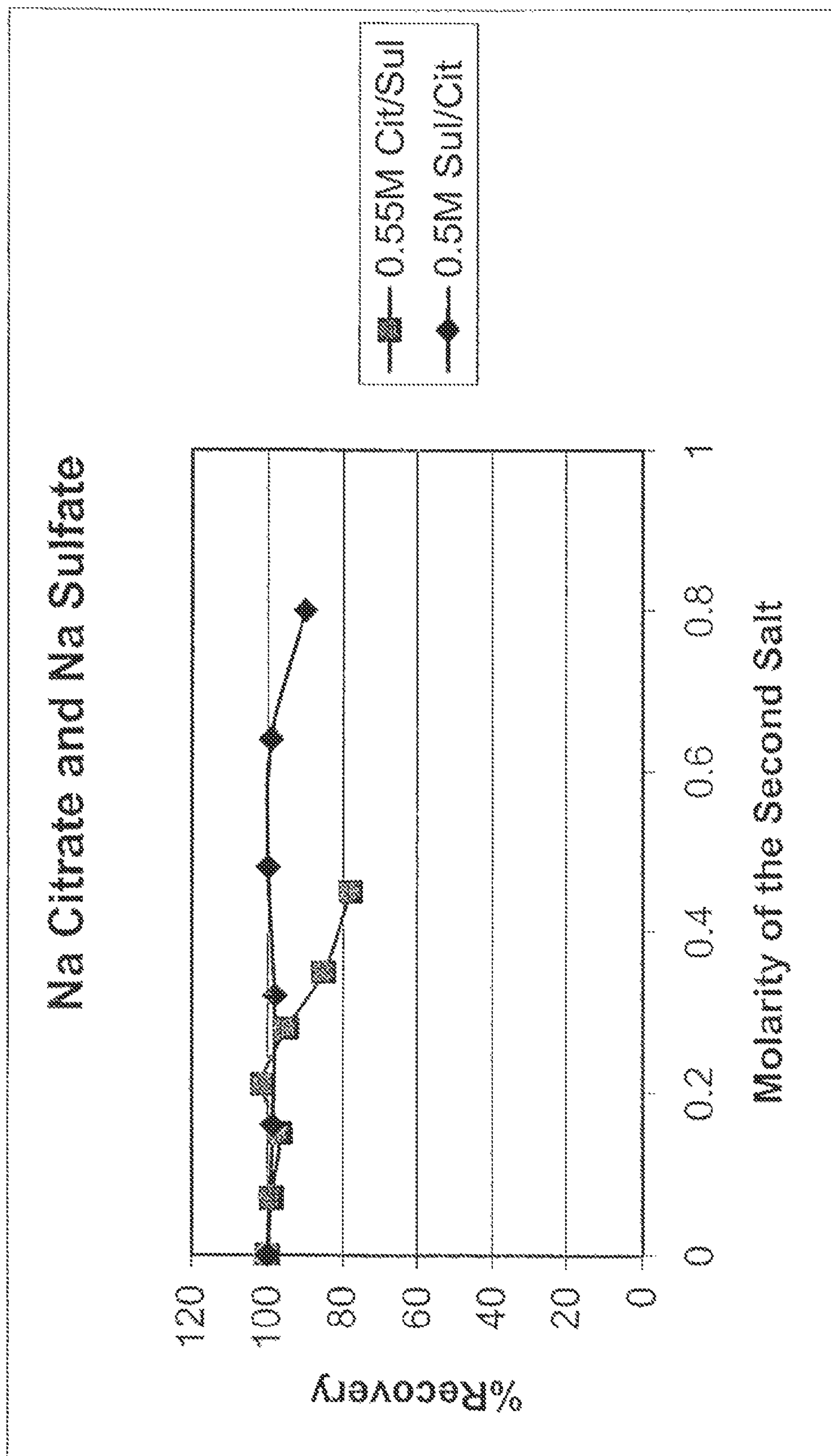

Figure 1E

PROCESS FOR PURIFYING PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional of U.S. application Ser. No. 10/895,581, filed Jul. 21, 2004, now allowed, which claims the benefit of U.S. provisional application No. 60/540,587, filed Jan. 30, 2004, the entire disclosure of which is relied on and incorporated by reference.

FIELD OF THE INVENTION

This invention relates to protein purification and specifically to a process for protein purification using hydrophobic interaction chromatography.

BACKGROUND OF THE INVENTION

The purification of proteins for the production of biological or pharmaceutical products from various source materials involves a number of procedures. Therapeutic proteins may be obtained from plasma or tissue extracts, for example, or may be produced by cell cultures using eukaryotic or procaryotic cells containing at least one recombinant plasmid encoding the desired protein. The engineered proteins are then either secreted into the surrounding media or into the perinuclear space, or made intracellularly and extracted from the cells. A number of well-known technologies are utilized for purifying desired proteins from their source material. Purification processes include procedures in which the protein of interest is separated from the source materials on the basis of solubility, ionic charge, molecular size, adsorption properties, and specific binding to other molecules. The procedures include gel filtration chromatography, ion-exchange chromatography, affinity chromatography, and hydrophobic interaction chromatography.

Hydrophobic interaction chromatography (HIC) is used to separate proteins on the basis of hydrophobic interactions between the hydrophobic moieties of the protein and insoluble, immobilized hydrophobic groups on the matrix. Generally, the protein preparation in a high salt buffer is loaded on the HIC column. The salt in the buffer interacts with water molecules to reduce the solvation of the proteins in solution, thereby exposing hydrophobic regions in the protein which are then adsorbed by hydrophobic groups on the matrix. The more hydrophobic the molecule, the less salt is needed to promote binding. Usually, a decreasing salt gradient is used to elute proteins from a column. As the ionic strength decreases, the exposure of the hydrophilic regions of the protein increases and proteins elute from the column in order of increasing hydrophobicity. See, for example, *Protein Purification,* 2d Ed., Springer-Verlag, New York, 176-179 (1988).

When developing processes for commercial production of therapeutically important proteins, increasing the efficiency of any intermediate purification steps is highly desirable. One way of improving the ease and efficiency of manufacturing is to increase the load capacity of one or more of the intermediate steps of the purification process to the point that the number of cycles required to purify a batch of protein is reduced without compromising the quality of the protein separation. The present invention improves the process of protein purification by increasing the capacity and efficiency of an intermediate step.

SUMMARY OF THE INVENTION

The present invention provides a process of purifying a protein comprising mixing a protein preparation with a solu-

tion containing a first salt and a second salt, forming a mixture which is loaded onto a hydrophobic interaction chromatography column, wherein the first and second salts have different lyotropic values, and at least one salt has a buffering capacity at a pH at which the protein is stable. In one embodiment, the pH of the mixture and equilibrium buffer is between about pH 5 and about pH 7. The process further comprises eluting the protein.

The present invention provides combinations of salts useful for increasing the dynamic capacity of an HIC column compared with the dynamic capacity of the column using separate salts alone. These combinations of salts allow for a decreased concentration of at least one of the salts to achieve a greater dynamic capacity, without compromising the quality of the protein separation. The first and second salt combinations are selected for each particular protein through a process of establishing precipitation curves for each salt individually, and precipitation curves for the combination of salts holding one salt constant and varying the second. The concentrations of the salt combinations can be optimized further, for example, to ensure protein stability at room temperature and to prevent formation of aggregates in the protein preparation.

Preferred first salts are those which form effective buffers at a pH at which the protein is stable. In one embodiment, the first and second salts are selected from acetate, citrate, phosphate, sulfate, or any mineral or organic acid salt thereof. In one embodiment the pH of the mixture is between about pH 5 and about pH 7. In one embodiment, the final salt concentrations of the first salt and second salts in the mixture are each between about 0.1 M and 1.0 M, in another embodiment between about 0.3 M and about 0.7 M. The cations can be selected from any non-toxic cations, including NH_4^+, K^+, and Na^+. Preferred cations are those which do not tend to denature the protein or to cause precipitation in combination with other ions, including NH_4^+ and Na^+.

The two salt buffers of the present invention result in an increase in dynamic capacity of an HIC column for a particular protein compared with the dynamic capacity achieved by single salts. This results in decreased number of cycles required for purifying a batch of protein. Therefore, the present invention has special applicability to commercial manufacturing practices for making and purifying commercially important proteins.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** shows dual salt precipitation curves for an antibody against EGFR performed as described in Example I below. FIG. **1**A shows the precipitation curve for 0.5 M sodium sulfate with increasing concentrations of sodium phosphate and the precipitation curve for 0.4 M sodium phosphate with increasing concentrations of sodium sulfate. FIG. **1**B shows the precipitation curves for 0.55 M sodium citrate with increasing concentrations of sodium phosphate, and 0.4 M sodium phosphate with increasing concentrations of sodium citrate. FIG. **1**C shows the precipitation curves for 0.6 M sodium acetate with increasing concentrations of sodium sulfate, and 0.5 M sodium phosphate with increasing concentrations of sodium sulfate. FIG. **1**D shows the precipitation curves for 0.6 M sodium acetate with increasing concentrations of sodium citrate, and 0.55 M sodium citrate with increasing concentrations of sodium acetate. FIG. **1**E shows the precipitation curves for 0.55 M sodium citrate with

increasing concentrations of sodium sulfate, and 0.5 M sodium sulfate with increasing concentrations of sodium citrate.

DETAILED DESCRIPTION OF THE INVENTION

Hydrophobic interaction chromatography (HIC) is now widely used as an important bioseparation tool in the purification of many types of proteins. The process relies on separation of proteins on the basis of hydrophobic interactions between non-polar regions on the surface of proteins and insoluble, immobilized hydrophobic groups on the matrix. The absorption increases with high salt concentration in the mobile phase and the elution is achieved by decreasing the salt concentration of the eluant (Fausnaugh et al. *J Chromatogr* 359, 131-146 (1986)). A protein preparation at any stage of purification is "conditioned" in preparation for HIC by mixing with high salt buffers to prepare the HIC "load" to be loaded onto the column. Generally, salt conditions are adjusted to individual proteins. Generally, requirements of between about 0.7 and about 2 M ammonium sulfate and between about 1.0 and 4.0 M NaCl salt concentration has been considered as useful for purifying proteins using HIC columns. The practice was to add a high concentration of salt to a low concentration buffer solution, such as, for example, 1.4 M NH_4SO_4 added to a 0.024 M phosphate buffer for the purification of monoclonal antibodies at pH 7.2 (Nau et al. *BioChromotography* 62 (5), 62-74 (1990)); or 1.7 M ammonium sulfate in 50 mM $NaPO_4$ for purifying yeast cell surface proteins (Singleton et al., *J. Bacteriology* 183 (12) 3582-3588 (2001)). The present invention differs from these practices in the use of an intermediate concentration of a buffering salt in combination with an intermediate concentration of a second buffering salt, or in combination with an intermediate concentration of a second non-buffering salt, to achieve increased dynamic capacity.

It has also been recognized that increasing salt concentrations can increase the "dynamic capacity" of a column, or the amount of protein that can be loaded onto a column without "breakthrough" or loss of protein to the solution phase before elution. At the same time, high salt can be detrimental to protein stability. High salt increases the viscosity of a solution, results in increased formation of aggregates, results in protein loss due to dilution and filtration of the protein after elution from the column, and can lead to reduced purity (Queiroz et al., *J. Biotechnology* 87:143-159 (2001), Sofer et al., *Process Chromatography*, Academic Press (1999)). The present invention, however, provides a process of purifying proteins that increases the dynamic capacity of an HIC column for a particular protein while reducing the concentration of the salts used, without reducing the quality of the protein separation or raising manufacturing issues.

As used herein, the term "hydrophobic interaction chromatography (HIC)" column refers to a column containing a stationary phase or resin and a mobile or solution phase in which the hydrophobic interaction between a protein and hydrophobic groups on the matrix serves as the basis for separating a protein from impurities including fragments and aggregates of the subject protein, other proteins or protein fragments and other contaminants such as cell debris, or residual impurities from other purification steps. The stationary phase comprises a base matrix or support such as a cross-linked agarose, silica or synthetic copolymer material to which hydrophobic ligands are attached.

As used herein the term "dynamic capacity" of a separation column such as a hydrophobic interaction chromatography column refers to the maximum amount of protein in solution which can be loaded onto a column without significant breakthrough or leakage of the protein into the solution phase of a column before elution. More formally, K' (capacity factor) =moles of solute in stationary phase divided by moles of solute in mobile phase=Vr−Vo/Vo, where Vr is the volume of the retained solute and Vo is the volume of unretarded solute. Practically, dynamic capacity of a given HIC column is determined by measuring the amount of protein loaded onto the column, and determining the resin load which is mg protein/column volume (mg/ml−r). The amount of protein leaving the column in the solution phase after the column is loaded ("breakthrough") but before elution begins can then be measured by collecting fractions during the loading process and first wash with equilibrium buffer. The load at which no significant breakthrough occurs is the dynamic capacity of the protein for those conditions.

As used herein, the term "buffer" or "buffered solution" refers to solutions which resist changes in pH by the action of its conjugate acid-base range. Examples of buffers that control pH at ranges of about pH 5 to about pH 7 include citrate, phosphate, and acetate, and other mineral acid or organic acid buffers, and combinations of these. Salt cations include sodium, ammonium, and potassium. As used herein the term "loading buffer" or "equilibrium buffer" refers to the buffer containing the salt or salts which is mixed with the protein preparation for loading the protein preparation onto the HIC column. This buffer is also used to equilibrate the column before loading, and to wash to column after loading the protein. The "elution buffer" refers to the buffer used to elute the protein from the column. As used herein, the term "solution" refers to either a buffered or a non-buffered solution, including water.

As used herein, the term "lyotropic" refers to the influence of different salts on hydrophobic interactions, more specifically the degree to which an anion increases the salting out effect on proteins, or for cations, increases the salting-in effect on proteins according to the Hofmeister series for precipitation of proteins from aqueous solutions (Queiroz et al. *J. Biotechnology* 87: 143-159 (2001), Palman et al. *J. Chromatography* 131, 99-108 (1977), Roe et al. *Protein Purification Methods: A Practical Approach*. IRL Press Oxford, pp. 221-232 (1989)). The series for anions in order of decreasing salting-out effect is: $PO_4^3- > SO_4^2- > CH_3COO- > Cl- > Br- > NO_3- > ClO_4- > I- > SCN-$, while the series for cations in order of increasing salting-in effect: $NH_4+ < Rb+ < K+ < Na+ < Li+ < Mg^2+ < Ca^2+ < Ba^2+$ (Queiroz et al., supra). According to the present invention, combining two different salts having different lyotrophic values with a protein preparation allows more protein to be loaded onto a column with no or negligible breakthrough compared with higher salt concentrations of each single salt.

It is an objective of the present invention to produce conditions for particular proteins which maximize the amount of protein which can be loaded and retained by an HIC column with little or no reduction in the quality of separation of the protein. The present invention is a process for purifying a protein comprising mixing a protein preparation with a buffered salt solution containing a first salt and a second salt, wherein each salt has a different lyotrophic value, and loading the protein salt mixture onto an HIC column.

It is now understood that several factors influence the hydrophobic interactions which control the retention of a native protein to the hydrophobic groups attached to the matrix. These include van der Waals forces, or electrostatic interactions between induced or permanent dipoles; hydrogen bonding, or electrostatic interactions between acidic donor and basic acceptor groups; the hydrophobicity of the

protein itself; and the influence of various salts on hydrophobic interactions. (Queiroz et al., *J Biotechnology* 87:143-159 (2001)). The Hofmeister ("lyotropic") series is an ordering of anions and cations in terms of their ability to precipitate proteins from aqueous solutions, as described above. The series for anions in order of decreasing salting-out effect is: $PO_4^{3-} > SO_4^{2-} > CH_3COO^- > Cl^- > Br^- > NO_3^- > ClO_4^- > I^- > SCN^-$, while the series for cations in order of increasing salting-in effect: $NH_4^+ < Rb^+ < K^+ < Na^+ < Li^+ < Mg^{2+} < Ca^{2+} < Ba^{2+}$ (Queiroz et al., supra)

The ions at the beginning of the series promote hydrophobic interactions and protein precipitation or salting out effects, and are called antichaotropic (Queiroz et al., supra). They are considered to be water structuring, whereas the ions at the end of the series are salting-in or chaotropic ions, and randomize the structure of water and tend to decrease the strength of hydrophobic interactions and result in denaturation (Porath et al., *Biotechnol Prog* 3: 14-21 (1987)). The tendency to promote hydrophobic interactions is the same tendency which promotes protein precipitation, and thus determining the salt concentration which causes a particular protein to begin to precipitate is a means of determining an appropriate concentration of that salt to use in an HIC column.

According to the present invention a first salt and a second salt are selected which have differing lyotropic values. This combination of salts acts together to increase the dynamic capacity of the HIC column for a particular protein. It has been found according to the present invention that each salt in combination can be provided at a lower concentration that the concentration of the salt alone to achieve a higher dynamic capacity for a protein compared with the dynamic capacity using a single salt. According to the present invention at least one salt has a buffering capacity at the desired pH.

According to the present invention, the appropriate concentrations of the salts are determined for a particular protein by generating precipitation curves for individual salts, then for combined salts. On the basis of individual salt precipitation curves, precipitation curves for combinations of salts are generated by holding one salt concentration constant, and varying the concentration of the second salt. Then the concentration of the second salt is held constant, and the concentration of the first salt is varied. From these two-salt precipitation curves, concentrations of salts useful for increasing the dynamic capacity of an HIC column can be determined. This is demonstrated in Examples 1 and 2 below, in which the concentrations of two salt combinations are determined using precipitation curves for each particular protein. In addition, the salt concentrations can be optimized to in order to confer additional stability on a protein at room temperature, for example, or to limit aggregate formation. Therefore, the present invention further provides a method of maximizing the dynamic capacity of a hydrophobic interaction chromatography column for a particular protein by selecting a combination of concentrations for a first and second salt having different lyotropic values by generating a series of precipitation curves for the salts alone, and then in combination holding a each salt constant while varying the second.

The salts of the present invention are selected from those having a buffering capacity at the pH at which the protein to be purified is stable. In one embodiment, salt combinations are chosen with a buffering capacity at between about pH 5 to about 7. These include, for example, citrate, phosphate, and acetate, and other mineral acid or organic acid buffers, and combinations of these. A second salt is selected from a salt which may or may not buffer at the desired pH, and can be added to the buffered solution, such as ammonium or sodium

sulfate. Cations are selected from those which are non-toxic and non-denaturing. Preferred cations according to the present invention are sodium, potassium, and ammonium, with sodium being the most preferred for manufacturing purposes. Preferred salts for purifying proteins according to the present invention include combinations of sodium citrate, sodium phosphate, sodium acetate, and sodium sulfate.

The concentration of the salts used according to the present invention will depend on the characteristics of the particular salts. In one embodiment, the salts are used at concentrations from about 0.1 M to about 1.0 M in the final concentration of the mixture of salt solution and protein preparation depending on the salt and protein, in another embodiment is in the range between about 0.3 M and about 0.7 M. The pH of the buffered solution may be varied depending on requirements of the protein separation. In one embodiment, the pH varies between about pH 5 to about pH 7.

Hydrophobic Interaction Chromatography Column

The present invention can be used with any type of HIC stationary phase. Stationary phases vary in terms of ligand, ligand chain length, ligand density, and type of matrix or support. Ligands used for HIC include linear chain alkanes with and without an amino group, aromatic groups such as phenyl and N-alkane ligands including methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl and octyl (Queiroz et al, supra). Many types of HIC columns are available commercially. These include, but are not limited to, SEPHAROSE™ columns such as Phenyl SEPHAROSE™ (Pharmacia LCK Biotechnology, AB, Sweden), FAST FLOW™ column with low or high substitution (Pharmacia LKB Biotechnology, AB, Sweden); Octyl SEPHAROSE™ High Performance column (Pharmacia LKB Biotechnology, AB, Sweden); FRACTOGEL™ EMD Propyl or FRACTOGEL™, EMD Phenyl columns (E. Merck, Germany); MACRO-PREP™ Methyl or MACRO-PREP™ t-Butyl Supports (Bio-Rad, Calif.); WP HI-Propyl (C_3)™ column (J. T. Baker, N.J.); and TOYOPEARL™ ether, phenyl or butyl columns (TosoHaas, Pa.).

In one embodiment, TOYOPEAR™ BUTYL-M columns have been used for purifying proteins as described in Examples 1 and 2.

The mobile phase of HIC according to the present invention is the two salt solution. Commercial applications processes for purifying large quantities of proteins require that the exact ion concentrations of the two salt solution be constant and consistent. Therefore, the adjustment of the dissolved salt solution is made with the acid form of the salt, such as citric acid mixed with citrate to get an exact ion concentration. The salts of the present invention are all commercially available from a number of vendors. At least one salt in the two salt solution will have a buffering effect at the pH at which the protein to be purified is stable. In one embodiment, the buffering capacity of at least one salt is between pH 5 to about pH 7 according to the present invention.

The protocol for using an HIC column according to the present invention is generally as follows. The column is first regenerated with several column volumes of sodium hydroxide, 0.5 N NaOH, for example, then washed with water. The column is then equilibrated with several column volumes of equilibration buffer, which is the same buffer containing the protein preparation for loading onto the column. The protein preparation is prepared by "conditioning" or mixing with the two salt buffered solution. Generally the salt solution is added slowly with the protein preparation at a rate of about 1-2% volume per minute, to avoid protein destabilization. Next, the protein/buffered salt solution mixture is loaded onto the column, and the column washed with several column volumes of equilibrium buffer. The HIC column is then eluted. Elution

can preferably be accomplished by decreasing the salt concentration of the buffer using a salt gradient or isocratic elution. The gradient or step starts at equilibrium buffer salt concentration, and is then reduced as a continuous gradient, or as discrete steps of successively lower concentrations. The elution generally concludes with washing the column with a solution such as a no-salt buffer, such as low ionic strength MES buffer, for example. Elution of the subject protein can also be accomplished by changing the polarity of the solvent, and by adding detergents to the buffer. The protein when purified can be diafiltered or diluted to remove any remaining excess salts.

The method of purifying a protein according to the present invention applies to protein preparations at any stage of purification. Protein purification of recombinantly produced proteins typically includes filtration and/or differential centrifugation to remove cell debris and subcellular fragments, followed by separation using a combination of different chromatography techniques.

A wide range of concentrations of protein can be loaded onto an HIC column using the two salt system of the present invention. The protein preparation to be purified according to the present invention may be of any concentration, however preferably may be varied from about 0.1 mg/ml to about 100 mg/ml or more, more preferably between about 2.5 mg/ml to about 20 mg/ml in an aqueous solution. As used herein the term "protein" is used interchangeably with the term "polypeptide" and is considered to be any chain of at least ten amino acids or more linked by peptide bonds. As used herein, the term "protein preparation" refers to protein in any stage of purification in an aqueous solution. The concentration of a protein preparation at any stage of purification 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, and the colloidal gold assay; 2) methods utilizing the UV absorption properties of proteins; and 3) visual estimation based on stained protein bands in gels relying on comparison with protein standards of known quantity on the same gel such as silver staining. See, for example, Stoschek *Methods in Enzymol.* 182:50-68 (1990).

For the purposes of the present invention a protein is "substantially similar" to another protein if they are at least 80%, preferably at least about 90%, more preferably at least about 95% identical to each other in amino acid sequence, and maintain or alter the biological activity of the unaltered protein. Amino acid substitutions which are conservative substitutions unlikely to affect biological activity are considered identical for the purposes of this invention and include 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 the reverse. (See, for example, Neurath et al., *The Proteins*, Academic Press, New York (1979)).

The method of purifying proteins according to the present invention is directed to all types of proteins. The present invention is particularly suitable for purifying protein-based drugs, also known as biologics. Typically biologics are produced recombinantly, using procaryotic or eukaryotic expression systems such as mammalian cells or yeasts, for example. Recombinant production refers to the production of the desired protein by transformed host cell cultures containing a vector capable of expressing the desired protein. Methods and vectors for creating cells or cell lines capable of expressing recombinant proteins are described for example, in Ausabel et al, eds. *Current Protocols in Molecular Biology*, (Wiley & Sons, New York, 1988, and quarterly updates).

The method of purifying proteins according to the present invention is particularly applicable to antibodies. As used herein, the term "antibody" refers to intact antibodies including polyclonal antibodies (see, for example *Antibodies: A Laboratory Manual*, Harlow and Lane (eds), Cold Spring Harbor Press, (1988)), and monoclonal antibodies (see, for example, U.S. Pat. Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993, and *Monoclonal Antibodies: A New Dimension in Biological Analysis*, Plenum Press, Kennett, McKearn and Bechtol (eds.) (1980)). As used herein, the term "antibody" also refers to a fragment of an antibody such as F(ab), F(ab'), $F(ab')_2$, Fv, Fc, and single chain antibodies which are produced by recombinant DNA techniques or by enzymatic or chemical cleavage of intact antibodies. The term "antibody" also refers to bispecific or bifunctional antibodies, which are an artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. (See Songsivilai et al, *Clin. Exp. Immunol.* 79:315-321 (1990), Kostelny et al., *J. Immuno* 1.148:1547-1553 (1992)). As used herein the term "antibody" also refers to chimeric antibodies, that is, antibodies having a human constant antibody immunoglobin domain is coupled to one or more non-human variable antibody immunoglobin domain, or fragments thereof (see, for example, U.S. Pat. No. 5,595,898 and U.S. Pat. No. 5,693,493). Antibodies also refers to "humanized" antibodies (see, for example, U.S. Pat. No. 4,816,567 and WO 94/10332), minibodies (WO 94/09817), and antibodies produced by transgenic animals, in which a transgenic animal containing a proportion of the human antibody producing genes but deficient in the production of endogenous antibodies are capable of producing human antibodies (see, for example, Mendez et al., *Nature Genetics* 15:146-156 (1997), and U.S. Pat. No. 6,300,129). The term "antibodies" also includes multimeric antibodies, or a higher order complex of proteins such as heterdimeric antibodies. "Antibodies" also includes anti-idiotypic antibodies including anti-idiotypic antibodies against an antibody targeted to the tumor antigen gp72; an antibody against the ganglioside GD3; or an antibody against the ganglioside GD2.

One exemplary antibody capable of being purified according to the present invention is an antibody that recognizes the epidermal growth factor receptor (EGFR), referred to as "an antibody against EGFR" or an "anti-EGFR antibody", described in U.S. Pat. No. 6,235,883, which is herein incorporated by reference in its entirety. An antibody against EGFR includes but is not limited to all variations of the antibody as described in U.S. Pat. No. 6,235,883. Many other antibodies against EGFR are well known in the art, and additional antibodies can be generated through known and yet to be discovered means. A preferred antibody against EGFR is a fully human monoclonal antibody capable of inhibiting the binding of EGF to the EGF receptor. The purification of an antibody against EGFR using a dual salt HIC according to the present invention is described herein in Example 1.

Additional exemplary proteins are three IgG monoclonal antibodies having the following designations: mAb1, mAb2, and mAb3. Purification of these monoclonal antibodies according to the present invention is described herein in Example 2.

The invention is also particularly applicable to proteins, in particular fusion proteins, containing one or more constant antibody immunoglobin domains, preferably an Fc domain of an antibody. The "Fc domain" refers to the portion of the antibody that is responsible for binding to antibody receptors on cells. An Fc domain can contain one, two or all of the

following: the constant heavy 1 domain (C_H1), the constant heavy 2 domain (C_H2), the constant heavy 3 domain (C_H3), and the hinge region. The Fc domain of the human IgG1, for example, contains the C_H2 domain, and the C_H3 domain and hinge region, but not the C_H1 domain. See, for example, C. A. Hasemann and J. Donald Capra, Immunoglobins: Structure and Function, in William E. Paul, ed. *Fundamental Immunology*, Second Edition, 209, 210-218 (1989). As used herein the term "fusion protein" refers to a fusion of all or part of at least two proteins made using recombinant DNA technology or by other means known in the art.

An example of an Fc-containing protein capable of being purified according to the present invention is tumor necrosis factor receptor-Fc fusion protein (TNFR:Fc). As used herein the term "TNFR" (tumor necrosis factor receptor) refers to a protein having an amino acid sequence that is identical or substantially similar to the sequence of a native mammalian tumor necrosis factor receptor, or a fragment thereof, such as the extracellular domain. Biological activity for the purpose of determining substantial similarity is the capacity to bind tumor necrosis factor (TNF), to transduce a biological signal initiated by TNF binding to a cell, and/or to cross-react with anti-TNFR antibodies raised against TNFR. A TNFR may be any mammalian TNRF, including murine and human, and are described in U.S. Pat. Nos. 5,395,760, 5,945,397, and 6,201,105, all of which are herein incorporated by reference. TNFR:Fc is a fusion protein having all or a part of an extracellular domain of any of the TNFR polypeptides including the human p55 and p75 TNFR fused to an Fc region of an antibody. An exemplary TNFR:Fc is a dimeric fusion protein made of the extracellular ligand-binding portion of the human 75 kDa tumor necrosis factor receptor linked to the Fc portion of the human IgG1 from natural (non-recombinant) sources. The purification of the exemplary TNFR:Fc according to the present invention is described in Example 2 below.

Additional proteins capable of being purified according to the present invention include differentiation antigens (referred to as CD proteins) or their ligands or proteins substantially similar to either of these. Such antigens are disclosed in *Leukocyte Typing VI* (*Proceedings of the VIth International Workshop and Conference*, Kishimoto, Kikutani et al., eds., Kobe, Japan, 1996). Similar CD proteins are disclosed in subsequent workshops. Examples of such antigens include CD27, CD30, CD39, CD40, and ligands thereto (CD27 ligand, CD30 ligand, etc.). Several of the CD antigens are members of the TNF receptor family, which also includes 41BB ligand and OX40. The ligands are often members of the TNF family, as are 41BB ligand and OX40 ligand.

An exemplary ligand capable of being purified according to the present invention is a CD40 ligand (CD40L). The native mammalian CD40 ligand is a cytokine and type II membrane polypeptide, having soluble forms containing the extracellular region of CD40L or a fragment of it. As used herein, the term "CD40L" refers to a protein having an amino acid sequence that is identical or substantially similar to the sequence of a native mammalian CD40 ligand or a fragment thereof, such as the extracellular region. As used herein, the term "CD40 ligand" refers to any mammalian CD40 ligand including murine and human forms, as described in U.S. Pat. No. 6,087,329, which is herein incorporated by reference in its entirety. Biological activity for the purpose of determining substantial similarity is the ability to bind a CD40 receptor. A preferred embodiment of a human soluble CD40L is a trimeric CD40L fusion protein having a 33 amino acid oligomerizing zipper (or "leucine zipper") in addition to an extracellular region of human CD40L as described in U.S. Pat. No. 6,087,329. The 33 amino acid sequence trimerizes spontaneously in solution.

In addition, a number of other proteins are capable of purified according to the improved purification methods of the present invention include a number of proteins of commercial, economic, pharmacologic, diagnostic, or therapeutic value. Such proteins may be monomeric or multimeric. These proteins include, but are not limited to, a protein or portion of a protein identical to, or substantially similar to, one of the following proteins: a flt3 ligand, erythropoietin, thrombopoeitin, calcitonin, Fas ligand, ligand for receptor activator of NF-kappa B (RANKL), TNF-related apoptosis-inducing ligand (TRAIL), thymic stroma-derived lymphopoietin, granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, mast cell growth factor, stem cell growth factor, epidermal growth factor, RANTES, growth hormone, insulin, insulinotropin, insulin-like growth factors, parathyroid hormone, interferons, nerve growth factors, glucagon, interleukins 1 through 18, colony stimulating factors, lymphotoxin-β, tumor necrosis factor, leukemia inhibitory factor, oncostatin-M, and various ligands for cell surface molecules ELK and Hek (such as the ligands for eph-related kinases or LERKS). Descriptions of proteins that can be stabilized according to the inventive methods may be found in, for example, *Human Cytokines: Handbook for Basic and Clinical Research, Vol. II* (Aggarwal and Gutterman, eds. Blackwell Sciences, Cambridge, Mass., 1998); *Growth Factors: A Practical Approach* (McKay and Leigh, eds., Oxford University Press Inc., New York, 1993); and *The Cytokine Handbook* (A. W. Thompson, ed., Academic Press, San Diego, Calif., 1991).

Additional proteins capable of being purified according to the present invention are receptors for any of the above-mentioned proteins or proteins substantially similar to such receptors or a fragment thereof such as the extracellular domains of such receptors. These receptors include, in addition to both forms of tumor necrosis factor receptor (referred to as p55 and p75) already described: interleukin-1 receptors (type 1 and 2), interleukin-4 receptor, interleukin-15 receptor, interleukin-17 receptor, interleukin-18 receptor, granulocyte-macrophage colony stimulating factor receptor, granulocyte colony stimulating factor receptor, receptors for oncostatin-M and leukemia inhibitory factor, receptor activator of NF-kappa B (RANK), receptors for TRAIL, and receptors that comprise death domains, such as Fas or apoptosis-inducing receptor (AIR). Proteins of interest also includes antibodies which bind to any of these receptors.

Proteins of interest capable of being purified according to the present invention also include enzymatically active proteins or their ligands. Examples include polypeptides which are identical or substantially similar to the following proteins or portions of the following proteins or their ligands: metalloproteinase-disintegrin family members, various kinases, glucocerebrosidase, superoxide dismutase, tissue plasminogen activator, Factor VIII, Factor IX, apolipoprotein E, apolipoprotein A-I, globins, an IL-2 antagonist, alpha-1 antitrypsin, TNF-alpha Converting Enzyme, ligands for any of the above-mentioned enzymes, and numerous other enzymes and their ligands. Proteins of interest also include antibodies that bind to the above-mentioned enzymatically active proteins or their ligands.

Additional proteins of interest capable of being purified according to the present invention are conjugates having an antibody and a cytotoxic or luminescent substance. Such substances include: maytansine derivatives (such as DM1); enterotoxins (such as a Staphlyococcal enterotoxin); iodine

isotopes (such as iodine-125); technium isotopes (such as Tc-99m); cyanine fluorochromes (such as Cy5.5.18); and ribosome-inactivating proteins (such as bouganin, gelonin, or saporin-S6). Examples of antibodies or antibody/cytotoxin or antibody/luminophore conjugates contemplated by the invention include those that recognize the following antigens: CD2, CD3, CD4, CD8, CD11a, CD14, CD18, CD20, CD22, CD23, CD25, CD33, CD40, CD44, CD52, CD80 (B7.1), CD86 (B7.2), CD147, IL-4, IL-5, IL-8, IL-10, IL-2 receptor, IL-6 receptor, PDGF-β, VEGF, TGF, TGF-β2, TGF-β1, VEGF receptor, C5 complement, IgE, tumor antigen CA125, tumor antigen MUC1, PEM antigen, LCG (which is a gene product that is expressed in association with lung cancer), HER-2, a tumor-associated glycoprotein TAG-72, the SK-1 antigen, tumor-associated epitopes that are present in elevated levels in the sera of patients with colon and/or pancreatic cancer, cancer-associated epitopes or proteins expressed on breast, colon, squamous cell, prostate, pancreatic, lung, and/or kidney cancer cells and/or on melanoma, glioma, or neuroblastoma cells, the necrotic core of a tumor, integrin alpha 4 beta 7, the integrin VLA-4, B2 integrins, TNF-α, the adhesion molecule VAP-1, epithelial cell adhesion molecule (EpCAM), intercellular adhesion molecule-3 (ICAM-3), leukointegrin adhesin, the platelet glycoprotein gp IIb/IIIa, cardiac myosin heavy chain, parathyroid hormone, rNAPc2 (which is an inhibitor of factor VIIa-tissue factor), MHC I, carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), tumor necrosis factor (TNF), CTLA-4 (which is a cytotoxic T lymphocyte-associated antigen), Fc-γ-1 receptor, HLA-DR 10 beta, HLA-DR antigen, L-selectin, IFN-γ, Respiratory Syncitial Virus, human immunodeficiency virus (HIV), hepatitis B virus (HBV), *Streptococcus mutans*, and *Staphlycoccus aureus*.

The present invention is particularly useful in the context of commercial production and purification of proteins, especially recombinantly produced proteins. By increasing the capacity of one step in the overall purification scheme of a commercially important protein, the present invention can reduce the number of cycles required to purify a batch of protein. The present invention therefore increases the efficiency of protein purification, without reducing the quality of the protein product. For large-scale production of commercially important biologics, for example, this represents a significant savings in cost and time.

The invention having been described, the following examples are offered by way of illustration, and not limitation.

Example I

Various combinations of salt solutions were tested for their ability to increase the dynamic capacity of an HIC column used for purifying an antibody against epidermal growth factor receptor (antibody against EGFR).

First the range of effective concentrations for single salts ("salts") and two salt buffers for the antibody against EGFR was determined by plotting precipitation curves for single salts and their combinations. The following salts were used: sodium citrate, sodium phosphate, sodium acetate, and sodium phosphate. All buffers were made by weighing out the appropriate chemicals, dissolving at approximately 80% of the final volume, and adjusting the pH using 11.2 N HCl or 10 NaOH to pH 6.0, at room temperature (21-23° C.), and bringing up to volume. For commercial applications, however, the buffered salts are prepared by mixing a salt with its acid form, such as sodium citrate with citric acid, to achieve an exact ion concentration, rather than adjusting to a pH with other acids or bases.

The antibody preparation used for testing was a partially purified eluant from a previous column having a concentration of approximately 5 mg/ml protein. Precipitation studies of this antibody using individual buffers were performed as follows: the antibody preparation was mixed with the buffer stock to make between 0 and 1.2 M final concentration of salt. The samples incubated for 20 minutes, centrifuged for 10 minutes at approximately 6000×g, filtered, and the supernatant assayed for protein. The control sample was diluted with water, and its supernatant reading was taken as 100% recovery. A salting out or precipitation curve was generated for the antibody by plotting amount of protein in the supernatant (percent recovery, compared with the control) versus salt molarity. The percent recovery decreased significantly at greater than about 0.6 M for sodium citrate, while the percent recovery decreased significantly at greater than about 0.8 M for sodium phosphate buffer, at greater than about 1.2 M for sodium acetate, and at greater than about 0.6 M for sodium sulfate. Using this information, a second series of salting out curves for two salt combinations was generated in which the concentration of the first salt was kept constant, while the concentration of the second salt was increased. The precipitation curves were generated by incubating the antibody and two salt mixture for twenty minutes and centrifuging as described for the single salts solutions. For example, sodium citrate was kept at 0.55 M while the concentration of sodium phosphate was increased, and the percent recovery of the antibody in the supernatant was measured and compared with that of the control. The reverse test was also performed keeping 0.4 M sodium phosphate constant while varying the concentration of sodium sulfate. The results are shown in FIG. **1**A through E. These results show that reduced concentrations of the salts together compared with a salt alone could precipitate the protein. This indicated that reduced concentrations of each salt in combination produced equivalent hydrophobic effects compared with higher concentrations of each salt alone.

The results of the single and two salt precipitations provided a range of single and combined salt concentrations for the determination of dynamic capacity for an HIC column for the antibody against EGFR. The dynamic capacity was determined according to the following protocol. An approximately 5 mg/ml antibody preparation was "conditioned" by diluting 1:1 with the appropriate buffered salt stock solution (2×). The salt stock was added to the antibody preparation at a rate of 1-2% volume per minute with stirring. Further salt dilution was performed as necessary to provide a range of salt concentrations, and the mixture of antibody preparation and salt buffer was filtered on a 0.2 um cellulose filter. This mixture was the hydrophobic interaction chromatography (HIC) load. The HIC column used to determine dynamic capacity for single and two salt combinations was a Millipore (Bellerica, Mass.) VANTAGE column having 1.1 cm diameter and packed to 8.5 mL column volume (CV) (9 cm bed height) with TOYOPEARL™ BUTYL 650 M resin (TosoHaas). The column was prepared by regenerating with 0.5N sodium hydroxide at 180 cm/hr for 3 column volumes (CV), washing for 3 CV at 180 cm/hr with water, then equilibrating the column at 180 cm/hr with the appropriate salt buffer or salt combination. Then the load mixture was loaded at 90 cm/hr and washed at 90 cm/hr with 3 CV of the same salt buffer (equilibrium buffer). For determining dynamic capacity, the columns were overloaded with protein, so that fractions were collected during the loading ("flow-through") and washing

steps. Protein content was determined by absorption at 280 nm, or by SDS-PAGE gels. The load concentration in mg/ml-resin at which the % breakthrough is zero is considered to be the dynamic capacity of the antibody at that salt concentration. The dynamic capacity was determined from plotting HIC load versus percent breakthrough (BT) (flow-through concentration/load concentration).

The antibody was then eluted at 180 cm/hr using a step elution or step gradient starting with the equilibrium conditions to a concentration of 0.2 M salt. Fractions were collected and SDS-PAGE analysis was performed on 4-20% Tris/Glycine Novex gels using silver stain (Pharmacia One-Plus™ kit) to visualize protein bands.

Two salt concentrations were optionally further modified in order to stabilize the monomer antibody preparation at room temperature, rather than 4-8° C., and also to minimize the formation of aggregates in the antibody sample. For example, the dynamic capacity of the column for the antibody using 0.4 M sodium phosphate buffer was 43/ml-r (ml-resin); the dynamic capacity of 0.35 M sodium phosphate was 40 mg/ml-r, and the dynamic capacity of 0.3 M sodium phosphate was 38 mg/ml-r. However, 25% protein loss was found to occur at 0.5 M phosphate at room temperature, while only 8% loss was found in 0.4 M for up to six days at room temperature. In addition, it was found that material that precipitated out between 0.3M and 0.4 M salt concentrations included almost all of the high molecular weight aggregates (HMW).

In addition, the rate at which the salt stock was mixed with the antibody preparation influenced the stability of the antibody. At a rate of 2% volume/minute, only about 2% of the antibody was lost as fragments of the monomer, as opposed to 12% lost at 10% volume/minute.

The dynamic capacities of the HIC column for the antibody against EGFR for the various single and combination salts were determined as described above and are shown in Table 1 below.

TABLE 1

Dynamic capacities of antibody against EGFR with four salts and their combinations. Only anions are listed; the cations were sodium for every salt

Experimental Conditions	Dynamic Capacity (mg/ml-r)
0.55M Citrate	24
0.5M Phosphate	12
0.8M Sulfate	24
1.2 M Acetate	5
0.55M Citrate/0.3M Sulfate	30
0.6M Acetate/0.5M Citrate	29
0.35M Phosphate/0.6M Citrate	39
0.6M Acetate/0.7M Sulfate	27
0.5M Citrate/1M Acetate	34
0.5M Sulfate/1M Acetate	33
0.4M Phosphate/0.3M Sulfate	15
0.5M Sulfate/0.3M Citrate	33
0.5M Sulfate/0.3M Phosphate	17
0.3M Citrate/0.6M Phosphate	35

Table 1 shows that the combinations of citrate/sulfate, acetate/citrate, phosphate/citrate, acetate/sulfate, citrate/acetate, sulfate/acetate, sulfate/citrate, and citrate/phosphate increased the dynamic capacity of the HIC column for the antibody by factors varying from approximately 1.5 to 2 times or more that of each salt alone. The phosphate/sulfate combination did not increase the dynamic capacity for the following reasons: sulfate in combination with phosphate resulted in a precipitate, so that lower concentrations of sul-

fate were required to prevent precipitation. These low concentrations proved too low to improve dynamic capacity. In addition, phosphate and acetate did not prove to be an effective combination due to the precipitation which resulted when the two salts were mixed.

Example 2

Using the same procedures as described in Example 1 the dynamic capacities of four additional proteins was determined for the single salts sodium phosphate and sodium citrate, and two salt combination 0.55 M sodium citrate with phosphate concentration varied. The additional proteins were the fusion protein TNFR:Fc described above, and three monoclonal antibodies designated mAb1, mAb2, and mAb3. The three monoclonal antibodies were partially purified and obtained as eluants from other types of chromatography columns. The TNFR:Fc fusion protein was obtained as a fully purified protein. The concentrations of the proteins used was between 4-5 mg/ml, for this particular experiment.

The precipitation curves for sodium citrate and sodium phosphate alone were first determined for each protein, and then a two salt precipitation curve for 0.55M sodium citrate with sodium phosphate varied was determined. The concentration at which each protein begins to precipitate is given in Table 2 below.

TABLE 2

Salt concentrations at which protein begins to precipitate (taken from the precipitation curves.)

Protein	Conc. Sodium Citrate	Conc. Sodium Phosphate	Combination Salt
mAb1	0.6M	0.9M	0.55M NaCitrate/ 0.4M Na Phosphate
mAb2	0.7M	1.1M	0.55M Na Citrate/ 0.4M Na Phosphate
mAb3	0.7M	1.0M	0.55M Na Citrate/ 0.2M Na Phosphate
TNFR:Fc	0.55M	1.0M	0.4M Na Citrate/ 0.2M Na Phosphate

It is clear from Table 2 that the combination of salts precipitated the proteins at lower concentrations compared to the concentrations of each salt alone.

The dynamic capacities of these proteins on TOYOPEARL™ BUTYL 650M (TosoHaas) gels was determined for the salt concentrations shown in Table 2, using the same procedure described above for the antibody against EGFR. The results are given in Table 3 below.

TABLE 3

Dynamic capacities under the salt conditions listed in Table 2.

Protein	Na Citrate	Na Phosphate	Combination
mAb1	37	20	49
mAb2	36	30	44
mAb3	21	12	25
TNFR:Fc	17	18	25

Again, it is clear that the combination of salts increased the dynamic capacity for all four proteins over that achieved using the single salts by 1.5 to 2 times.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are

within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

What is claimed is:

1. A process for purifying a protein on a hydrophobic interaction chromatography column such that the dynamic capacity of the column is increased for the protein comprising mixing a preparation containing the protein with a combination of a first salt and a second salt, loading the mixture onto a hydrophobic interaction chromatography column, and eluting the protein, wherein the first and second salts are selected from the group consisting of citrate and sulfate, citrate and acetate, and sulfate and acetate, respectively, and wherein the concentration of each of the first salt and the second salt in the mixture is between about 0.1 M and about 1.0.

2. The process of claim **1** wherein the pH of the mixture loaded onto the column is between about pH 5 and about pH 7.

3. The process of claim **1** wherein the column is eluted with a solution having a pH between about pH 5 and pH 7.

4. The process of claim **1** wherein the first and second salts are selected from the group consisting of sodium, potassium and ammonium salts.

5. The process of claim **1** wherein the protein is a fusion protein or an antibody.

6. The process of claim **1**, further comprising diluting the protein.

7. The process of claim **1**, further comprising filtering the protein.

8. The process of claim **1**, further comprising formulating the protein.

9. The process of claim **1**, further comprising lyophilizing the protein.

10. A method of increasing the dynamic capacity of a hydrophobic interaction chromatography column for a protein, comprising mixing a preparation containing the protein with a combination of a first salt and a second salt, and loading the mixture onto a hydrophobic interaction chromatography column, wherein the first and second salts are selected from the group consisting of citrate and sulfate, citrate and acetate and sulfate and acetate, respectively, and wherein the concentration of each of the first and second salts in the mixture is between about 0.1 M and about 1.0 M.

11. The method of claim **10** wherein the pH of the mixture loaded onto the column is between about pH 5 and about pH 7.

12. The method process of claim **10**, wherein the first and second salts are selected from the group consisting of sodium, potassium and ammonium salts.

13. The method of claim **10** wherein the protein is a fusion protein or an antibody.

* * * * *

Exhibit 5

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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This patent is subject to a terminal disclaimer.

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

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(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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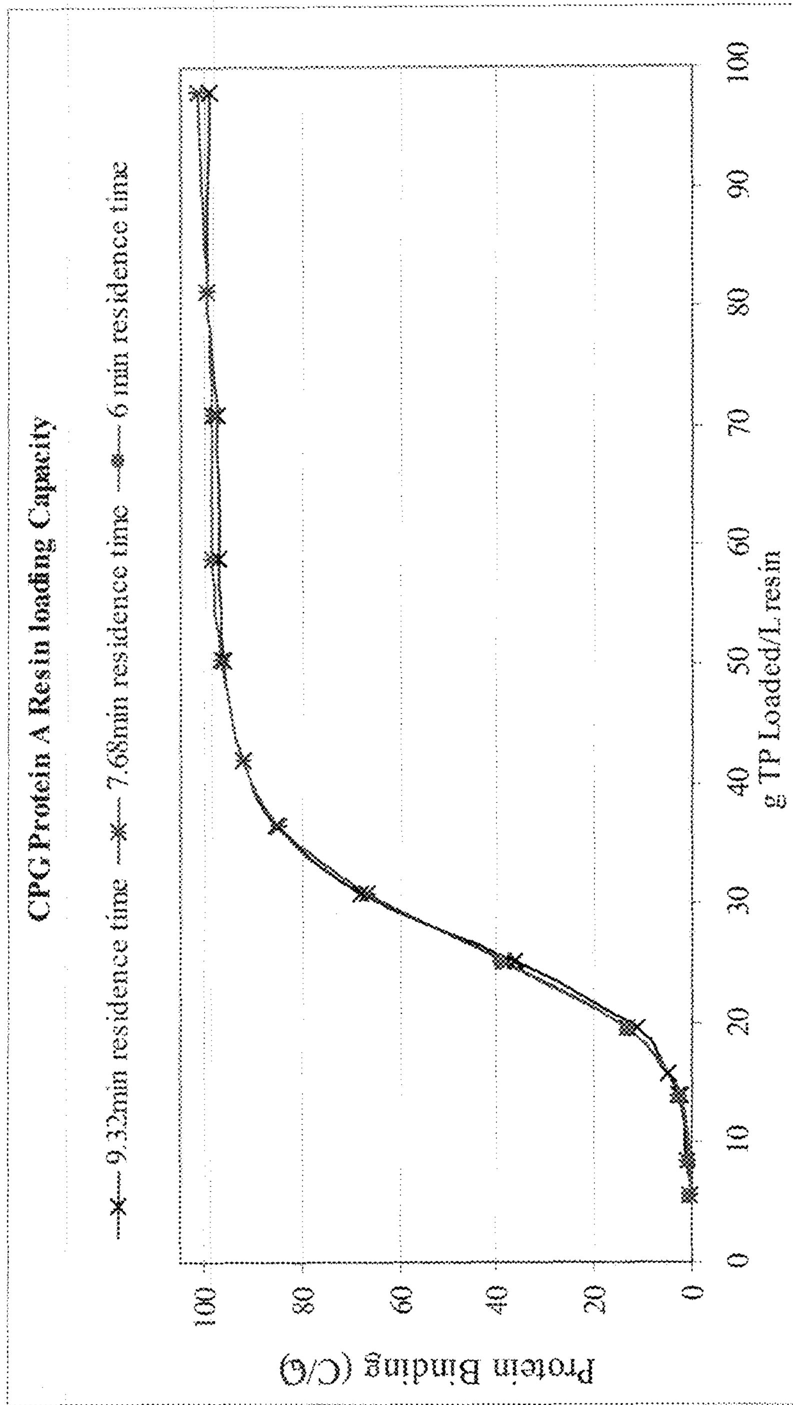

Figure 1

		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

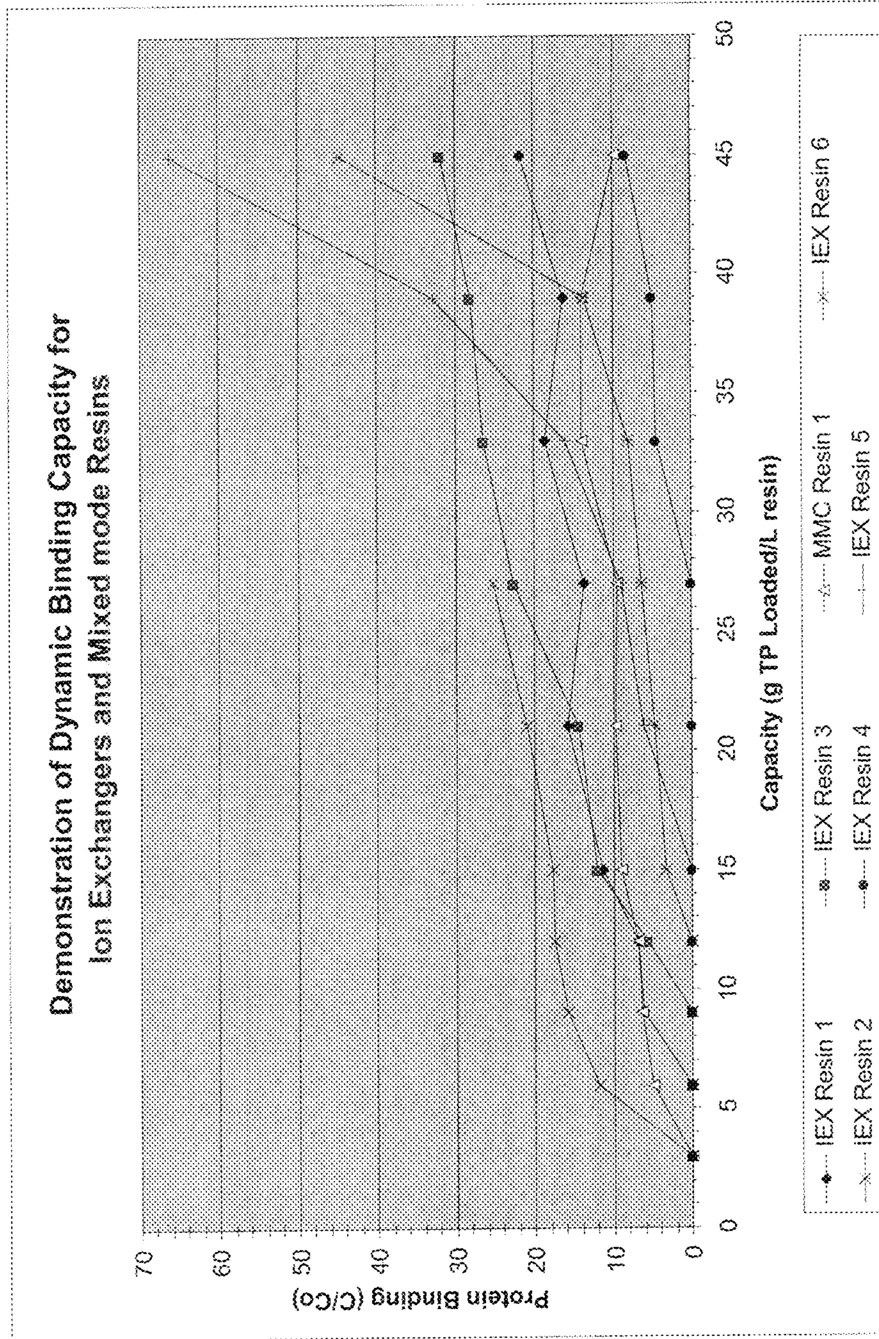

Figure 4

	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 exchange 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 $S0_3^-$ 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.

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