IN THE UNITED STATES DISTRICT COURT
FOR THE SOUTHERN DISTRICT OF FLORIDA

AMGEN INC. and AMGEN MANUFACTURING LIMITED, Plaintiff, v. APOTEX INC. and APOTEX CORP., Defendant.	Case No. _____

COMPLAINT FOR PATENT INFRINGEMENT

Plaintiffs Amgen Inc. and Amgen Manufacturing Ltd. (together, "Amgen") for its Complaint against Defendants Apotex Inc. and Apotex Corp. (together, "Apotex") allege as follows:

THE PARTIES

1. Amgen Inc. 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 Inc. discovers, develops, manufactures, and sells innovative therapeutic products based on advances in molecular biology, recombinant DNA technology, and chemistry.

2. Amgen Manufacturing, Limited ("AML") is a corporation existing under the laws of Bermuda with its principal place of business in Juncos, Puerto Rico. AML manufactures and sells biologic medicines for treating particular diseases in humans.

3. On information and belief, Apotex Inc. is a corporation existing under the laws of Canada, with its principal place of business at 150 Signet Drive, Toronto, Ontario M9L 1T9, Canada. Upon information and belief, acting in concert with Defendant Apotex Corp.,

Apotex Inc. is in the business of developing, manufacturing, and marketing biopharmaceutical products that are distributed and sold throughout the United States and in the State of Florida.

4. On information and belief, Apotex Corp. is a corporation existing under the laws of Delaware, with its principle place of business at 2400 North Commerce Parkway, Suite 400, Weston, Florida 33326. Upon information and belief, acting in concert with Defendant Apotex Inc., Apotex Corp. is in the business of developing, manufacturing, and marketing biopharmaceutical products that are distributed and sold throughout the United States and in the State of Florida. Upon information and belief, Apotex Corp. is also the United States agent for Apotex Inc. for purposes including, but not limited to, filing regulatory submissions to and corresponding with FDA.

5. Upon information and belief, Apotex Corp. is a wholly owned affiliate of Apotex Inc. Upon information and belief, Apotex Corp. acts at the direction of, under the control of, and for the direct benefit of Apotex Inc. and is controlled and/or dominated by Apotex Inc.

6. Upon information and belief, Apotex Inc. and Apotex Corp. share common officers, including, but not limited to, Dr. Bernard C. Sherman.

NATURE OF THE ACTION

7. This is an action for patent infringement arising under the patent laws of the United States, Title 35, United States Code, involving United States Patent Nos. 8,952,138 ("the '138 Patent") and 5,824,784 ("the '784 Patent").

8. This is one of the first actions for patent infringement under 35 U.S.C. § 271(e)(2)(C), which was enacted in 2010 as part of the Biologics Price Competition and Innovation Act ("the BPCIA").

9. The BPCIA created an abbreviated pathway for the approval of biosimilar versions of approved biologic drugs. The abbreviated pathway (also known as "the (k) pathway") allows a biosimilar applicant (the "subsection (k) applicant") to rely on the prior licensure and approval status of the innovative biological product (called the "reference product") that the biosimilar purports to copy.

10. In addition to creating an abbreviated pathway for approval, the BPCIA created an intricate and carefully orchestrated set of information exchanges to facilitate the resolution of patent disputes before a biosimilar product enters the market. 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).

11. Pursuant to the BPCIA, specifically 42 U.S.C. § 262(k), defendants Apotex submitted Biologic License Application (BLA) no. 761026 (the "Apotex BLA") seeking authorization from FDA to market a biosimilar version of Amgen's Neulasta® (pegfilgrastim) product ("the Apotex Pegfilgrastim Product").

12. Beginning in December 2014, the parties engaged in the exchange of information and statements as required by the BPCIA. As a result of these exchanges, the parties have agreed to the inclusion of two U.S. patents in this action: the '138 Patent and the '784 Patent ("Patents in Suit").

13. Under 35 U.S.C. § 271(e)(2)(C)(i), it is an act of infringement, with respect to patents listed pursuant to 42 U.S.C. § 262(*l*)(3)(A), to submit an application seeking approval of a biological product.

14. With respect to the patents that Amgen identified in 42 U.S.C. § 262(*l*)(3)(A), including the Patents in Suit, Apotex committed an act of infringement, under 35

U.S.C. § 271(e)(2)(C)(i), when it submitted the Apotex BLA seeking FDA approval to commercially manufacture, use, offer for sale, sell, distribute in, or import into the United States the Apotex Pegfilgrastim Product prior to the expiration of each aforementioned patent, or any extensions thereof.

15. Apotex will infringe one or more claims of the Patents in Suit, under 35 U.S.C. § 271(a), (b), (c), or (g), should it engage in the commercial manufacture, use, offer for sale, sale, distribution in, or importation into the United States of the Apotex Pegfilgrastim Product prior to the expiration of each aforementioned patent, or any extensions thereof.

JURISDICTION AND VENUE

16. This action arises under the patent laws of the United states, Title 35 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.

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

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

Apotex Inc.

19. Upon information and belief, Apotex Inc. develops, manufactures, seeks regulatory approval for, markets, distributes, and sells biopharmaceuticals for sale and use throughout the United States, including in the State of Florida.

20. This Court has personal specific jurisdiction over Apotex Inc. because Apotex Inc. has committed, or aided, abetted, contributed to and/or participated in the commission of, the tortious act of patent infringement that has led to foreseeable harm and injury to Amgen. In particular, Apotex Inc. collaborates with Apotex Corp. to develop, manufacture,

seek approval for, and sell the disputed biosimilar product, which will cause tortious injury to Plaintiffs.

21. Moreover, upon information and belief, Apotex Inc., following any FDA approval of the biosimilar product, will sell the Apotex Pegfilgrastim Product that is the subject of the patent infringement claims in this action in Florida and throughout the United States.

22. This Court has personal general jurisdiction over Apotex Inc. by virtue of, *inter alia*, its having conducted business in this District, having availed itself of the rights and benefits of Florida law, and having engaged in substantial and continuing contacts with Florida. Upon information and belief, Apotex Inc. has regular and continuous commercial business dealings with representatives, agents, distributors, and customers located in Florida and in this District, including with its subsidiary Apotex Corp.

23. Upon information and belief, Apotex Inc. exercises considerable control over Apotex Corp. with respect to biosimilar products, and approves significant decisions of Apotex Corp., including designating Apotex Corp. as the agent for Apotex Inc. in connection with preparing and filing the Apotex BLA.

24. In addition, Apotex Inc. has previously submitted to the jurisdiction of this Court and has previously availed itself of this Court by filing suit in this jurisdiction and/or by asserting counterclaims in other civil actions initiated in this jurisdiction. *See, e.g., Apotex, Inc. et al v. Mylan Pharmaceuticals, Inc.*, Case No. 12-cv-60704 (S.D. Fla., Apr. 20, 2012). Further, Apotex previously admitted that this Court has personal jurisdiction over both Apotex Corp. and Apotex Inc. *See Alcon v. Apotex Inc. & Apotex Corp.*, C.A. No. 1:06-cv-01642, D.I. 23 at 7 (S.D. Ind. Dec. 13, 2006) ("Plaintiffs could have brought this action in the S.D. Fla. because the S.D. Fla. has personal jurisdiction over both Defendants. Apotex Corp. has a principal place of

business in Weston, Florida, while Apotex Inc. is a Canadian corporation that regularly conducts business in Florida. Thus, venue in the S.D. Fla. would also be proper.").

25. In the alternative, should Apotex Inc. contest jurisdiction in this forum, this Court has personal jurisdiction over Apotex Inc. under Fed. R. Civ. P. 4(k)(2) because, on information and belief, Apotex Inc. "is not subject to jurisdiction in any state's courts of general jurisdiction," and because "exercising jurisdiction is nevertheless consistent with the United States Constitution and laws" given that Apotex Inc. has filed the Apotex BLA in the United States for a product that it intends to market in the United States.

Apotex Corp.

26. This Court has personal jurisdiction over Apotex Corp. by virtue of the fact that, *inter alia*, Apotex Corp. has a principle place of business within this judicial district, in Weston, Florida.

27. Upon information and belief, Apotex Corp. develops, manufactures, seeks regulatory approval for, markets, distributes, and sells biopharmaceuticals for sale and use throughout the United States, including in the State of Florida.

28. This Court has personal specific jurisdiction over Apotex Corp. because Apotex Corp. has committed, or aided, abetted, contributed to and/or participated in the commission of, the tortious act of patent infringement that has led to foreseeable harm and injury to Amgen. In particular, on information and belief, Apotex Corp. collaborated with Apotex Inc. to develop, manufacture, and seek approval for the Apotex Pegfilgrastim Product, and on information, ApoBiologix®, a division of Apotex Corp., will market the Apotex Pegfilgrastim Product in the United States, which will cause tortious injury to Plaintiffs.

29. This Court has personal general jurisdiction over Apotex Corp. by virtue of, *inter alia*, its having conducted business in this District, having availed itself of the rights and benefits of Florida law, and having engaged in substantial and continuing contacts with Florida. Upon information and belief, Apotex Corp. has regular and continuous commercial business dealings with representatives, agents, distributors, and customers located in Florida and in this District.

30. In addition, Apotex Inc. has previously submitted to the jurisdiction of this Court and has previously availed itself of this Court by filing suit in this jurisdiction and/or by asserting counterclaims in other civil actions initiated in this jurisdiction. *See, e.g., Apotex, Inc. et al v. Mylan Pharmaceuticals, Inc.*, Case No. 12-cv-60704 (S.D. Fla., Apr. 20, 2012). Further, Apotex previously admitted that this Court has personal jurisdiction over both Apotex Corp. and Apotex Inc. *See Alcon v. Apotex Inc. & Apotex Corp.*, C.A. No. 1:06-cv-01642, D.I. 23 at 7 (S.D. Ind. Dec. 13, 2006) ("Plaintiffs could have brought this action in the S.D. Fla. because the S.D. Fla. has personal jurisdiction over both Defendants. Apotex Corp. has a principal place of business in Weston, Florida, while Apotex Inc. is a Canadian corporation that regularly conducts business in Florida. Thus, venue in the S.D. Fla. would also be proper.").

31. On information and belief, following FDA approval of the Apotex BLA, Apotex Corp. will sell the Apotex Pegfilgrastim Product that is the subject of the infringement claims in this action in the State of Florida and throughout the United States.

THE PATENTS-IN-SUIT

U.S. PATENT NO. 8,952,138

32. Amgen is the owner of all rights, title, and interest in the '138 Patent.

33. The '138 Patent is titled "Refolding Proteins Using a Chemically Controlled Redox State." The '138 Patent was duly and legally issued on February 10, 2015 by

the United States Patent and Trademark Office ("USPTO"). The inventors of the '138 Patent are Joseph Edward Shultz, Roger Hart, and Ronald Nixon Keener III. A true and correct copy of the '138 Patent is attached to this Complaint as Exhibit A.

34. The '138 Patent covers improved redox chemistry-based methodologies for efficiently refolding cysteine-containing proteins expressed in non-mammalian cells at high protein concentrations.

U.S. PATENT NO. 5,824,784

35. Amgen is the owner of all rights, title, and interest in the '784 Patent.

36. The '784 Patent is titled "N-Terminally Chemically Modified Protein Compositions and Methods." The '784 Patent was duly and legally issued on October 20, 1998 by the USPTO. The inventors of the '784 Patent are Olaf B. Kinstler, Nancy E. Gabriel, Christrine E. Farrer, and Randolf B. DePrince. A true and correct copy of the '784 Patent is attached to this Complaint as Exhibit B.

37. The '784 Patent relates, in part, to novel compositions of N-terminally chemically modified G-CSF, and to preparations of the same compositions, *e.g.* a substantially homogenous preparation of N-terminally PEGylated G-CSF, and methods of N-terminally modifying G-CSF and analogs thereof.

AMGEN'S NEULASTA® PRODUCT

38. The active ingredient in Amgen's Neulasta® 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 the G-CSF.

39. 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.

40. The availability of Neulasta® represented a major advance in cancer treatment by protecting chemotherapy patients from the harmful effects of neutropenia and by thus facilitating more effective chemotherapy regimes.

THE APOTEX PEGFILGRASTIM PRODUCT

41. On information and belief, Apotex filed the Apotex BLA under Section 351(k) of the Public Health Service Act to obtain approval to commercially manufacture, use, offer to sell, and sell, and import into the United States, a pegylated filgrastim product, the Apotex Pegfilgrastim Product, that is a biosimilar version of Amgen's Neulasta®.

42. On information and belief, the Apotex BLA listed Amgen's Neulasta® as a reference product.

43. On information and belief, Apotex has represented to FDA that its Pegfilgrastim Product is biosimilar to Amgen's Neulasta®. As such, the Apotex Pegfilgrastim Product should utilize the same mechanism of action as Neulasta® for the conditions of use prescribed, recommended, or suggested in Neulasta®'s approved label and the route of administration, the dosage form, and the strength of the Apotex Pegfilgrastim Product are the same as those of Amgen's Neulasta®. *See* 42 U.S.C. § 262(k)(2)(A)(i).

INFORMATION EXCHANGE UNDER 42 U.S.C. § 262(*l*)

44. On information and belief, Apotex filed a BLA 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 Apotex Pegfilgrastim Product, a biosimilar version of Amgen's Neulasta® (pegfilgrastim) product.

45. On information and belief, Apotex's BLA references and relies on the approval and licensure of Amgen's Neulasta® product in support of Apotex's request for FDA approval.

46. On December 16, 2014, Amgen received a letter from in-house counsel for Apotex Inc., notifying Amgen that the Apotex BLA had been accepted for review by FDA and that Apotex intended to provide Amgen the Apotex BLA pursuant to 42 U.S.C. § 262(*l*)(2).

47. Subsequently, Amgen received a copy of the Apotex BLA under the confidentiality provisions set forth in 42 U.S.C. § 262(*l*)(1).

48. Pursuant to 42 U.S.C. § 262(*l*)(3)(A), on February 27, 2015, Amgen provided Apotex a list of patents for which it believed a claim of patent infringement could reasonably be asserted against the Apotex Pegfilgrastim Product ("Amgen's (*l*)(3)(A) list"). Amgen's (*l*)(3)(A) list included the Patents in Suit.

49. On April 17, 2015, Apotex provided Amgen with its statements designated as being in accordance with 42 U.S.C. § 262(*l*)(3)(B).

50. On June 16, 2015, Amgen provided Apotex with a detailed statement, pursuant to 42 U.S.C. § 262(*l*)(3)(C).

51. Between June 22, 2015 and July 7, 2015, Amgen and Apotex engaged in good faith negotiations, pursuant to 42 U.S.C. § 262(*l*)(4). On July 7, 2015, Amgen and Apotex

agreed that the Patents in Suit should be the subject of any patent infringement action brought pursuant to 42 U.S.C. § 262(*l*)(6)(A).

NOTICE OF COMMERCIAL MARKETING UNDER 42 U.S.C. § 262(*l*)(8)

52. On April 17, 2015, Apotex sent Amgen a letter purporting to be Apotex's Notice of Commercial Marketing pursuant to 42 U.S.C. § 262(*l*)(8)(A).

53. Apotex's purported Notice of Commercial Marketing failed to specify a date on or after which it intends to commence commercial marketing of the Apotex Pegfilgrastim Product.

54. 42 U.S.C. § 262(*l*)(8)(A) states that the "subsection (k) applicant shall provide notice to the reference product sponsor not later than 180 days before the date of the first commercial marketing of the biological product licensed under subsection (k)." The Court of Appeals for the Federal Circuit has held that under subsection 262(*l*)(8)(A) "a subsection (k) applicant may only give effective notice of commercial marketing after FDA has licensed its product." *Amgen v. Sandoz*, No. 2015-1499, 2015 WL 4430108, at *9 (Fed. Cir. Jul. 21, 2015).

55. On information and belief, the Apotex Pegfilgrastim Product has not yet been licensed by FDA.

56. On information and belief, Apotex intends to market its Pegfilgrastim Product immediately upon receiving FDA approval.

57. On information and belief, Apotex will not provide Amgen with an effective Notice of Commercial Marketing under 42 U.S.C. § 262(*l*)(8)(A) after it receives FDA licensure and 180 days before it begins to commercially market the Apotex Pegfilgrastim Product.

58. Amgen brings this action to lift the cloud created by the imminent threat of Apotex's refusal to provide a legally effective Notice of Commercial Marketing pursuant to

42 U.S.C. § 262(*l*)(8)(A). Without declaratory relief, the threat of Apotex's violation of 42 U.S.C. § 262(*l*)(8)(A) poses a substantial risk to Amgen, and impedes Amgen's ability to exercise its rights provided under 42 U.S.C. § 262(*l*) and 35 U.S.C. § 271.

FIRST COUNT
(INFRINGEMENT OF THE '138 PATENT)

59. The allegations of ¶¶ 1-58 are incorporated herein by reference.

60. On information and belief, Apotex seeks FDA approval under Section 351(k) of the Public Health Service Act to manufacture and sell the Apotex Pegfilgrastim Product, a biosimilar version of Amgen's Neulasta® (pegfilgrastim) product.

61. On information and belief, Apotex intends to manufacture, use, sell, offer for sale, and/or import the Apotex Pegfilgrastim Product prior to the expiration of the '138 Patent.

62. The submission and filing of Apotex's subsection (k) application for the purpose of obtaining approval to engage in the commercial manufacture, use, sale, offer for sale, and/or importation of the Apotex Pegfilgrastim Product before the expiration of the '138 Patent is an act of infringement of one or more claims of the '138 Patent under 35 U.S.C. § 271(e)(2)(C).

63. Apotex Corp.'s participation in, contribution to, inducement of, aiding or abetting the submission of the Apotex BLA to FDA constitutes direct, contributory, or induced infringement of one or more claims of the '138 Patent under 35 U.S.C. § 271(e)(2)(C)(i).

64. On information and belief, the manufacture, use, sale, offer for sale, and/or importation of the Apotex Pegfilgrastim Product will infringe one or more claims of the '138 Patent.

65. Amgen will be irreparably harmed if Apotex is not enjoined from infringing or actively inducing or contributing to infringement of one or more claims of the '138 Patent. Amgen is entitled to injunctive relief under 35 U.S.C. § 271(e)(4)(B) preventing Apotex from any further infringement. Amgen does not have an adequate remedy at law.

66. To the extent Apotex commercializes its product prior to the expiration of the '138 Patent, Amgen will also be entitled to damages under 35 U.S.C. § 284.

SECOND COUNT
(INFRINGEMENT OF THE '784 PATENT)

67. The allegations of ¶¶ 1-66 are incorporated herein by reference.

68. On information and belief, Apotex seeks FDA approval under Section 351(k) of the Public Health Service Act to manufacture and sell the Apotex Pegfilgrastim Product, a biosimilar version of Amgen's Neulasta® (pegfilgrastim) product.

69. The submission and filing of Apotex's subsection (k) application for the purpose of obtaining approval to engage in the commercial manufacture, use, sale, offer for sale, and/or importation of the Apotex Pegfilgrastim Product before the expiration of the '784 Patent is an act of infringement of one or more claims of the '784 Patent under 35 U.S.C. § 271(e)(2)(C).

70. Apotex Corp.'s participation in, contribution to, inducement of, aiding or abetting the submission of the Apotex BLA to FDA constitutes direct, contributory, or induced infringement of one or more claims of the '784 Patent under 35 U.S.C. § 271(e)(2)(C)(i).

71. On information and belief, the manufacture, use, sale, offer for sale, and/or importation of the Apotex Pegfilgrastim Product will infringe one or more claims of the '784 Patent.

72. Amgen will be irreparably harmed if Apotex is not enjoined from infringing or actively inducing or contributing to infringement of one or more claims of the '784 Patent. Amgen is entitled to injunctive relief under 35 U.S.C. § 271(e)(4)(B) preventing Apotex from any further infringement. Amgen does not have an adequate remedy at law.

73. To the extent Apotex commercializes its product prior to the expiration of the '784 Patent, Amgen will also be entitled to damages under 35 U.S.C. § 284.

THIRD COUNT
(DECLARATORY JUDGMENT OF INFRINGEMENT OF THE '138 PATENT)

74. The allegations of ¶¶ 1-73 are incorporated herein by reference.

75. On information and belief, Apotex seeks FDA approval under Section 351(k) of the Public Health Service Act to manufacture and sell the Apotex Pegfilgrastim Product, a biosimilar version of Amgen's Neulasta® (pegfilgrastim) product.

76. Upon information and belief, Apotex intends to, and will, manufacture, use, offer to sell, or sell within the United States, or import into the United States, the Apotex Pegfilgrastim Product immediately and imminently upon FDA licensure of the Apotex BLA.

77. If Apotex manufactures, uses, offers to sell, or sells within the United States, or imports into the United States, the Apotex Pegfilgrastim Product prior to the expiration of the '138 Patent, Apotex will infringe one or more claims of the '138 Patent under 35 U.S.C. § 271 (a) and/or (g).

78. An actual controversy has arisen and now exists between the parties concerning whether the Apotex Pegfilgrastim Product will infringe one or more claims of the '138 Patent.

79. Amgen is entitled to a declaratory judgment that Apotex will infringe one or more claims of the '138 Patent by making, using, offering to sell, or selling within the United

States, or importing into the United States, the Apotex Pegfilgrastim Product prior to the expiration of the '138 Patent.

80. Amgen is entitled to injunctive relief preventing Apotex from making, using, offering to sell, or selling within the United States, or importing into the United States, the Apotex Pegfilgrastim Product prior to the expiration of the '138 Patent. Amgen does not have an adequate remedy at law.

FOURTH COUNT
(DECLARATORY JUDGMENT THAT APOTEX'S NOTICE OF COMMERCIAL MARKETING VIOLATES 42 U.S.C. § 262(*l*)(8)(A))

81. The allegations of ¶¶ 1-80 are incorporated herein by reference.

82. To comply with 42 U.S.C. § 262(*l*)(8)(A), Apotex must provide notice to Amgen "not later than 180 days before the date of the first commercial marketing of the biological product licensed under subsection (k)."

83. Amgen received a letter from Apotex dated April 17, 2015, in which Apotex purported to provide notice of commercial marketing of the Apotex Pegfilgrastim Product, *which has not yet been approved for licensure by FDA*. This purported notice is ineffective because, *inter alia*, a subsection (k) applicant may only give effective notice of commercial marketing after FDA has licensed its product, and the purported notice failed to specify a date on or after which Apotex intends to commence commercial marketing of the Apotex Pegfilgrastim Product.

84. Upon information and belief, Apotex intends to rely upon its April 17, 2015 notice, and will not provide Amgen with a notice *after* the Apotex Pegfilgrastim Product has been licensed by FDA.

85. Upon information and belief, Apotex intends to begin commercial marketing of the Apotex Pegfilgrastim Product less than 180 days after the Apotex Pegfilgrastim Product has been licensed by FDA.

86. An actual controversy has arisen and now exists between the parties concerning whether Apotex's purported notice of April 17, 2015 is legally effective.

87. Apotex's purported notice of April 17, 2015 is legally ineffective, and Amgen is entitled to a declaratory judgment that Apotex is in violation of 42 U.S.C. § 262(*l*)(8)(A).

88. Amgen is entitled to injunctive relief under 42 U.S.C. § 262(*l*)(8)(A) preventing Apotex from engaging in commercial marketing of the Apotex Pegfilgrastim Product until a date that is at least 180 days after Apotex provides effective notice to Amgen under 42 U.S.C. § 262(*l*)(8)(A). Amgen does not have an adequate remedy at law.

PRAYER FOR RELIEF

WHEREFORE, Amgen respectfully requests that this Court enter judgment in its favor against Apotex and grant the following relief:

A. a judgment that Apotex has infringed directly, contributed to, or induced the infringement of one or more claims of the '138 Patent under 35 U.S.C. § 271(e)(2)(C)(i), by submitting to FDA the Apotex BLA to obtain approval for the commercial manufacture, use, offer for sale, sale, distribution in, or importation into the United States of the Apotex Pegfilgrastim Product before the expiration of the '138 Patent;

B. a preliminary and/or permanent injunction that enjoins Apotex, their officers, partners, agents, servants, employees, attorneys, affiliates, divisions, subsidiaries, other related business entities, and those persons in active concert or participation with any of them from infringing the '138 Patent, or contributing to or inducing anyone to do the same, by acts

including the manufacture, use, offer to sell, sale, distribution, or importation of any current or future versions of a product that infringes, or the use or manufacture of which infringes the ’138 Patent;

C. a judgment declaring that the manufacture, use, offer to sell, sale, distribution, or importation of the products described in the Apotex BLA would constitute infringement of one or more claims of the ’138 Patent, or inducement of or contribution to such conduct, by Apotex pursuant to 35 U.S.C. § 271 (a), (b), (c), or (g);

D. a judgment that Apotex has infringed directly, contributed to, or induced the infringement of one or more claims of the ’784 Patent under 35 U.S.C. § 271(e)(2)(C)(i), by submitting to FDA the Apotex BLA to obtain approval for the commercial manufacture, use, offer for sale, sale, distribution in, or importation into the United States of the Apotex Pegfilgrastim Product before the expiration of the ’784 Patent;

E. a preliminary and/or permanent injunction that enjoins Apotex, their officers, partners, agents, servants, employees, attorneys, affiliates, divisions, subsidiaries, other related business entities, and those persons in active concert or participation with any of them from infringing the ’784 Patent, or contributing to or inducing anyone to do the same, by acts including the manufacture, use, offer to sell, sale, distribution, or importation of any current or future versions of a product that infringes, or the use or manufacture of which infringes the ’784 Patent;

F. a judgment declaring that the manufacture, use, offer to sell, sale, distribution, or importation of the products described in the Apotex BLA would constitute infringement of one or more claims of the ’784 Patent, or inducement of or contribution to such conduct, by Apotex pursuant to 35 U.S.C. § 271 (a), (b), (c), or (g);

G. a declaration that the notice of commercial marketing that Apotex provided on April 17, 2015 is ineffective under 42 U.S.C. § 262(*l*)(8)(A);

H. a declaration that Apotex will be in violation of 42 U.S.C. § 262(*l*)(8)(A) by not providing Amgen with an effective notice of commercial marketing after the Apotex Pegfilgrastim Product is licensed by FDA and at least 180 days before Apotex begins commercial marketing of the Apotex Pegfilgrastim Product;

I. a preliminary and/or permanent injunction that enjoins Apotex, its officers, partners, agents, servants, employees, attorneys, affiliates, divisions, subsidiaries, other related business entities, and those persons in active concert or participation with any of them from commencing commercial marketing of the Apotex Pegfilgrastim Product until a date that is at least 180 days after Apotex provides effective notice to Amgen under 42 U.S.C. § 262(*l*)(8)(A);

J. a judgment compelling Apotex to pay to Amgen damages adequate to compensate for Apotex's infringement, in accordance with 35 U.S.C. § 284;

K. a declaration that this is an exceptional case and an award to Amgen of its attorneys' fees and costs pursuant to 35 U.S.C. § 285; and

L. such other relief as this Court may deem just and proper.

Dated: August 6, 2015

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EXHIBIT A

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Shultz et al.

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

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

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C07K 1/22 (2006.01)
C07K 1/113 (2006.01)

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

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

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(57) **ABSTRACT**

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

24 Claims, 8 Drawing Sheets

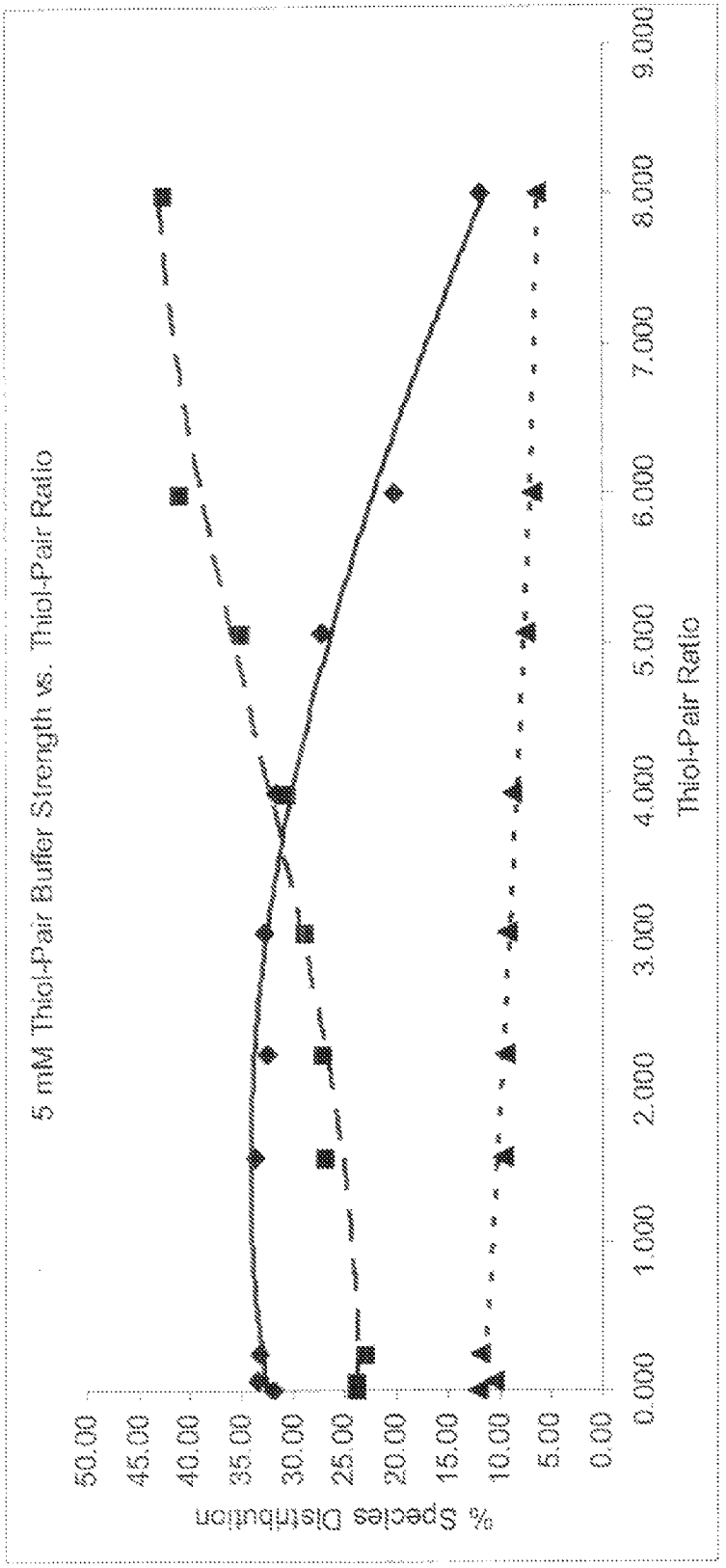
5 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
% Species Distribution
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0.000
1.000
2.000
3.000
4.000
5.000
6.000
7.000
8.000
9.000
Thiol-Pair Ratio

Figure 1a

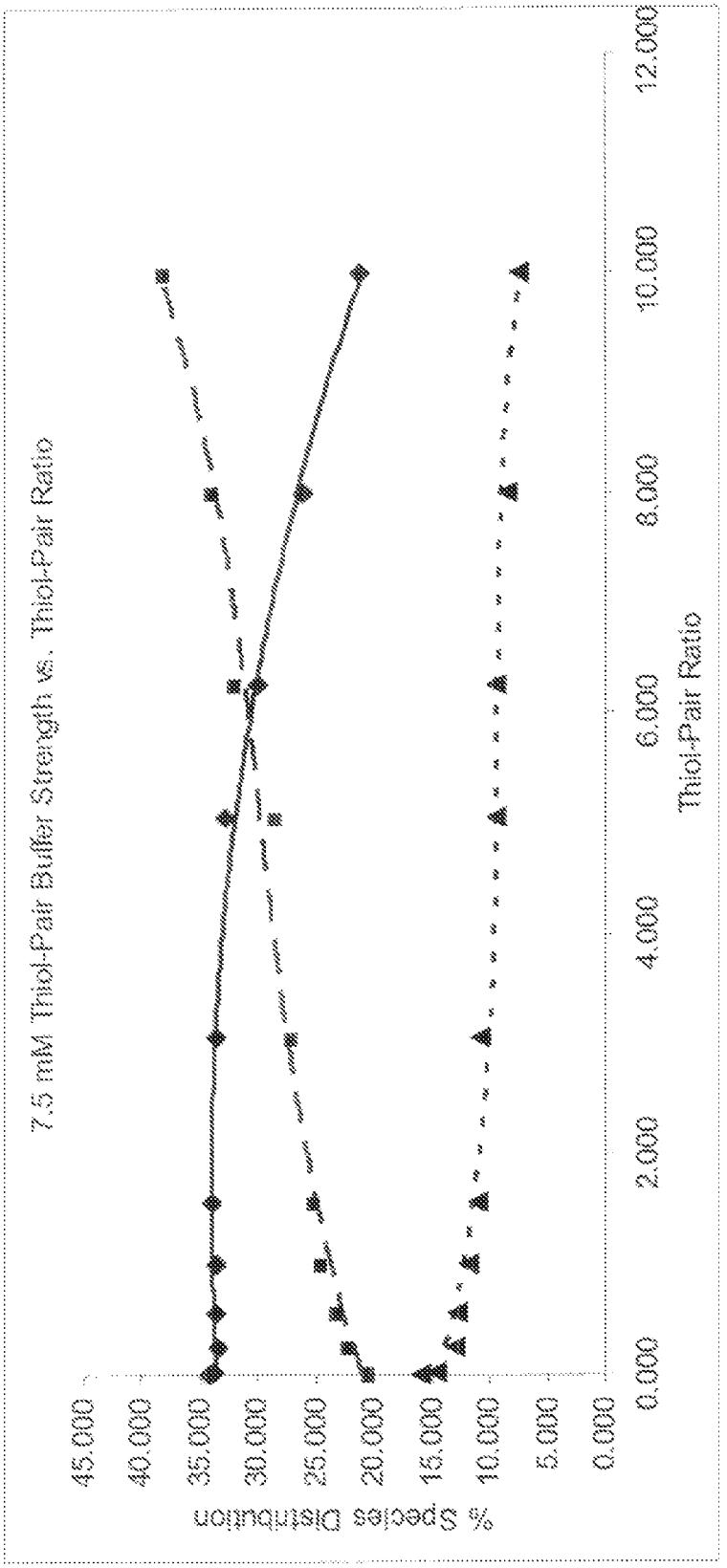
7.5 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
% Species Distribution
45.000
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
0.000
2.000
4.000
6.000
8.000
10.000
12.000
Thiol-Pair Ratio

Figure 1b

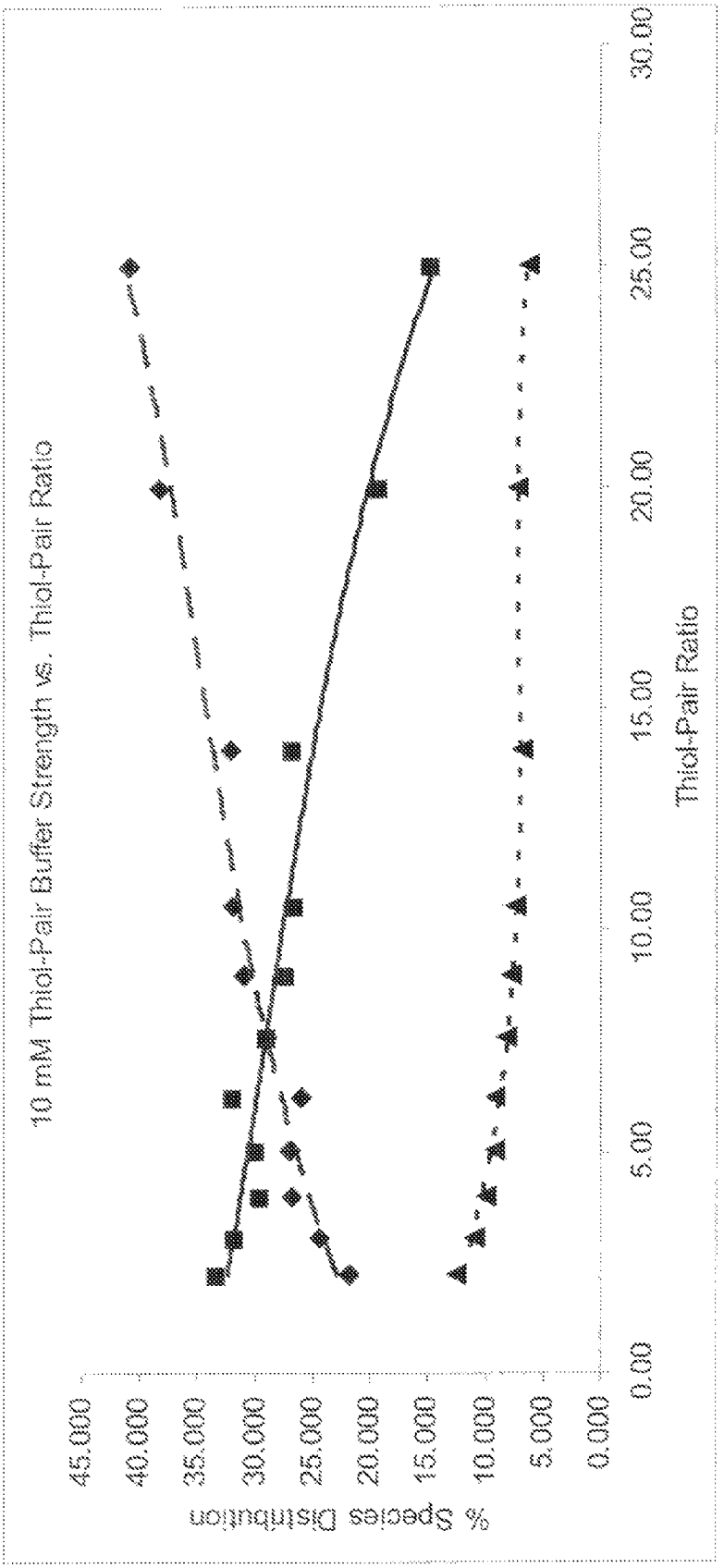
10 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
% Species Distribution
45.000
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
0.00
5.00
10.00
15.00
20.00
25.00
30.00
Thiol-Pair Ratio

Figure 1C

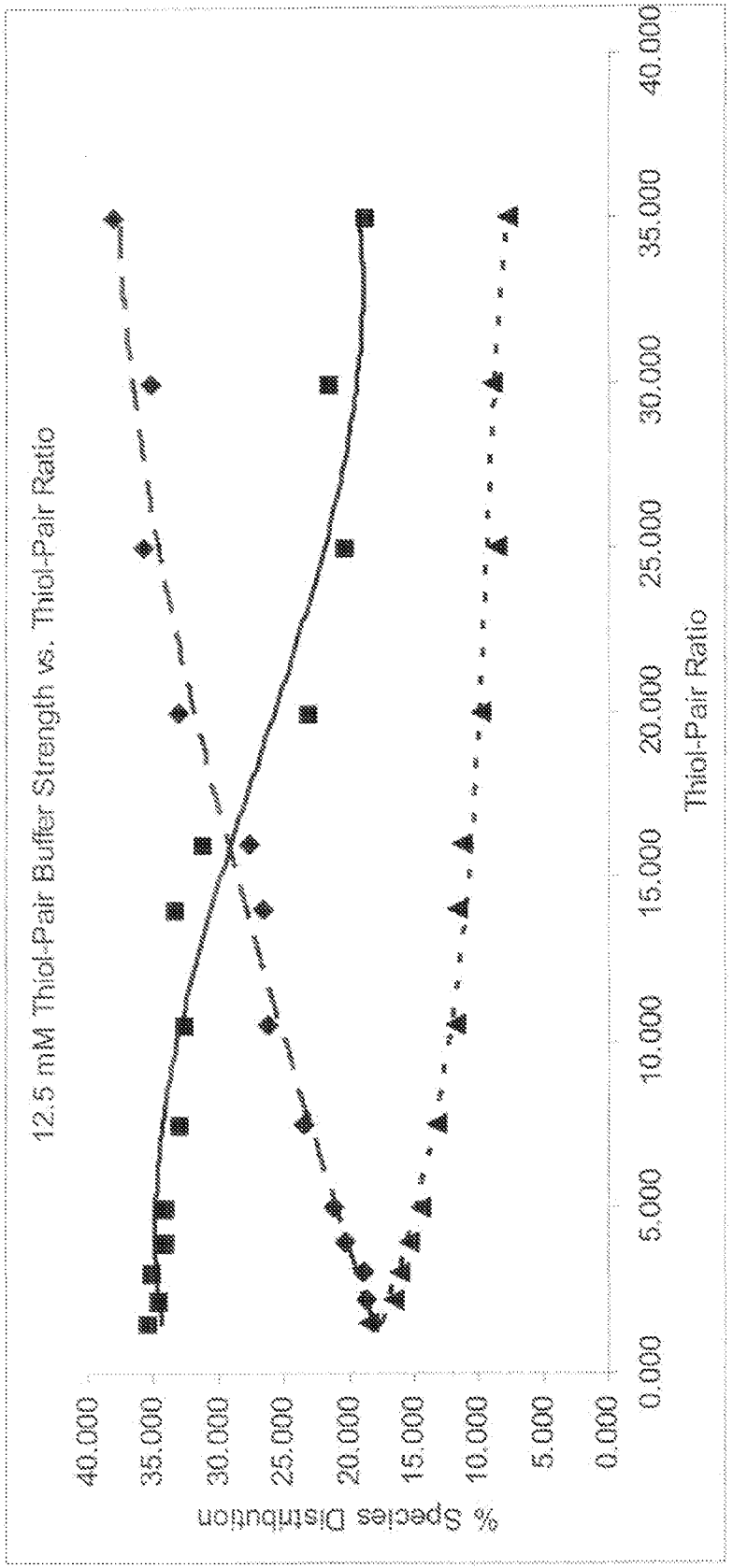
12.5 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
% Species Distribution
Thiol-Pair Ratio
0.000
5.000
10.000
15.000
20.000
25.000
30.000
35.000
40.000

Figure 1d

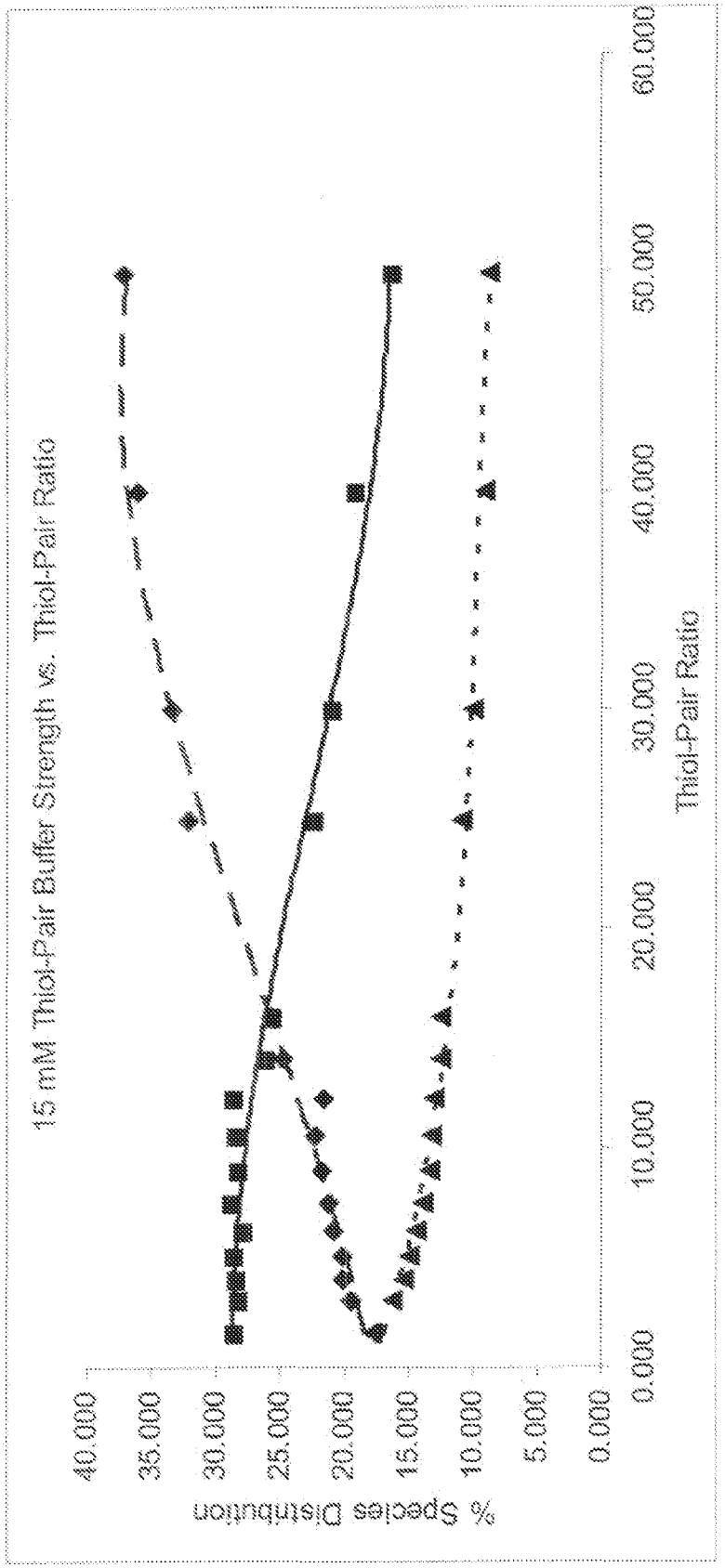
15 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
% Species Distribution
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
0.000
10.000
20.000
30.000
40.000
50.000
60.000
Thiol-Pair Ratio

Figure 1e

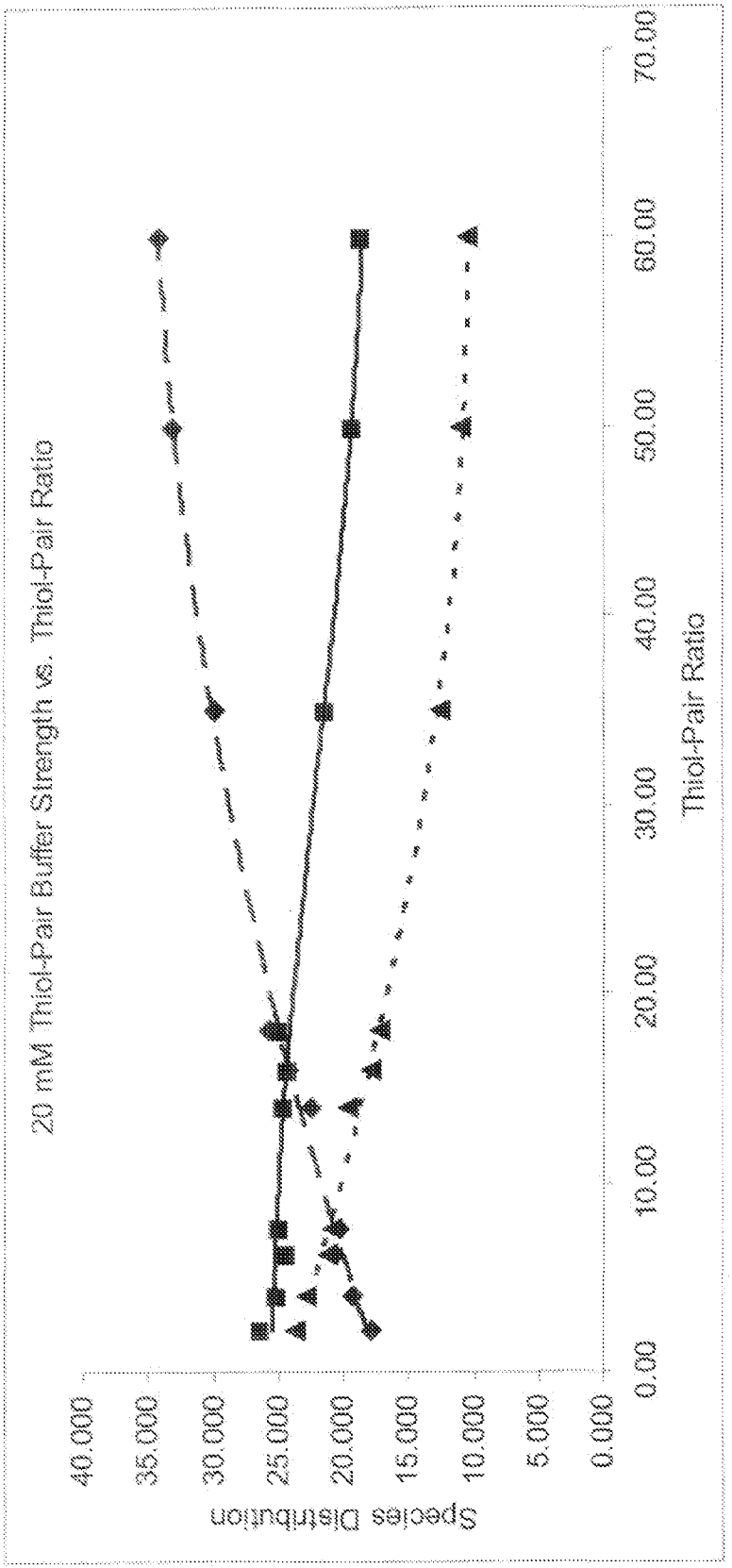
20 mM Thiol-Pair Buffer Strength vs. Thiol-Pair Ratio
Species Distribution
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
Thiol-Pair Ratio

Figure 1f

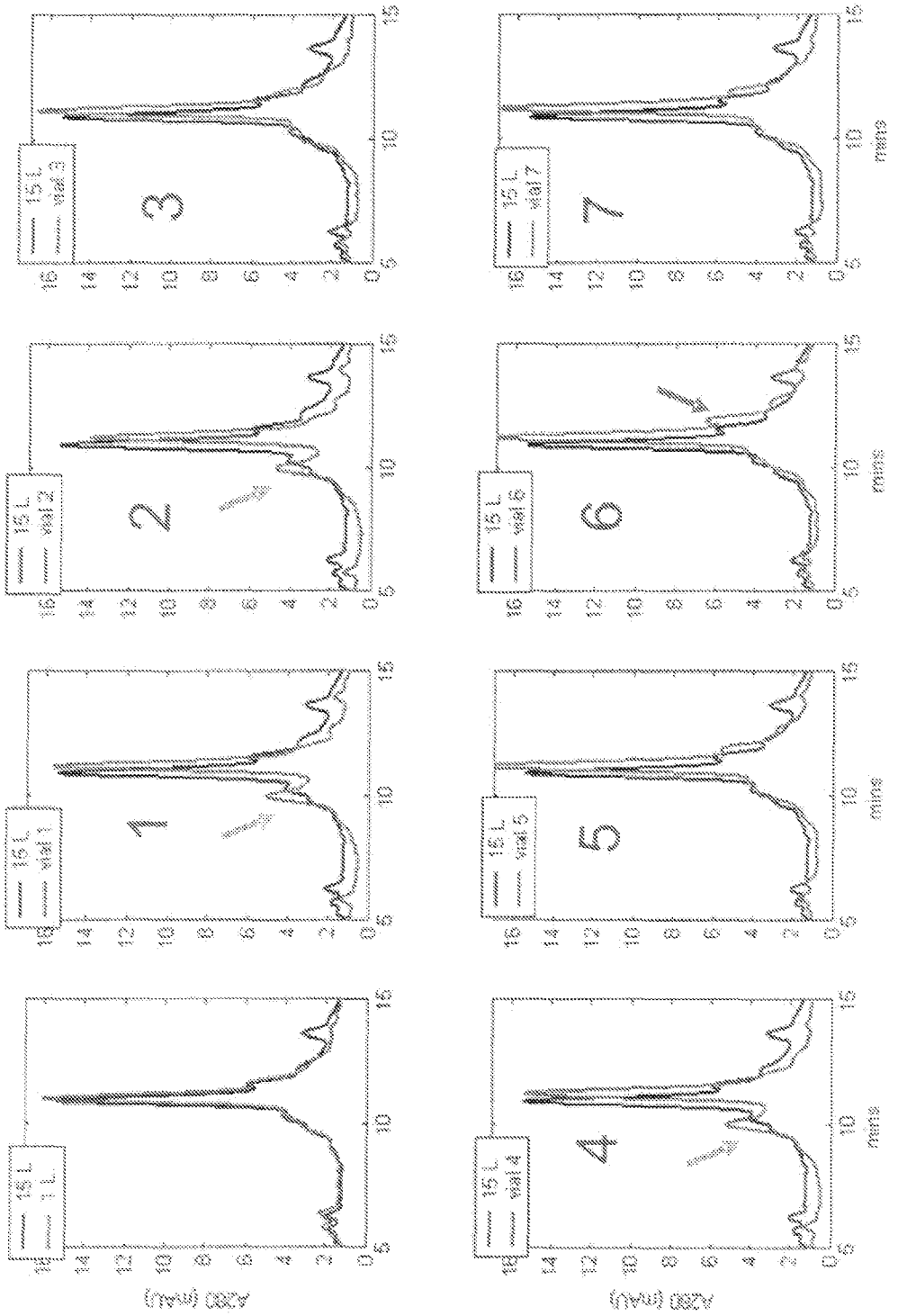

Figure 2

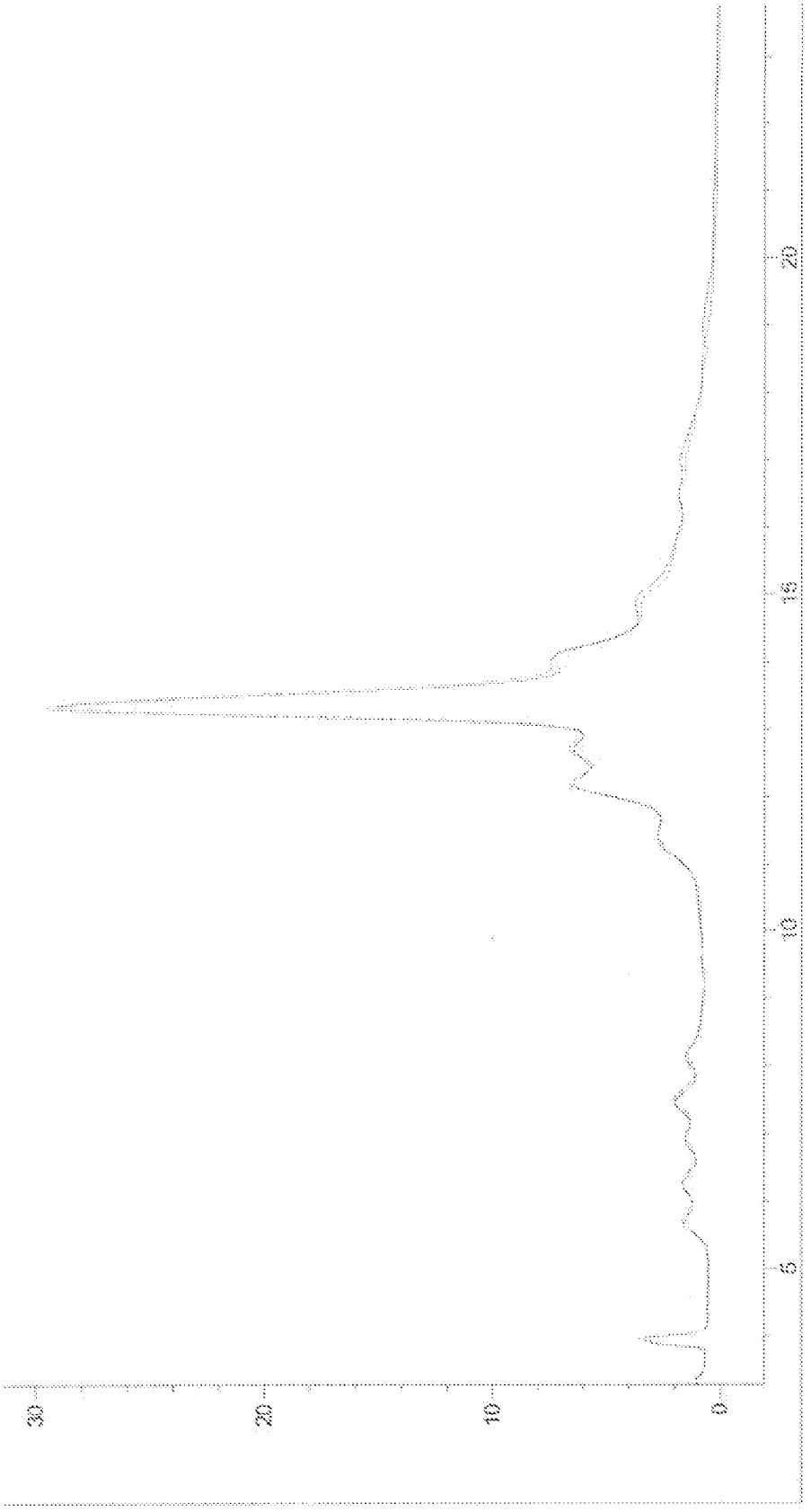

Figure 3

REFOLDING PROTEINS USING A CHEMICALLY CONTROLLED REDOX STATE

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

FIELD OF THE INVENTION

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

BACKGROUND OF THE INVENTION

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

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

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

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

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

BRIEF DESCRIPTION OF THE DRAWINGS

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

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

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

SUMMARY OF THE INVENTION

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

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

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

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

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

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

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

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

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

DETAILED DESCRIPTION OF THE INVENTION

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

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

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

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

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

I. Definitions

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Definition of Buffer Thiol-Pair Buffer Strength/Thiol Buffer Strength (BS) Thiol-Pair Buffer Strength=2[oxidant]+[reductant]=2[cystamine]+[cysteine] Equation 2.

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

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

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

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

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

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

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

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

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

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

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

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

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

II. Theory

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

EXAMPLES

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

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

Example 1

Expression of Recombinant Protein

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

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

Example 2

Identification of Refold Conditions/Redox Components

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

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

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

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

Example 3

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

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

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

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

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

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

Example 4

High Concentration Refolding from Inclusion Bodies

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

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

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

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

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

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

Example 5

Effect of Thiol-Pair Oxidation State on Disulfide Pairings

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

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

Example 6

Effect of Non-Aerobic Conditions on Refolding Efficiency

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

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

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

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

What is claimed is:

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

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

(i) a denaturant;

(ii) an aggregation suppressor; and

(iii) a protein stabilizer;

to form a refold mixture;

(b) incubating the refold mixture; and

(c) isolating the protein from the refold mixture.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

* * * * *

EXHIBIT B

US005824784A

United States Patent [19]

Kinstler et al.

[11] **Patent Number: 5,824,784**

[45] **Date of Patent: Oct. 20, 1998**

[54] **N-TERMINALLY CHEMICALLY MODIFIED PROTEIN COMPOSITIONS AND METHODS**

[75] Inventors: **Olaf B. Kinstler**, Thousand Oaks; **Nancy E. Gabriel**; **Christine E. Farrar**, both of Newbury Park, all of Calif.; **Randolph B. DePrince**, Raleigh, N.C.

[73] Assignee: **Amgen Inc.**, Thousand Oaks, Calif.

[21] Appl. No.: **321,510**

[22] Filed: **Oct. 12, 1994**

[51] **Int. Cl.**[6] **A61K 38/18**; C07K 1/00

[52] **U.S. Cl.** **530/399**; 530/350; 530/402; 530/410

[58] **Field of Search** 530/351, 410, 530/350, 399, 402; 424/85.1, 85.4

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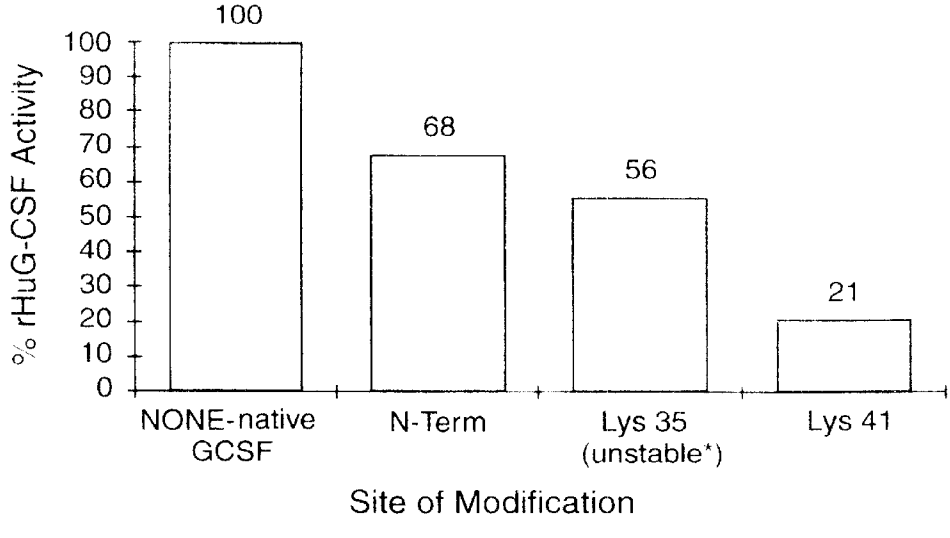
Primary Examiner—Ponnathapura Achutamurthy

Attorney, Agent, or Firm—Robert B. Winter; Karol M. Pessin; Steven M. Odre

[57] **ABSTRACT**

Provided herein are methods and compositions relating to the attachment of water soluble polymers to proteins. Provided are novel methods for N-terminally modifying proteins or analogs thereof, and resultant compositions, including novel N-terminally chemically modified G-CSF compositions and related methods of preparation. Also provided is chemically modified consensus interferon.

12 Claims, 15 Drawing Sheets



* contains de-Pegylated rHuG-CSF, generated during storage.

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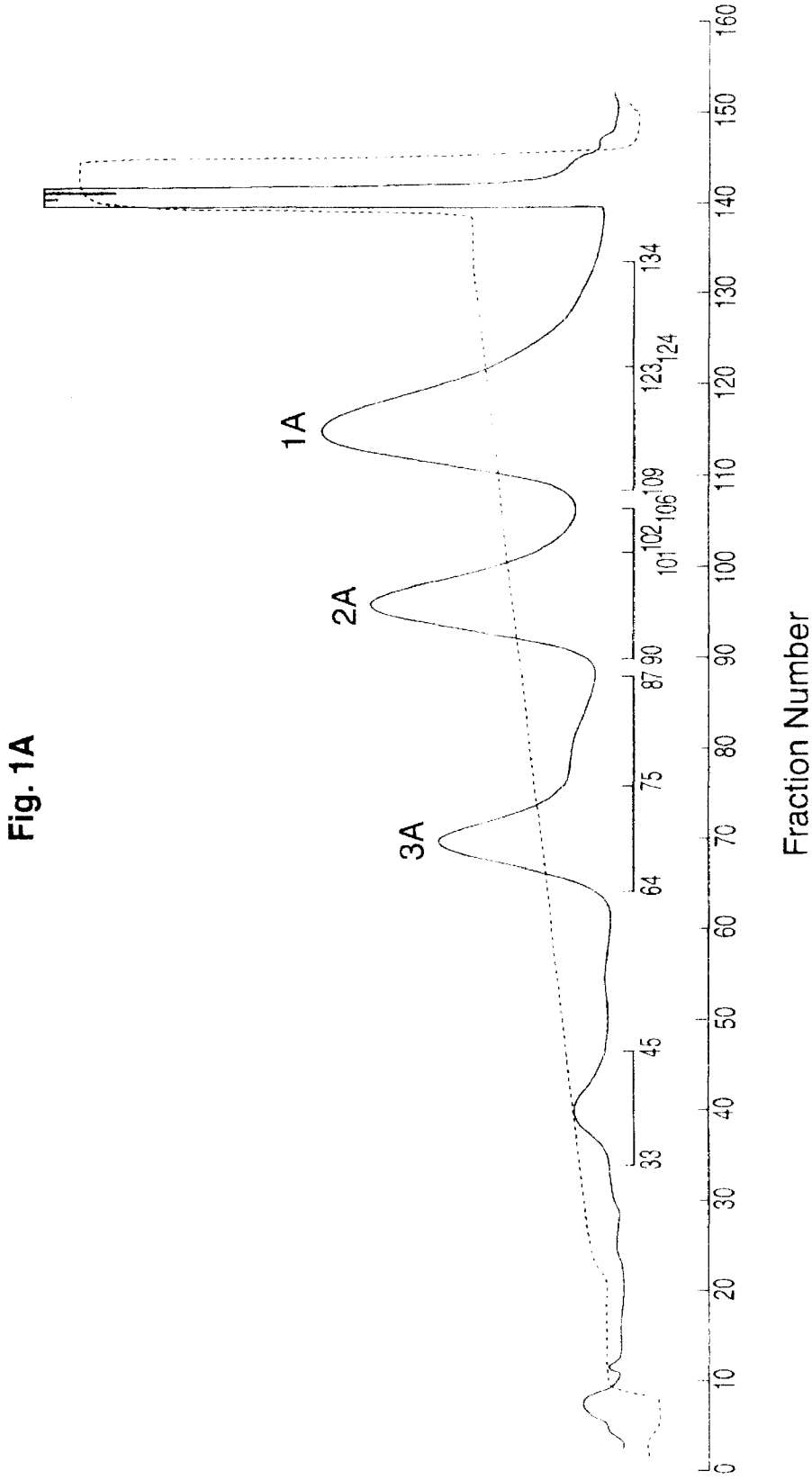
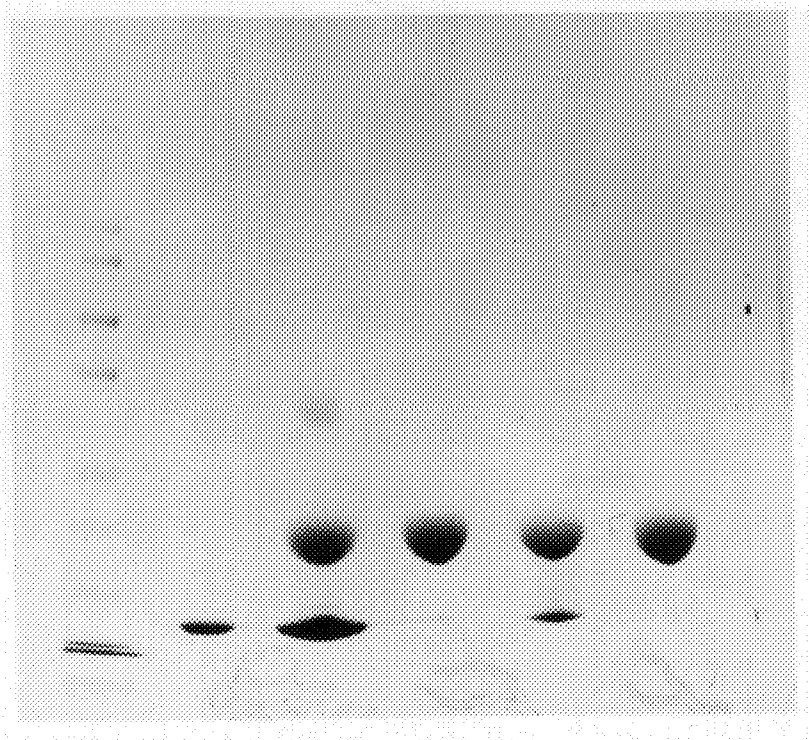

1A
2A
3A
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
Fraction Number
33
45
64
75
87
90
101
102
106
109
123
124
134

Fig. 1A

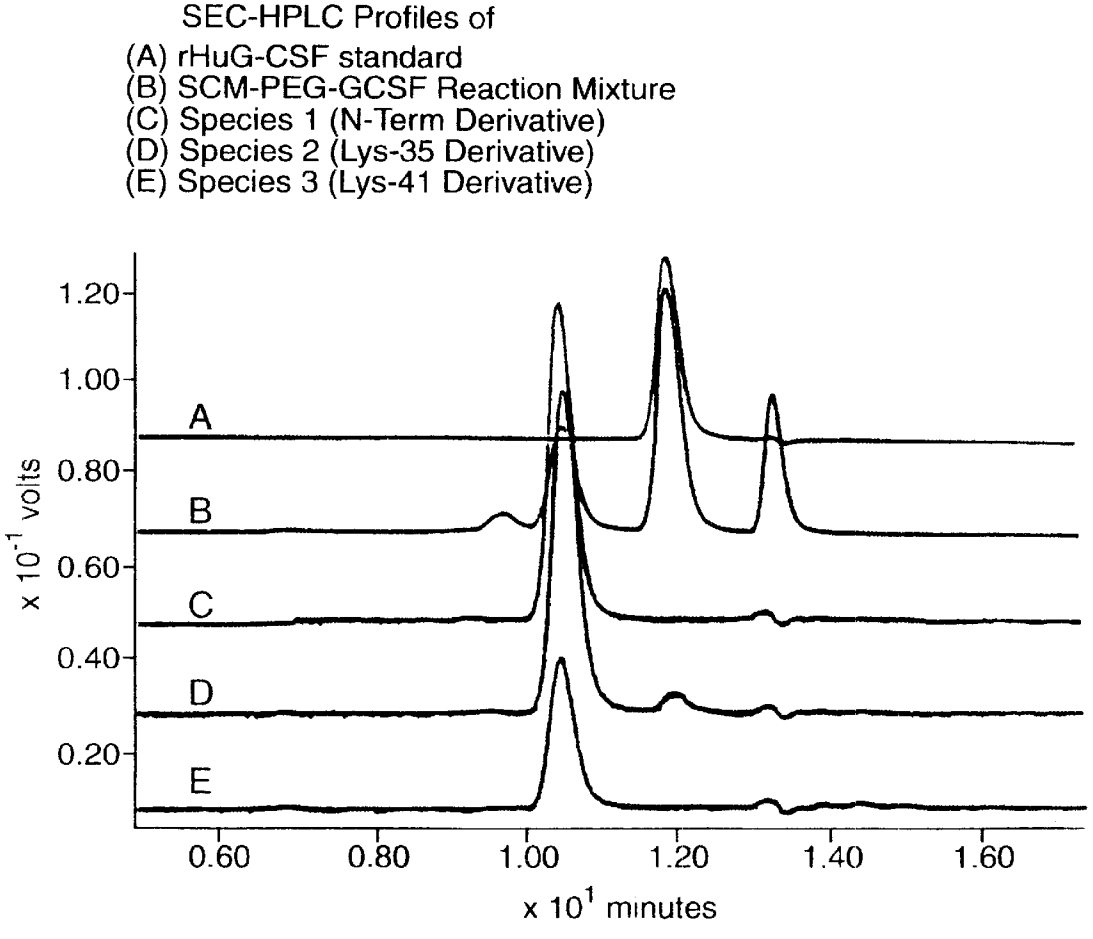
Fig. 1B



Lane No. 1 2 3 4 5 6

Lane No.	Sample	ug loaded
1	MW Protein Standards	-
2	rHuG-CSF Std	3.0
3	SCM-PEG-GCSF Reaction Mix	10.0
4	Species 1 (N-Term)	10.0
5	Species 2 (Lys-35)	10.0
6	Species 3 (Lys-41)	10.0

Fig. 2


SEC-HPLC Profiles of
(A) rHuG-CSF standard
(B) SCM-PEG-GCSF Reaction Mixture
(C) Species 1 (N-Term Derivative)
(D) Species 2 (Lys-35 Derivative)
(E) Species 3 (Lys-41 Derivative)
1.20
1.00
0.80
0.60
0.40
0.20
x 10-1 volts
A
B
C
D
E
0.60
0.80
1.00
1.20
1.40
1.60
x 10¹ minutes

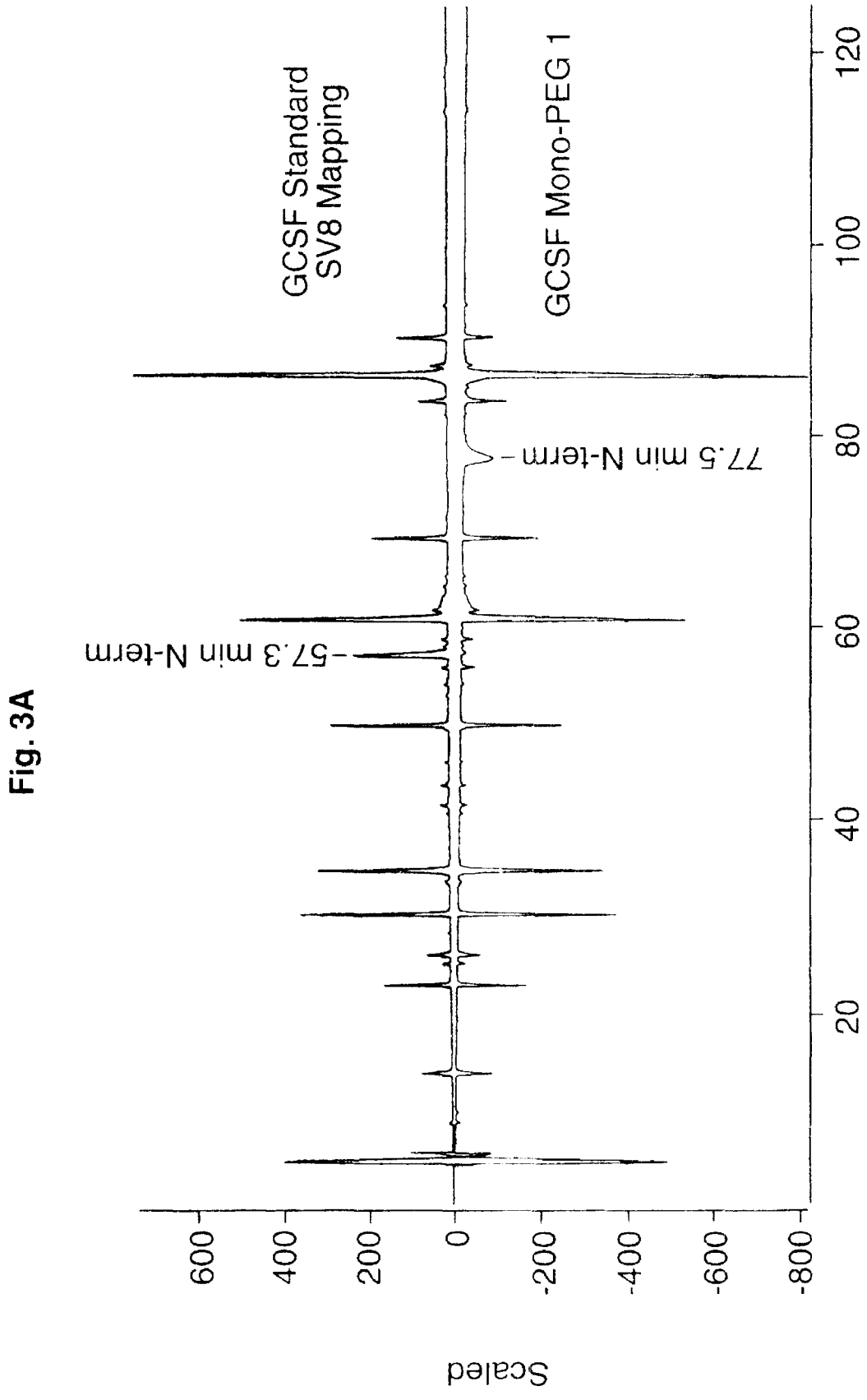

GCSF Standard
SV8 Mapping
GCSF Mono-PEG 1
57.3 min N-term
77.5 min N-term
Scaled
600
400
200
0
-200
-400
-600
-800
20
40
60
80
100
120

Fig. 3A

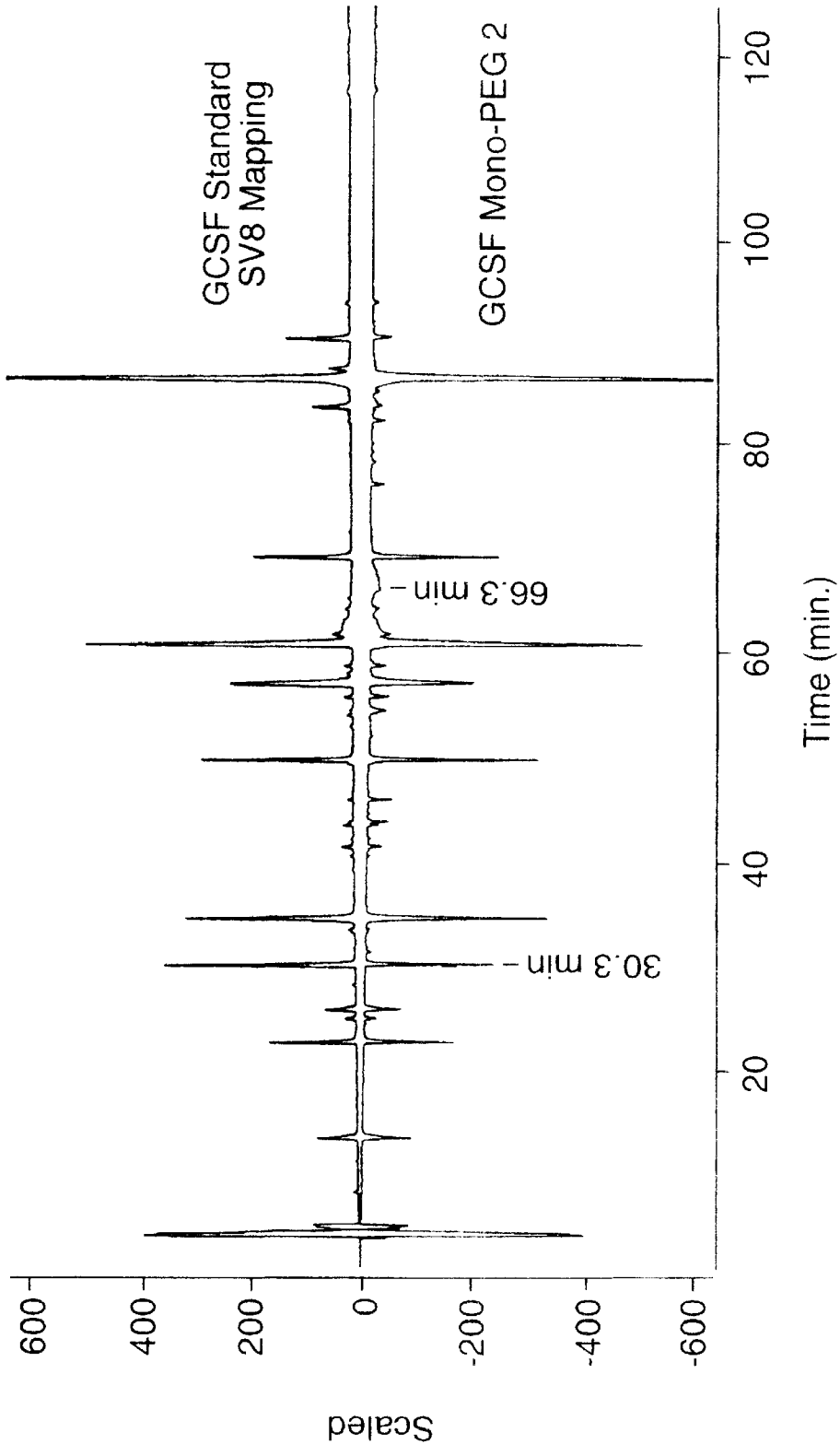

GCSF Standard
SV8 Mapping
GCSF Mono-PEG 2
66.3 min
30.3 min
Scaled
600
400
200
0
-200
-400
-600
20
40
60
80
100
120
Time (min.)

Fig. 3B

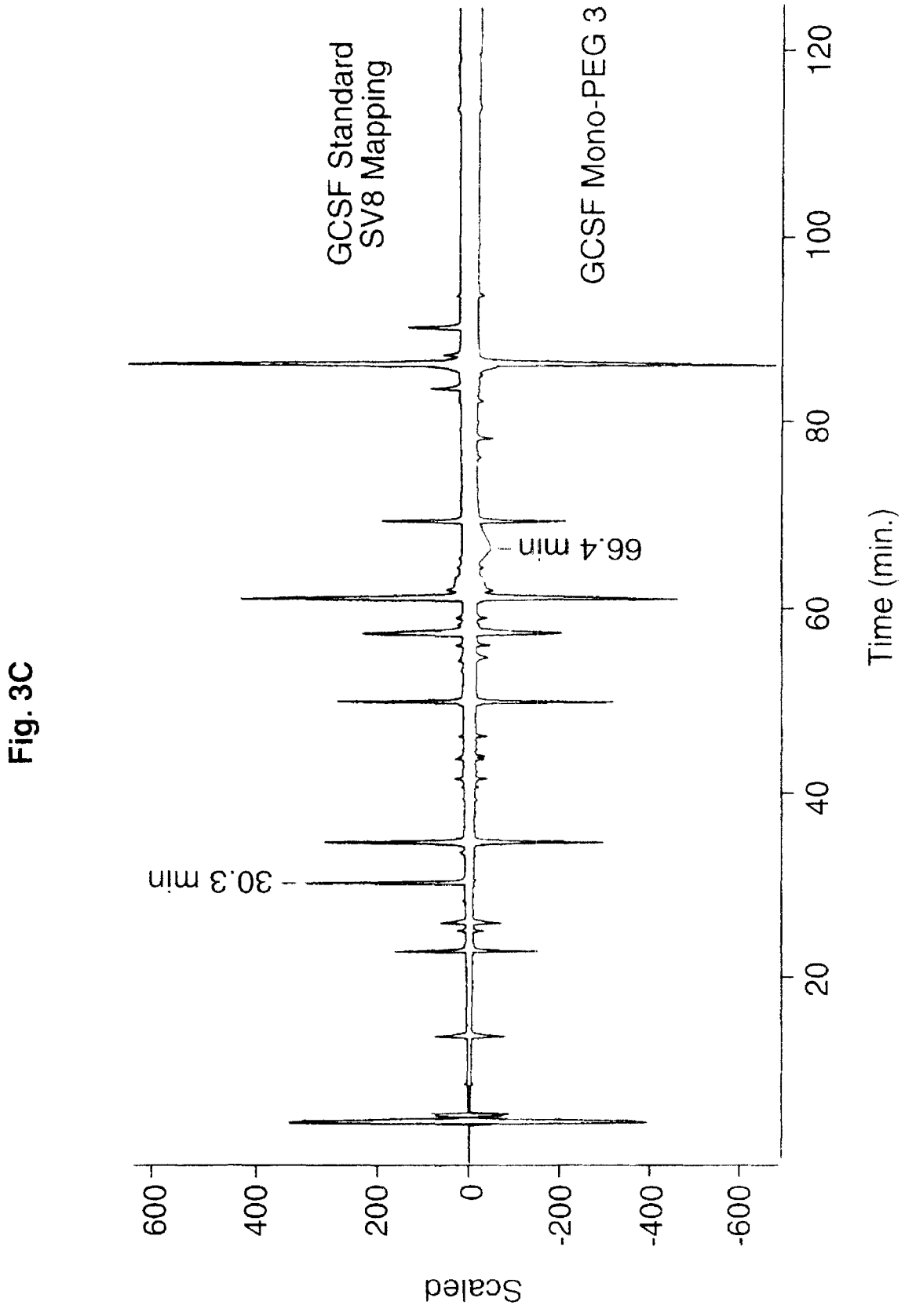
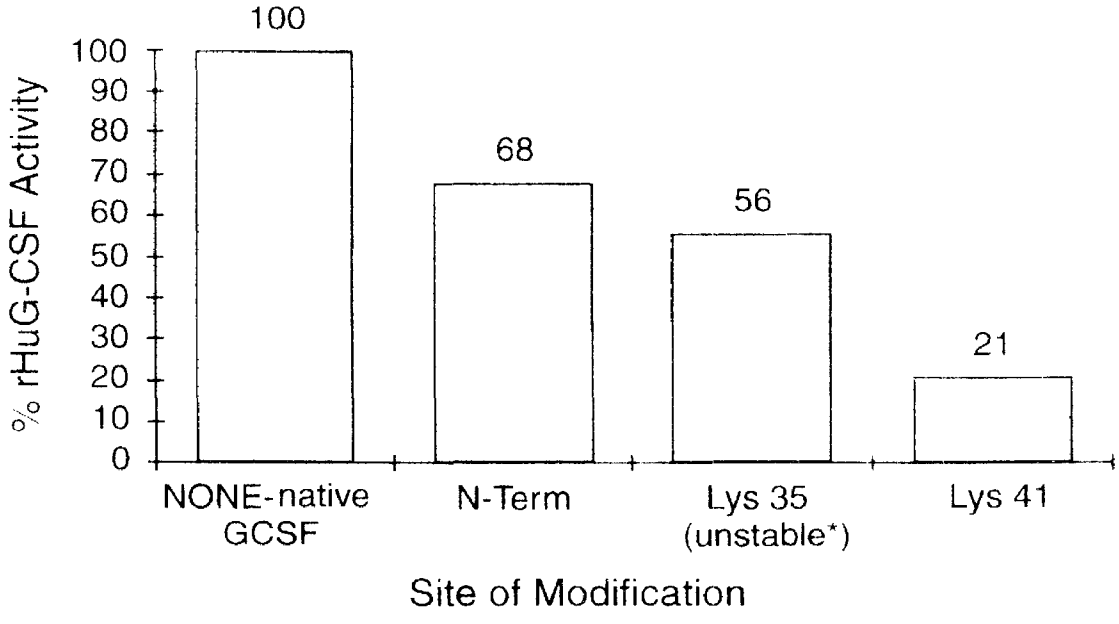

GCSF Standard
SV8 Mapping
GCSF Mono-PEG 3
66.4 min
30.3 min
Scaled
600
400
200
0
-200
-400
-600
20
40
60
80
100
120
Time (min.)

Fig. 3C

Fig. 4



* contains de-Pegylated rHuG-CSF, generated during storage.

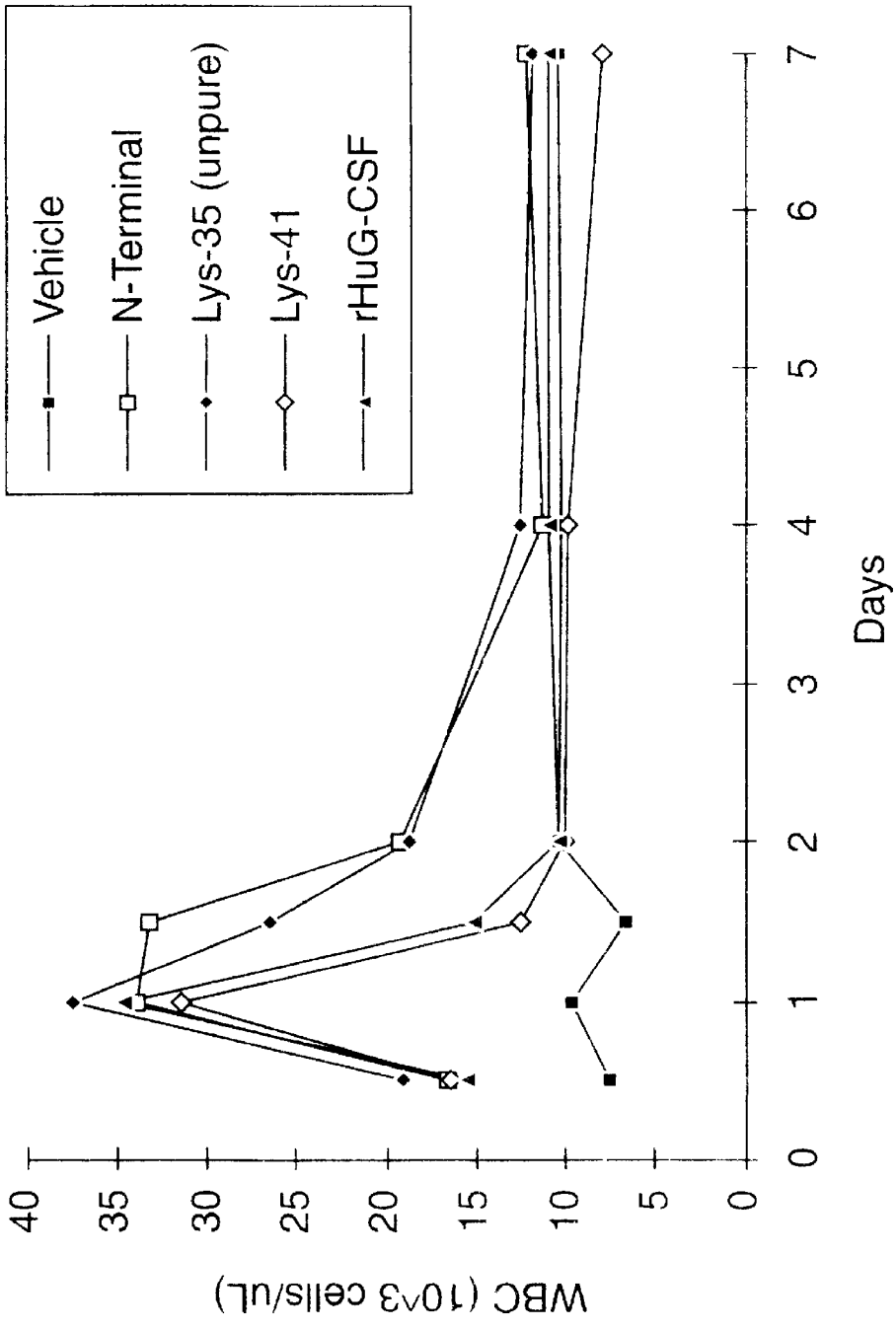

Vehicle
N-Terminal
Lys-35 (unpure)
Lys-41
rHuG-CSF
WBC (10^3 cells/uL)
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
Days

Fig. 5A

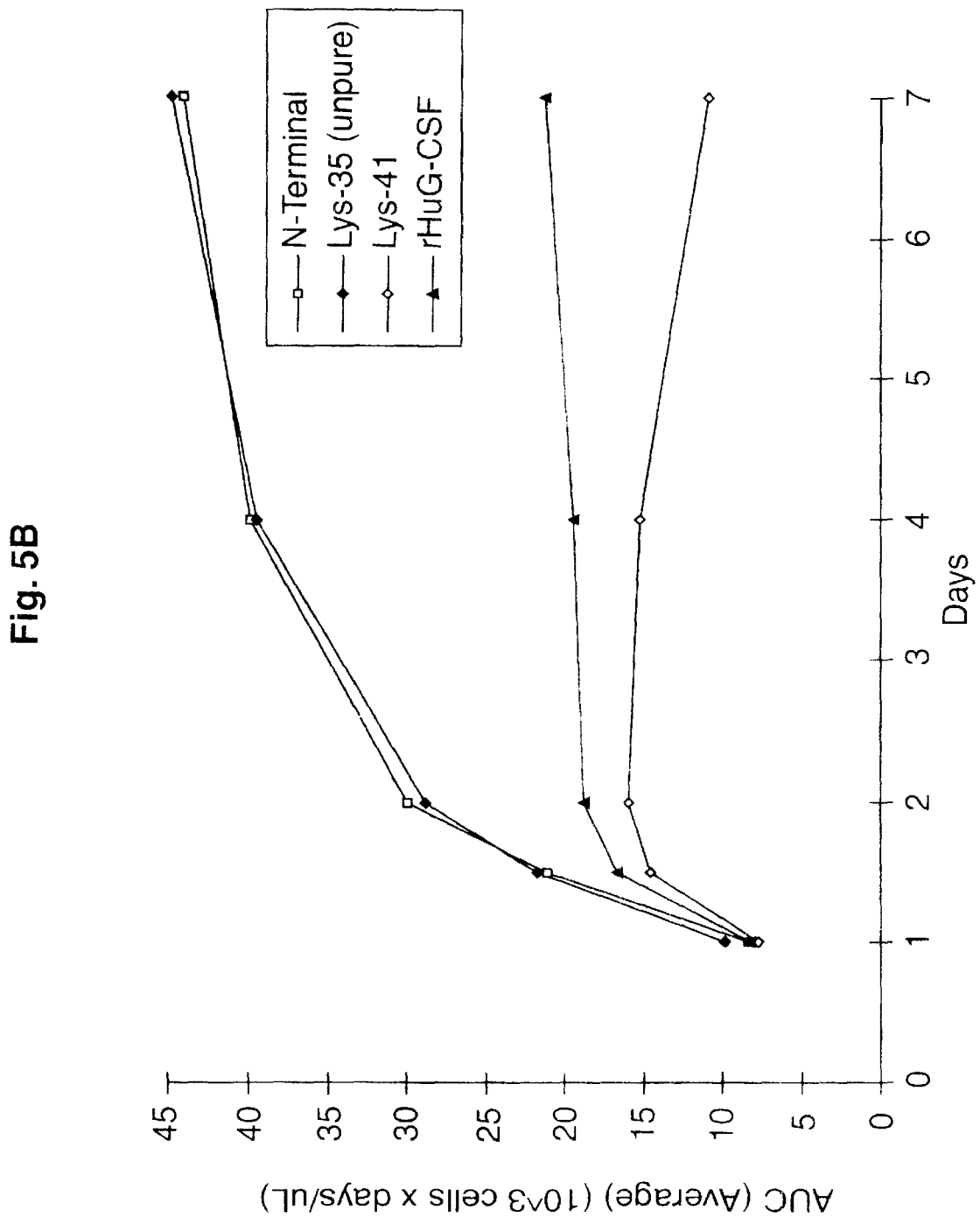

N-Terminal
Lys-35 (unpure)
Lys-41
rHuG-CSF
AUC (Average) (10^3 cells x days/uL)
0
5
10
15
20
25
30
35
40
45
Days
0
1
2
3
4
5
6
7

Fig. 5B

Fig. 6A

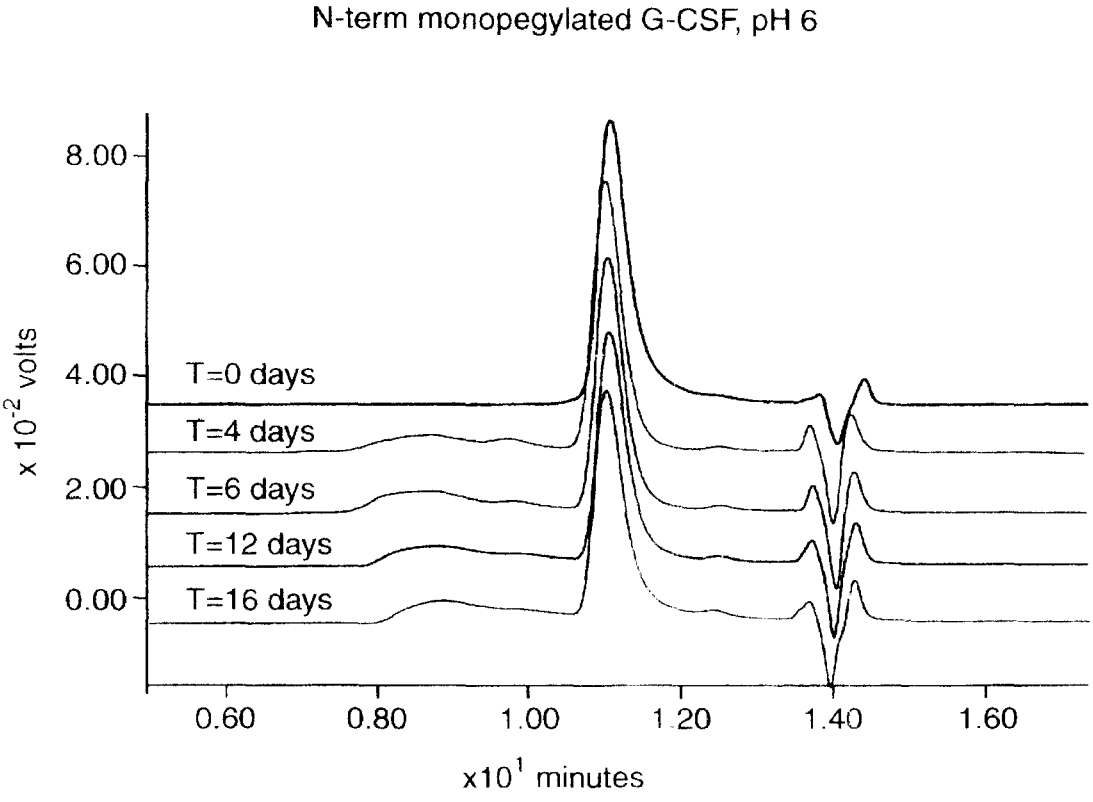

N-term monopegylated G-CSF, pH 6
8.00
6.00
4.00
2.00
0.00
x 10-2 volts
T=0 days
T=4 days
T=6 days
T=12 days
T=16 days
0.60
0.80
1.00
1.20
1.40
1.60
x10¹ minutes

Fig. 6B

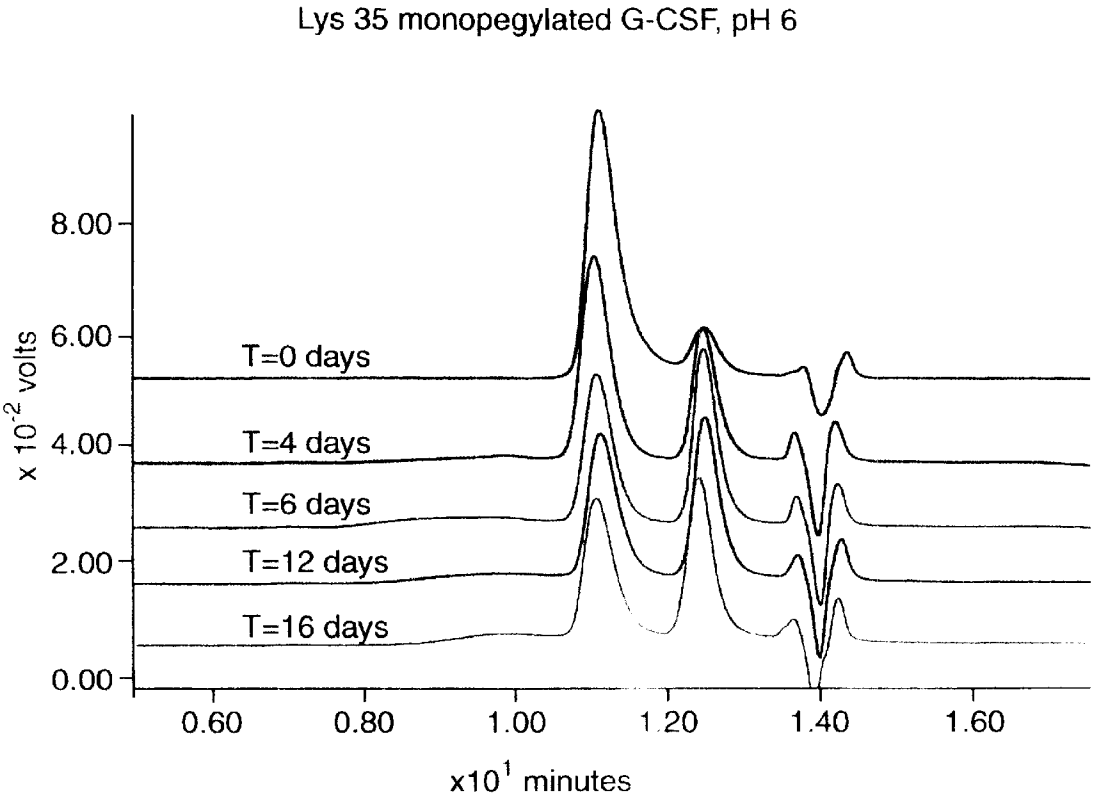

Lys 35 monopegylated G-CSF, pH 6
8.00
6.00
4.00
2.00
0.00
x 10-2 volts
T=0 days
T=4 days
T=6 days
T=12 days
T=16 days
0.60
0.80
1.00
1.20
1.40
1.60
x10¹ minutes

Fig. 6C

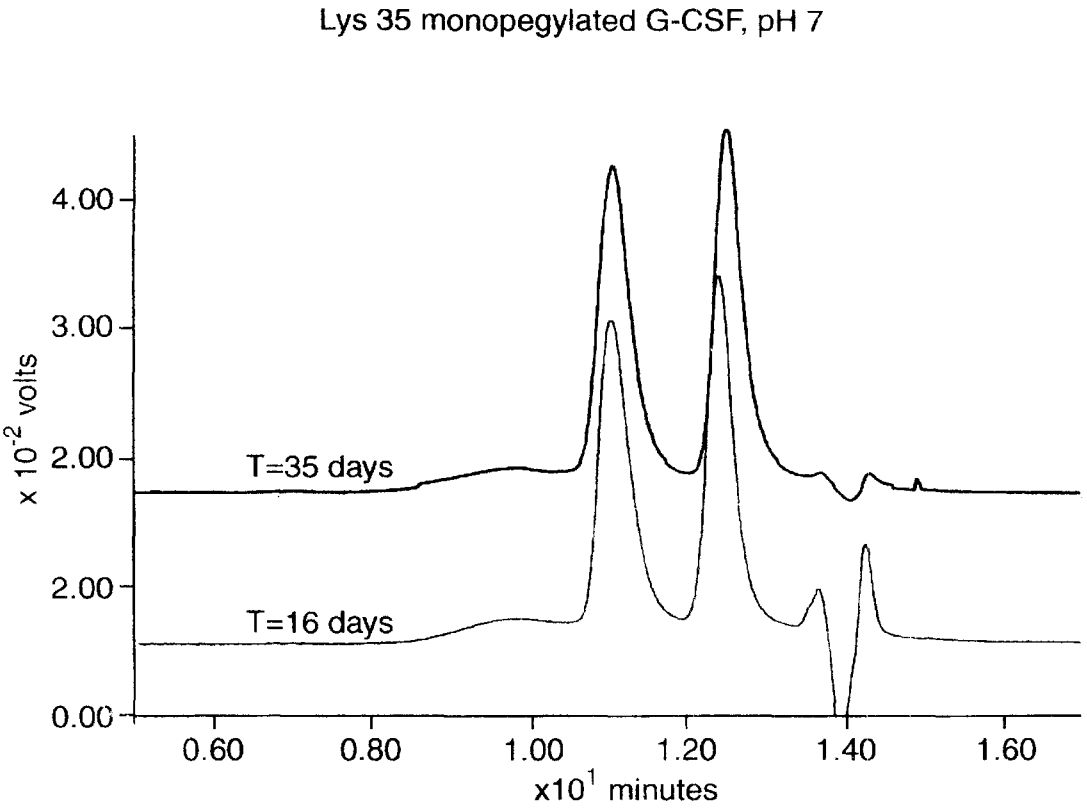

Lys 35 monopegylated G-CSF, pH 7
4.00
3.00
2.00
2.00
0.00
x 10-2 volts
T=35 days
T=16 days
0.60
0.80
1.00
1.20
1.40
1.60
x10¹ minutes

Fig. 7

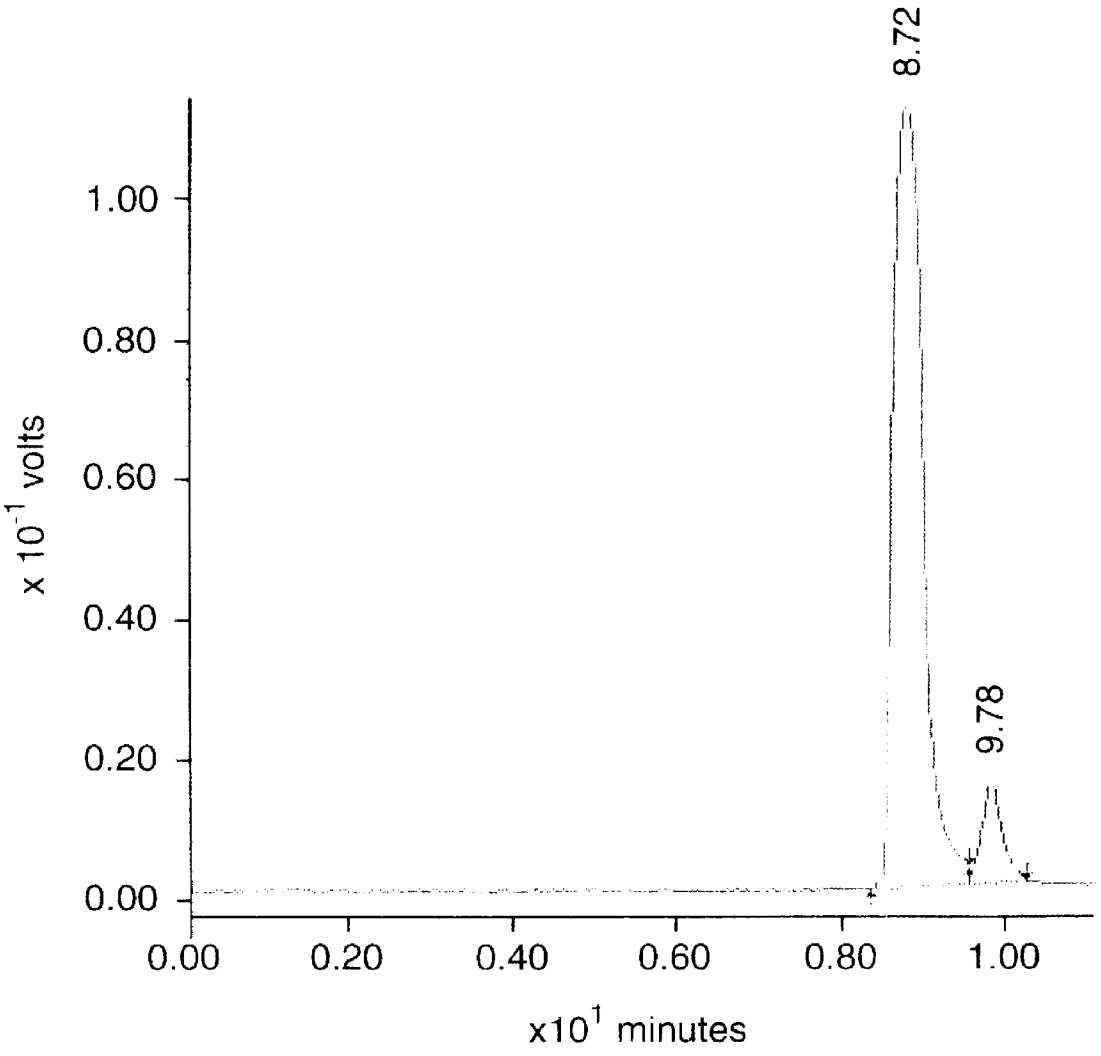

8.72
9.78
1.00
0.80
0.60
0.40
0.20
0.00
x 10^-1 volts
0.00
0.20
0.40
0.60
0.80
1.00
x10^1 minutes

Fig. 8

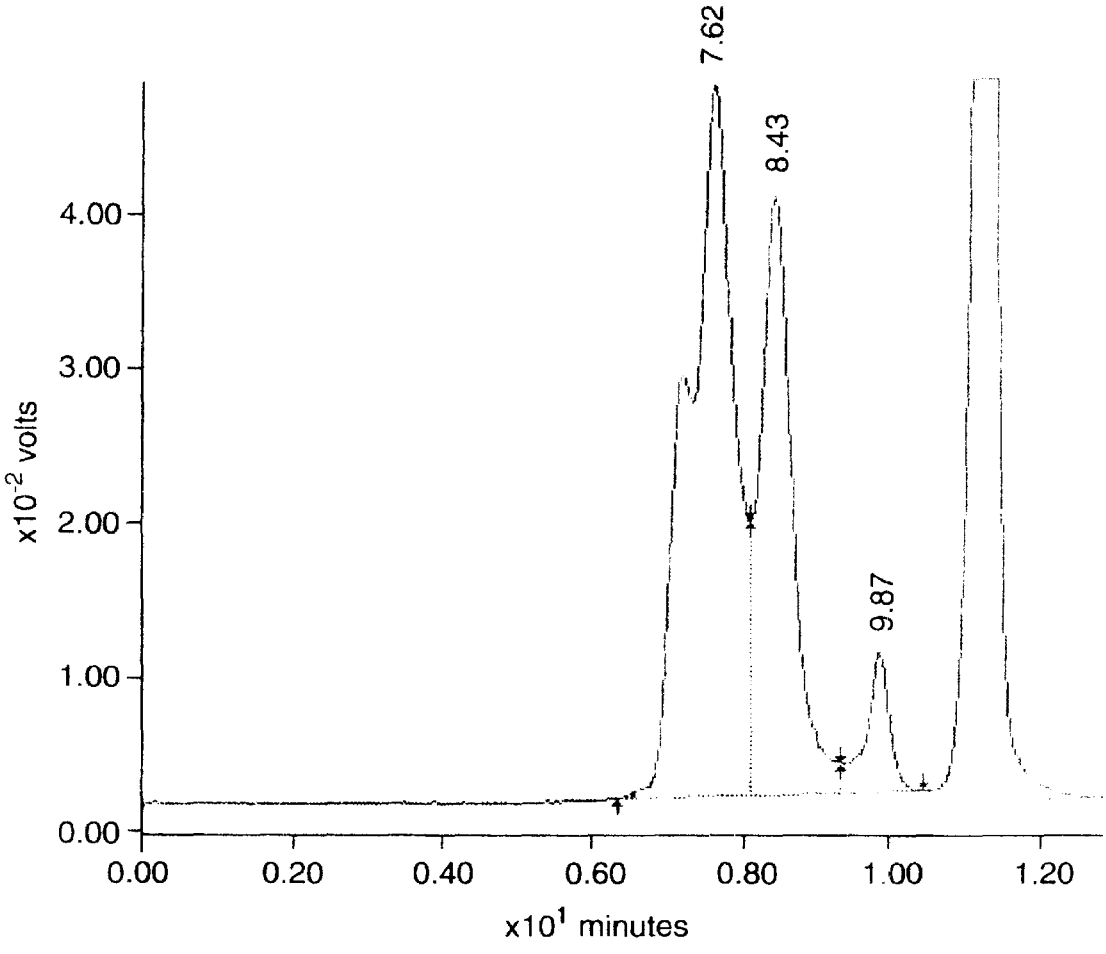
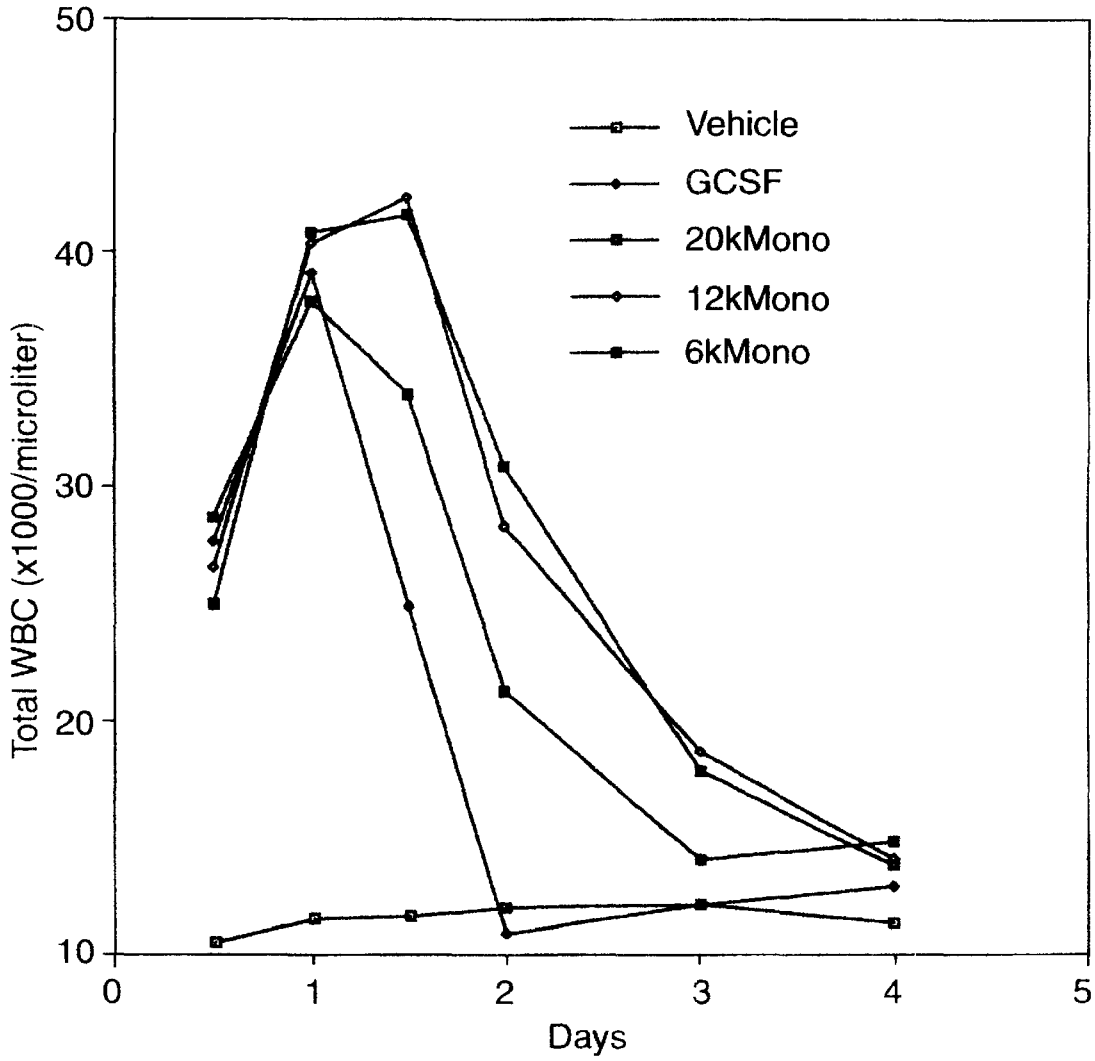

7.62
8.43
9.87
4.00
3.00
2.00
1.00
0.00
x10-2 volts
0.00
0.20
0.40
0.60
0.80
1.00
1.20
x101 minutes

Fig. 9


Vehicle
GCSF
20kMono
12kMono
6kMono
Total WBC (x1000/microliter)
Days
50
40
30
20
10
0
1
2
3
4
5

N-TERMINALLY CHEMICALLY MODIFIED PROTEIN COMPOSITIONS AND METHODS

FIELD OF THE INVENTION

The present invention broadly relates to the field of protein modification, and, more specifically, the attachment of water soluble polymers to proteins or analogs thereof (the term "protein" as used herein is synonymous with "polypeptide" or "peptide" unless otherwise indicated). The present invention also relates to novel methods for N-terminally modifying proteins or analogs thereof, and resultant compositions. In another aspect, the present invention relates to novel N-terminally chemically modified G-CSF compositions and related methods of preparation. The present invention also relates to chemically modified consensus interferon.

BACKGROUND

Proteins for therapeutic use are currently available in suitable forms in adequate quantities largely as a result of the advances in recombinant DNA technologies. The availability of recombinant proteins has engendered advances in protein formulation and chemical modification. One goal of such modification is protein protection. Chemical attachment may effectively block a proteolytic enzyme from physical contact with the protein backbone itself, and thus prevent degradation. Additional advantages include, under certain circumstances, increasing the stability and circulation time of the therapeutic protein and decreasing immunogenicity. A review article describing protein modification and fusion proteins is Francis, *Focus on Growth Factors* 3: 4–10 (May 1992) (published by Mediscript, Mountview Court, Friern Barnet Lane, London N20, OLD, UK).

Polyethylene glycol ("PEG") is one such chemical moiety which has been used in the preparation of therapeutic protein products (the verb "pegylate" meaning to attach at least one PEG molecule). For example Adagen, a pegylated formulation of adenosine deaminase is approved for treating severe combined immunodeficiency disease; pegylated superoxide dismutase has been in clinical trials for treating head injury; pegylated alpha interferon has been tested in phase I clinical trials for treating hepatitis; pegylated glucocerebrosidase and pegylated hemoglobin are reported to have been in preclinical testing. The attachment of polyethylene glycol has been shown to protect against proteolysis, Sada, et al., J. Fermentation Bioengineering 71: 137–139 (1991), and methods for attachment of certain polyethylene glycol moieties are available. See U.S. Pat. No. 4,179,337, Davis et al., "Non-Immunogenic Polypeptides," issued Dec. 18, 1979; and U.S. Pat. No. 4,002,531, Royer, "Modifying enzymes with Polyethylene Glycol and Product Produced Thereby," issued Jan. 11, 1977. For a review, see Abuchowski et al., in Enzymes as Drugs. (J. S. Holcerberg and J. Roberts, eds. pp. 367–383 (1981)).

Other water soluble polymers have been used, such as copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers).

For polyethylene glycol, a variety of means have been used to attach the polyethylene glycol molecules to the protein. Generally, polyethylene glycol molecules are connected to the protein via a reactive group found on the protein. Amino groups, such as those on lysine residues or at the N-terminus, are convenient for such attachment. For

example, Royer (U.S. Pat. No. 4,002,531, above) states that reductive alkylation was used for attachment of polyethylene glycol molecules to an enzyme. EP 0 539 167, published Apr. 28, 1993, Wright, "Peg Imidates and Protein Derivates Thereof" states that peptides and organic compounds with free amino group(s) are modified with an immediate derivative of PEG or related water-soluble organic polymers. U.S. Pat. No. 4,904,584, Shaw, issued Feb. 27, 1990, relates to the modification of the number of lysine residues in proteins for the attachment of polyethylene glycol molecules via reactive amine groups.

One specific therapeutic protein which has been chemically modified is granulocyte colony stimulating factor, "G-CSF." G-CSF induces the rapid proliferation and release of neutrophilic granulocytes to the blood stream, and thereby provides therapeutic effect in fighting infection.

European patent publication EP 0 401 384, published Dec. 12, 1990, entitled, "Chemically Modified Granulocyte Colony Stimulating Factor," describes materials and methods for preparing G-CSF to which polyethylene glycol molecules are attached.

Modified G-CSF and analogs thereof are also reported in EP 0 473 268, published Mar. 4, 1992, entitled "Continuous Release Pharmaceutical Compositions Comprising a Polypeptide Covalently Conjugated To A Water Soluble Polymer," stating the use of various G-CSF and derivatives covalently conjugated to a water soluble particle polymer, such as polyethylene glycol.

A modified polypeptide having human granulocyte colony stimulating factor activity is reported in EP 0 335 423 published Oct. 4, 1989.

Another example is pegylated IL-6, EP 0 442 724, entitled, "Modified hIL-6," (see U.S. Pat. No. 5,264,209) which discloses polyethylene glycol molecules added to IL-6.

EP 0 154 316, published Sep. 11, 1985 reports reacting a lymphokine with an aldehyde of polyethylene glycol.

Many methods of attaching a polymer to a protein involve using a moiety to act as a linking group. Such moieties may, however, be antigenic. A tresyl chloride method involving no linking group is available, but this method may be difficult to use to produce therapeutic products as the use of tresyl chloride may produce toxic by-products. See Francis et al., In: Stability of protein pharmaceuticals: in vivo pathways of degradation and strategies for protein stabilization (Eds. Ahern., T. and Manning, M. C.) Plenum, N.Y., 1991) Also, Delgado et al., "Coupling of PEG to Protein By Activation With Tresyl Chloride, Applications In Immunoaffinity Cell Preparation", In: Fisher et al., eds., Separations Using Aqueous Phase Systems, Applications In Cell Biology and Biotechnology, Plenum Press, N.Y. N.Y.,1989 pp. 211–213

Chamow et al., Bioconjugate Chem. 5: 133–140 (1994) report the modification of CD4 immunoadhesin with monomethoxylpoly(ethylene glycol) aldehyde via reductive alkylation. The authors report that 50% of the CD4-Ig was MePEG-modified under conditions allowing the control over the extent of pegylation. Id. at page 137. The authors also report that the in vitro binding capability of the modified CD4-Ig (to the protein gp 120) decreased at a rate correlated to the extent of MePEGylation. Ibid. See also, Rose et al., Bioconjugate Chemistry 2: 154–159 (1991) which reports the selective attachment of the linker group carbohydrazide to the C-terminal carboxyl group of a protein substrate (insulin).

None of the methods in the general state of the art, or the art relating to particular proteins, allow for selective attach-

ment of a water soluble polymer to the N-terminus of a protein such as G-CSF, however. Rather, the currently existing methods provide for non-selective attachment at any reactive group, whether located within the protein, such as a lysine side group, or at the N-terminus. This results in a heterogenous population. For example, for pegylated G-CSF molecules, some molecules have a different number of polyethylene glycol moieties than others. As an illustration, protein molecules with five lysine residues reacted in the above methods may result in a heterogenous mixture, some having six polyethylene glycol moieties, some five, some four, some three, some two, some one and some zero. And, among the molecules with several, the polyethylene glycol moieties may not be attached at the same location on different molecules.

This is disadvantageous when developing a therapeutic pegylated protein product. In such development, predictability of biological activity is crucial. For example, it has been shown that in the case of nonselective conjugation of superoxide dismutase with polyethylene glycol, several fractions of the modified enzyme were completely inactive (P. McGoff et al. Chem. Pharm. Bull. 36:3079–3091 (1988)). One cannot have such predictability if the therapeutic protein differs in composition from lot to lot. Some of the polyethylene glycol moieties may not be bound as stably in some locations as others, and this may result in such moieties becoming dissociated with the protein. Of course, if such moieties are randomly attached and therefore become randomly dissociated, the pharmacokinetics of the therapeutic protein cannot be precisely predictable. From a consumer's point of view, the circulation time may vary from lot to lot, and thus dosing may be inaccurate. From a producer's point of view, garnering regulatory approval for sale of the therapeutic protein may have added complexities. Additionally, none of the above methods provide for selective N-terminal chemical modification without a linking moiety (between the protein and the polymer). If a linking moiety is used, there may be disadvantages due to possible antigenicity.

Thus, there exists a need for methods allowing for selectively N-terminally chemically modified proteins and analogs thereof, including G-CSF and consensus interferon (two chemically modified proteins exemplified below). The present invention addresses this need in a number of aspects.

SUMMARY OF THE INVENTION

The present invention relates to substantially homogenous preparations of N-terminally chemically modified proteins, and methods therefor. Unexpectedly, chemical modification at the N-terminus of G-CSF demonstrated advantages in stability which are not seen in other G-CSF species containing one chemical modification at another location on the molecule. Also unexpectedly, in the present process for making N-terminally chemically modified G-CSF, it was found that using reductive alkylation, one could provide conditions for selectively modifying the N-terminus, and this method is broadly applicable to other proteins (or analogs thereof), as well as G-CSF. Also surprisingly, using reductive alkylation, the end product—protein with an amine linkage to the water soluble polymer—was found to be far more stable than identical polymer/protein conjugate having an amide linkage. One other protein so modified (as described in a working example below) is consensus interferon. Thus, as described below in more detail, the present invention has a number of aspects relating to chemically modifying proteins (or analogs thereof) as well as specific modifications of specific proteins.

In one aspect, the present invention relates to a substantially homogenous preparation of N-terminally chemically modified G-CSF (or analog thereof) and related methods. One working example below demonstrates that N-terminally monopegylated G-CSF more stable than other types of monopegylated C-CSF. Additionally, since the N-terminus of the G-CSF molecule is more available during reaction with polyethylene glycol, a higher proportion of the N-termini are pegylated, and therefore, this species provides processing advantages.

The present invention also relates to a type of reductive alkylation which selectively activates α-amino group of the N-terminal residue of a protein or analog thereof, thereby providing for selective attachment of a water soluble polymer moiety at the N-terminus. This provides for a substantially homogenous preparation of polymer/protein conjugate molecules as well as (if polyethylene glycol is used) a preparation of pegylated protein molecules having the polyethylene glycol moiety directly coupled to the protein moiety. This method is described below for G-CSF and for consensus interferon, and these provide for additional aspects of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**A is a reproduction of the chromatogram of the peaks from ion exchange chromatography of pegylated G-CSF.

FIG. **1**B is an SDS-PAGE of various species of monopegylated G-CSF.

FIG. **2** is an SEC-HPLC profile of (Line A) recombinant human methionyl G-CSF standard; (Line B) SCM-PEG-GCSF reaction mix; (Line C) N-terminally pegylated G-CSF; (Line D) lysine 35 monopegylated G-CSF; (Line E) lysine 41 monopegylated G-CSF.

FIGS. **3**A, **3**B, and **3**C are HPLC endoproteinase SV8 peptide mapping tracings of (**3**A) N-terminally pegylated G-CSF; (**3**B) lysine 35 monopegylated G-CSF; (**3**C) lysine 41 monopegylated G-CSF.

FIG. **4** is a bar graph illustrating a comparison of in vitro bioactivity of monopegylated G-CSF species compared to an unpegylated standard.

FIGS. **5**A and **5**B are graphs illustrating results of in vivo bioactivity assays of monopegylated G-CSF derivatives, with **5**A illustrating the average hamster white blood cell count after a single subcutaneous injection of N-terminally pegylated G-CSF, lysine 35 monopegylated G-CSF, or lysine 41 monopegylated G-CSF, and **5**B illustrating the net average white blood cell count area under the curve after a single subcutaneous injection of the various monopegylated G-CSF derivatives listed above.

FIGS. **6**A, **6**B, and **6**C are SEC-HPLC profiles for stability studies of N-terminally pegylated G-CSF or lysine 35 monopegylated G-CSF. FIGS. **6**A and **6**B are the profiles for stability studies conducted at pH 6.0 at 4° C. for (**6**A) N-terminally monopegylated G-CSF or (**6**B) lysine 35 monopegylated G-CSF. FIG. **6**C shows the profiles for extended stability studies at pH 6.0 and 4° C. for lysine 35 monopegylated G-CSF. Time ("T") indicates days.

FIG. **7** illustrates size exclusion HPLC analysis of the reaction mixture in the process of reductive alkylation of rh-G-CSF with methoxypolyethylene glycol aldehyde (MW 6 kDa).

FIG. **8** illustrates size exclusion HPLC analysis of the reaction mixture using N-hydroxysuccinimidyl ester of MPEG, also at MW=6kDa.

FIG. **9** illustrates the total white blood cell response after a single subcutaneous dose to mono-N terminal MPEG-GCSF conjugates prepared by reductive alkylation of rh-G-CSF with MPEG aldehydes of different molecular weights (6 kDa,12kDa and 20 kDa).

DETAILED DESCRIPTION

The present invention relates to substantially homogenous preparations of N-terminally chemically modified proteins, and methods therefor.

In one aspect, the present invention relates to N-terminally chemically modified G-CSF compositions and methods therefor.

The present methods (for both N-terminally modified G-CSF as well as the present reductive alkylation methods) provide for a substantially homogenous mixture of monopolymer/protein conjugate. "Substantially homogenous" as used herein means that the only polymer/protein conjugate molecules observed are those having one polymer moiety. The preparation may contain unreacted (i.e., lacking polymer moiety) protein. As ascertained by peptide mapping and N-terminal sequencing, one example below provides for a preparation which is at least 90% monopolymer/protein conjugate, and at most 10% unreacted protein. Preferably, the N-terminally monopegylated material is at least 95% of the preparation (as in the working example below) and most preferably, the N-terminally monopegylated material is 99% of the preparation or more. The monopolymer/protein conjugate has biological activity. The present "substantially homogenous" N-terminally pegylated G-CSF preparations provided herein are those which are homogenous enough to display the advantages of a homogenous preparation, e.g., ease in clinical application in predictability of lot to lot pharmacokinetics.

One may choose to prepare a mixture of polymer/protein conjugate molecules, and the advantage provided herein is that one may select the proportion of monopolymer/protein conjugate to include in the mixture. Thus, if desired, one may prepare a mixture of various protein with various numbers of polymer moieties attached (i.e., di-, tri-, tetra-, etc.) and combine with the monopolymer/protein conjugate material prepared using the present methods, and have a mixture with a predetermined proportion of monopolymer/protein conjugate.

Provided below is a working example using G-CSF, which, as described above, is a therapeutic protein used to treat hematopoietic disorders. In general, G-CSF useful in the practice of this invention may be a form isolated from mammalian organisms or, alternatively, a product of chemical synthetic procedures or of prokaryotic or eukaryotic host expression of exogenous DNA sequences obtained by genomic or cDNA cloning or by DNA synthesis. Suitable prokaryotic hosts include various bacteria (e.g., E. coli); suitable eukaryotic hosts include yeast (e.g., S. cerevisiae) and mammalian cells (e.g., Chinese hamster ovary cells, monkey cells). Depending upon the host employed, the G-CSF expression product may be glycosylated with mammalian or other eukaryotic carbohydrates, or it may be non-glycosylated. The G-CSF expression product may also include an initial methionine amino acid residue (at position −1). The present invention contemplates the use of any and all such forms of G-CSF, although recombinant G-CSF, especially *E. coli* derived, is preferred, for, among other things, greatest commercial practicality.

Certain G-CSF analogs have been reported to be biologically functional, and these may also be chemically modified, by, for example, the addition of one or more polyethylene glycol molecules. G-CSF analogs are reported in U.S. Pat. No. 4,810,643. Examples of other G-CSF analogs which have been reported to have biological activity are those set forth in AU-A-76380/91, EP 0 459 630, EP 0 272 703, EP 0 473 268 and EP 0 335 423, although no representation is made with regard to the activity of each analog reportedly disclosed. See also AU-A-10948/92, PCT US94/00913 and EP 0 243 153.

Generally, the G-CSFs and analogs thereof useful in the present invention may be ascertained by practicing the chemical modification procedures as provided herein to selectively chemically modify the N-terminal α-amino group, and testing the resultant product for the desired biological characteristic, such as the biological activity assays provided herein. Of course, if one so desires when treating non-human mammals, one may use recombinant non-human G-CSF's, such as recombinant murine, bovine, canine, etc. See PCT WO 9105798 and PCT WO 8910932, for example.

Thus, another aspect of the present invention includes N-terminally chemically modified G-CSF analog compositions. As described above, G-CSF analogs may include those having amino acid additions, deletions and/or substitutions (as compared to the G-CSF amino acid sequence set forth in Example 1, below). Those G-CSF analogs which are predicted to function when N-terminally pegylated to selectively stimulate the production of neutrophils are those with an N-terminus which is not necessary for binding to a G-CSF receptor. See Hill et al., PNAS-USA 90: 5167–5171 (1993); see also PCT US94/00913.

The polymer molecules used may be selected from among water soluble polymers. (For the reductive alkylation procedure described herein, the polymers should have a single reactive aldehyde.) The polymer selected should be water soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. For reductive alkylation, the polymer selected should have a single reactive aldehyde so that the degree of polymerization may be controlled as provided for in the present methods. The polymer may be branched or unbranched. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. For G-CSF, these may be ascertained using the assays provided herein, and one skilled in the art should select the appropriate assays for other therapeutic proteins. The water soluble polymer may be selected from the group consisting of, for example, those listed above (in the Background section), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols and polyvinyl alcohol.

Subject to considerations for optimization as discussed below, the polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 2kDa and about 100kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight). Examples 1 and 2 below involve the use of PEG 6000, which was selected for ease in purification and for providing an adequate model system. Other sizes may be used, depending

on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

One specific aspect of the present invention is N-terminally monopegylated G-CSF comprised of a polyethylene glycol moiety and a G-CSF moiety. For the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to G-CSF protein molecules in the reaction mix, the type of pegylation reaction to be performed, the method of obtaining the selected N-terminally pegylated G-CSF, and the type of G-CSF to be used. Further, the present compositions and methods include formulation of pharmaceutical compositions, methods of treatment and manufacture of medicaments.

The proportion of polyethylene glycol molecules to protein molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted protein or polymer) will be determined by the molecular weight of the polyethylene glycol selected. In addition, as one example of the present methods involves non-specific pegylation and later purification of N-terminally monopegylated species, the ratio may depend on the number of available reactive groups (typically ∝or ∋ amino groups) available. One working example herein involved a fairly low reaction ratio of protein:PEG molecules to obtain monopegylated material generally (1.5 PEG molecules per protein molecules).

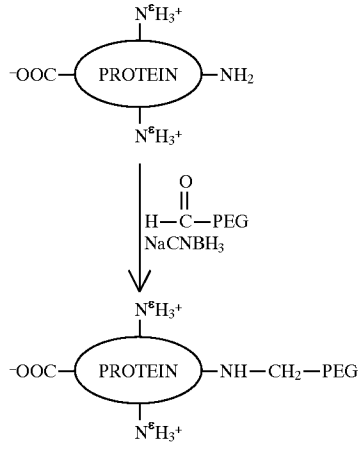
For obtaining N-terminally pegylated G-CSF, the method for pegylation may also be selected from among various methods, as discussed above, or the present reductive alkylation as described in Example 2, below. A method involving no linking group between the polyethylene glycol moiety and the protein moiety is described in Francis et al., In: Stability of protein pharmaceuticals: in vivo pathways of degradation and strategies for protein stabilization (Eds. Ahern., T. and Manning, M. C.) Plenum, N.Y., 1991) Also, Delgado et al., "Coupling of PEG to Protein By Activation With Tresyl Chloride, Applications In Immunoaffinity Cell Preparation", In: Fisher et al., eds., Separations Using Aqueous Phase Systems, Applications In Cell Biology and Biotechnology, Plenum Press, N.Y. N.Y.,1989 pp. 211–213, involves the use of tresyl chloride, which results in no linkage group between the polyethylene glycol moiety and the protein moiety. This method may be difficult to use to produce therapeutic products as the use of tresyl chloride may produce toxic by-products. One of the present working examples involves the use of N-hydroxy succinimidyl esters of carboxymethyl methoxy polyethylene glycol. As will be discussed in more detail below, another working example involves the use of the present reductive alkylation methods.

The method of obtaining the N-terminally pegylated G-CSF preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated G-CSF molecules. For example, presented below is an example where pegylated G-CSF is first separated by ion exchange chromatography to obtain material having a charge characteristic of monopegylated material (other multi-pegylated material having the same apparent charge may be present), and then the monopegylated materials are separated using size exclusion chromatography. In this way, N-terminally monopegylated G-CSF was separated from other monopegylated species, as well as other multi-pegylated species. Other methods are reported. For example, PCT WO 90/04606, published May 3, 1990, reports a process for fractionating a mixture of PEG-protein adducts comprising partitioning the PEG/protein adducts in a PEG-containing aqueous biphasic system.

In a different aspect, the present invention provides a method for selectively obtaining an N-terminally chemically modified protein (or analog). Provided below is a method of protein modification by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved. The reaction is performed at pH which allows one to take advantage of the pK_a differences between the ϵ-amino groups of the lysine residues and that of the α-amino group of the N-terminal residue of the protein. By such selective derivatization attachment of a water soluble polymer to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs.

Importantly, and surprisingly, the present invention provides for a method of making a substantially homogenous preparation of monopolymer/protein conjugate molecules, in the absence of further extensive purification as is required using other chemical modification chemistries. Additionally, the product having an amine linkage is unexpectedly more stable than a product produced with an amide linkage, and this is demonstrated in the aggregation studies below. More specifically, if polyethylene glycol is used, the present invention also provides for N-terminally pegylated protein lacking possibly antigenic linkage groups, and having the polyethylene glycol moiety directly coupled to the protein moiety without toxic by-products.

The reaction may be diagrammed as follows (indicating sodium cyanohydroboride as an illustrative reducing agent):



Thus, one aspect of the present invention is a method for preparing a polymer/protein conjugate comprised of (a) reacting a protein moiety having more than one amino group with a water soluble polymer moiety under reducing alkylation conditions, at a pH suitable to selectively activate the α-amino group at the amino terminus of said protein moiety so that said water soluble polymer selectively attaches to said α-amino group; and (b) obtaining the reaction product.

One may optionally, and preferably for a therapeutic product, separate the reaction products from unreacted moieties.

Another aspect of the present invention is that such reductive alkylation will provide for selective attachment of the polymer to any protein having an α-amino group at the amino terminus, and provide for a substantially homogenous preparation of monopolymer/protein conjugate. The term "monopolymer/protein conjugate" is used here to mean a composition comprised of a single polymer moiety attached to a protein moiety (also encompassed are those conjugates using protein analogs as described herein). The monopolymer/protein conjugate will have a polymer moiety located at the N-terminus, but not on amino side groups, such as those for lysine. The preparation will preferably be greater than 80% monopolymer/protein conjugate, and more preferably greater than 95% monopolymer protein conjugate.

For a substantially homogenous population of monopolymer/protein conjugate molecules, the reaction conditions are those which permit the selective attachment of the water soluble polymer moiety to the N-terminus of the desired protein. Such reaction conditions generally provide for pK_a differences between the lysine amino groups and the α-amino group at the N-terminus (the pK being the pH at which 50% of the amino groups are protonated and 50% are not). In general, for different proteins, different pH's may be used for optimally modifying the α-amino groups of the N-terminus.

The pH also affects the ratio of polymer to protein to be used. In general, if the pH is lower than the pK, a larger excess of polymer to protein will be desired (i.e., the less reactive the N-terminal α-amino group, the more polymer needed to achieve optimal conditions). If the pH is higher than the pK, the polymer:protein ratio need not be as large (i.e., more reactive groups are available, so fewer polymer molecules are needed).

Another important consideration is the molecular weight of the polymer. In general, the higher the molecular weight of the polymer, the fewer number of polymer molecules which may be attached to the protein. Similarly, branching of the polymer should be taken into account when optimizing these parameters. Generally, the higher the molecular weight (or the more branches) the higher the polymer:protein ratio.

For the present reductive alkylation, the reducing agent should be stable in aqueous solution and preferably be able to reduce only the Schiff base formed in the initial process of reductive alkylation. Preferred reducing agents may be selected from the group consisting of sodium borohydride, sodium cyanoborohydride, dimethylamine borate, trimethylamine borate and pyridine borate. Sodium cyanoborohydride was used in the working examples below.

The water soluble polymer may be of the type described above, and should have a single reactive aldehyde for coupling to the protein. For polyethylene glycol, use of PEG 6000 for coupling to G-CSF and PEG 12000 for consensus interferon are described below. It is noted, that for G-CSF, PEG 12000, 20000 and 25000 have also been used successfully in the present methods. Polyethylene glycol propionaldenhyde (see, e.g., U.S. Pat. No. 5,252,714) is advantageous for its stability in water.

As indicated above, the present methods are broadly applicable to any protein or analog thereof having an N-terminal α-amino group. For example, proteins which are the product of an exogenous DNA sequence expressed in bacteria may have, as a result of bacterially expression, an N-terminal methionyl residue with an α-amino group. As indicated above, peptides are included, as are peptidomimetics and other modified proteins. Protein analogs, such as the G-CSF analogs described above, and the non-naturally occurring consensus interferon are also suitable for the present methods.

Thus, for the present N-terminally chemically modified G-CSF, any of the G-CSF's or analogs as described herein may be used (e.g., those described supra). The working examples below use recombinant G-CSF produced in bacteria, having 174 amino acids and an extra N-terminal methionyl residue. As described herein, the chemical modification may be performed with any of the water soluble polymers described herein, and the present working examples describe the use of polyethylene glycol.

Consensus interferon is another protein used in the present working examples. Demonstrated below is the preparation of chemically modified consensus interferon using the present reductive alkylation methods for N-terminal monopegylation. Thus, other aspects of the present invention relate to these preparations. As employed herein, consensus human leukocyte interferon, referred to here as "consensus interferon," or "IFN-con", means a nonnaturally-occurring polypeptide, which predominantly includes those amino acid residues that are common to all naturally-occurring human leukocyte interferon subtype sequences and which include, at one or more of those positions where there is no amino acid common to all subtypes, an amino acid which predominantly occurs at that position and in no event includes any amino acid residue which is not extant in that position in at least one naturally-occurring subtype. IFN-con encompasses the amino acid sequences designated IFN-con_1, IFN-con_2 and IFN-con_3 which are disclosed in commonly owned U.S. Pat. Nos. 4,695,623 and 4,897,471, the entirety of which are hereby incorporated by reference. (U.S. Pat. Nos. 4,897,471 and 4,695,623 use the denomination "α" which is not used herein.) DNA sequences encoding IFN-con may be synthesized as described in the above-mentioned patents or other standard methods. IFN-con polypeptides are preferably the products of expression of manufactured DNA sequences, transformed or transfected into bacterial hosts, especially *E. coli*. That is, IFN-con is recombinant IFN-con. IFN-con is preferably produced in *E. coli* may be purified by procedures known to those skilled in the art and generally described in Klein et al.,J. Chromatog. 454: 205–215 (1988) for IFN-con_1. Purified IFN-con may comprise a mixture of isoforms, e.g., purified IFN-con_1 comprises a mixture of methionyl IFN-con_1, des-methionyl IFN-con_1 and des-methionyl IFN-con_1 with a blocked N-terminus (Klein et al., Arc. Biochem. Biophys. 276: 531–537 (1990)). Alternatively, IFN-con may comprise a specific, isolated isoform. Isoforms of IFN-con are separated from each other by techniques such as isoelectric focusing which are known to those skilled in the art.

Thus, another aspect of the present invention is a chemically modified consensus interferon wherein said consensus interferon moiety is selected from the group consisting of IFN-con_1, IFN-con_2, and IFN-con_3. The chemical modification is using a water soluble polymer as described herein, such as PEG, and the present reductive alkylation methods may be used for selective N-terminal chemical modification. Example 3 herein illustrates a chemically modified IFN con_1 comprised of an IFN con_1 moiety connected at the N-terminus to a polyethylene glycol moiety (PEG 12000).

In another aspect, the present methods yield pegylated proteins where the polyethylene glycol moiety is directly

attached to a protein moiety, and a separate linking group is absent and no toxic by-products are present. The examples include G-CSF and consensus interferon as described herein. For a population of pegylated G-CSF protein molecules wherein the polyethylene glycol moiety is directly attached to the G-CSF protein moiety (not necessarily a population of N-terminally pegylated G-CSF molecules), one may perform the above reductive alkylation with or without an acidic pH.

In yet another aspect of the present invention, provided are pharmaceutical compositions of the above. Such pharmaceutical compositions may be for administration for injection, or for oral, pulmonary, nasal or other forms of administration. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of monopolymer/protein conjugate products of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimersol, benzyl alcohol) and bulking substances (e.g., lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present N-terminally chemically modified proteins. See, e.g., Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435–1712 which are herein incorporated by reference.

In yet another aspect of the present invention, methods of treatment and manufacture of a medicament are provided. Conditions alleviated or modulated by the administration of the present polymer/G-CSF conjugates (or analogs having the hematopoietic biological properties of naturally occurring G-CSF) are typically those characterized by a reduced hematopoietic or immune function, and, more specifically, a reduced neutrophil count. Such conditions may be induced as a course of therapy for other purposes, such as chemotherapy or radiation therapy. Such conditions may result from infectious disease, such as bacterial, viral, fungal or other infectious disease. For example, sepsis results from bacterial infection. Or, such condition may be hereditary or environmentally caused, such as severe chronic neutropenia or leukemias. Age may also play a factor, as in the geriatric setting, patients may have a reduced neutrophil count or reduced neutrophil mobilization. Some of such conditions are reviewed in Filgrastim (r-met Hu G-CSF) in Clinical Practice, Morstyn, G. and T. M. Dexter, eds., Marcel Dekker, Inc., N.Y., N.Y. (1993), 351 pp. Other less-studied conditions which may be alleviated or modulated by administration of the present polymer/G-CSF conjugates may include the reduction of lipids (or cholesterol) in the blood stream, and certain cardiovascular conditions, as G-CSF may induce production of plasminogen activators. The mode of action of G-CSF (or analogs) in these settings is not well understood at present. The addition of a water soluble polymer, such as polyethylene glycol, may provide practical patient benefits in that the sustained duration of biological activity may allow for fewer G-CSF injections per course of treatment.

Generally, conditions which may be alleviated or modulated by administration of the present polymer/consensus interferon are those to which consensus interferon is applicable and include cell proliferation disorders, viral infections, and autoimmune disorders such as multiple sclerosis. Cf., McManus Balmer, DICP, The Annals of Pharmacotherapy 24: 761–767 (1990)(Clinical use of biologic response modifiers in cancer treatment: an overview. Part I. The Interferons). Methods and compositions for the treatment of cell proliferation disorders using consensus interferon are described in PCT WO 92/06707, published Apr. 30, 1992, which is herein incorporated by reference. For example, hepatitis (A, B, C, D, E) may be treatable using the present pegylated consensus interferon molecules. The working example below demonstrates that, in vitro, chemically modified consensus interferon has 20% of the biological activity of non-chemically modified consensus interferon.

For all of the above molecules, as further studies are conducted, information will emerge regarding appropriate dosage levels for treatment of various conditions in various patients, and the ordinary skilled worker, considering the therapeutic context, age and general health of the recipient, will be able to ascertain proper dosing. Generally, for injection or infusion, dosage will be between 0.01 μg/kg body weight, (calculating the mass of the protein alone, without chemical modification), and 100 μg/kg (based on the same).

The below examples illustrate the various aspects discussed above. In Example 1, the advantages of N-terminally pegylated G-CSF are demonstrated as compared to G-CSF monopegylated at lysine-35 or lysine 41 (of the G-CSF met+174 amino acid version). Example 2 illustrates the present reductive alkylation in N-terminally pegylating G-CSF. The method provides for a substantially homogenous preparation of N-terminally pegylated G-CSF. Example 3 illustrates the present reductive alkylation in N-terminally pegylating consensus interferon.

EXAMPLE 1

A. Preparation of Recombinant Human met-G-CSF

Recombinant human met-G-CSF (referred to as "rhG-CSF" or "r-met-hu-G-CSF" from time to time herein) was prepared as described above according to methods in the Souza patent, U.S. Pat. No. , 4,810,643, which is herein incorporated by reference. The rhG-CSF employed was an *E. coli* derived recombinant expression product having the amino acid sequence (encoded by the DNA sequence) shown below (Seq.ID NOs. 1 and 2):

ATG	ACT	CCA	TTA	GGT	CCT	GCT	TCT	TCT	CTG	CCG	CAA	AGC	TTT	CTG
M	T	P	L	G	P	A	S	S	L	P	Q	S	F	L
CTG	AAA	TGT	CTG	GAA	CAG	GTT	CGT	AAA	ATC	CAG	GGT	GAC	GGT	GCT
L	K	C	L	E	Q	V	R	K	I	Q	G	D	G	A
GCA	CTG	CAA	GAA	AAA	CTG	TGC	GCT	ACT	TAC	AAA	CTG	TGC	CAT	CCG
A	L	Q	E	K	L	C	A	T	Y	K	L	C	H	P

-continued

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GAA GAG CTG GTA CTG CTG GGT CAT TCT CTT GGG ATC CCG TGG GCT
 E   E   L   V   L   L   G   H   S   L   G   I   P   W   A

CCG CTG TCT TCT TGT CCA TCT CAA GCT CTT CAG CTG GCT GGT TGT
 P   L   S   S   C   P   S   Q   A   L   Q   L   A   G   C

CTG TCT CAA CTG CAT TCT GGT CTG TTC CTG TAT CAG GGT CTT CTG
 L   S   Q   L   H   S   G   L   F   L   Y   Q   G   L   L

CAA GCT CTG GAA GGT ATC TCT CCG GAA CTG GGT CCG ACT CTG GAC
 Q   A   L   E   G   I   S   P   E   L   G   P   T   L   D

ACT CTG CAG .CTA GAT GTA GCT GAC TTT GCT ACT ACT ATT TGG CAA
 T   L   Q   L   D   V   A   D   F   A   T   T   I   W   Q

CAG ATG GAA GAG CTC GGT ATG GCA CCA GCT CTG CAA CCG ACT CAA
 Q   M   E   E   L   G   M   A   P   A   L   Q   P   T   Q

GGT GCT ATG CCG GCA TTC GCT TCT GCA TTC CAG CGT CGT GCA GGA
 G   A   M   P   A   F   A   S   A   F   Q   R   R   A   G

GGT GTA CTG GTT GCT TCT CAT CTG CAA TCT TTC CTG GAA GTA TCT
 G   V   L   V   A   S   H   L   Q   S   F   L   E   V   S

TAC CGT GTT CTG CGT CAT CTG GCT CAG CCG TAA TAG
 Y   R   V   L   R   H   L   A   Q   P   *   *
```

(This was also the non-pegylated composition used for the control animals.) Alternatively one may use purchased NEUPOCOEN® for the following pegylation procedures (the package insert for which is herein incorporated by reference).

B. Preparation of Pegylated G-CSF

A 10 mg/ml solution of the above rh-G-CSF, in 100 mM Bicine pH 8.0, was added to solid SCM-MPEG (N-hydroxy succinimidyl esters of carboxymethyl methoxy polyethylene glycol) (Union Carbide) with an average molecular weight of 6000 Daltons. This gave a 1.5 molar excess of SCM-MPEG to rh-G-CSF. After one hour with gentle stirring, the mixture was diluted to 2 mg/ml with sterile water, and the pH was adjusted to 4.0 with dilute HCl. The reaction was carried out at room temperature. At this stage, the reaction mixture consisted mainly of three forms of mono-pegylated rh-G-CSF, some di-pegylated rh-G-CSF, unmodified rh-G-CSF and reaction bi-product (N-hydroxy succinimide).

C. Preparation of N-terminally Pegylated rh-G-CSF

The three forms of monopegylated rh-G-CSF were separated from each other using ion exchange chromatography. The reaction mixture was loaded (1 mg protein/ml resin) onto a Pharmacia S SEPHAROSE FF column (Pharmacia XK50/30 reservoir, bed volume of 440 ml) equilibrated in buffer A (20 mM sodium acetate, pH 4.0). The column was washed with 3 column volumes of buffer A. The protein was eluted using a linear gradient from 0–23% buffer B (20 mM sodium acetate, pH 4.0, 1M NaCl) in 15 column volumes. The column was then washed with one column volume of 100% buffer B and reequilibrated with 3 column volumes of buffer A. The flow rate for the entire run was maintained at 8 ml/min. The eluent was monitored at 280 nm and 5 ml fractions were collected. Fractions containing the individual monopegylated species were pooled according to FIG. **1**A. These pools were concentrated with a 350 mL Amicon stirred cell using a YM10 76 mm membrane.

Pooled fractions from the ion exchange chromatography were subjected to size exclusion chromatography to separate di-pegylated species from monopegylated species. Typically, 5–10 mg in 2–5 ml of solution were loaded onto a 120 ml Pharmacia SUPERDEX 75 HR 16/60 column equilibrated with 20 mM sodium acetate pH 4.0. The column was run at 1.5 ml/min for 100 min. Two ml fractions were collected. The protein content of the eluent was monitored at 280 nm. Fractions from separated peaks were pooled and subjected to analysis. The table below compares the proportional yields for each peak.

TABLE 1

Relative Yields and Site of Modification

Site of Modification	Figure 1A Reference	Relative Yields
N-Terminus	Peak 1A	3
Lysine-35	Peak 2A	2
Lysine-41	Peak 3A	1

Under these conditions, the lysines at positions 17 and 24 probably were not significantly pegylated.

D. Characterization

Five analyses were done to characterize each sample: (1) SDS-Page (FIG. **1**B), (2) Size exclusion chromatography HPLC ("SEC HPLC")(FIG. **2**), (3) peptide mapping analysis (FIGS. **3**A, **3**B, and **3**C), (4) in vitro G-CSF bioassay (FIG. **4**), and (5) in vivo testing in hamster (FIGS. **5**A and **5**B).

With regard to the composition of each sample, results demonstrate that, of the N-terminally monopegylated G-CSF, the samples showed a greater than 95% N-terminally pegylated composition, with the remainder probably being unpegylated material (although the remainder of the samples is lower than the detection limit of the assay). With regard to the percent monopegylated for each of the three types of monopegylated material (N-terminal, pegylated at lysine 35, and pegylated at lysine 41), the N-terminal and the lysine 41 demonstrated greater than 97% monopegylated, and the lysine 35 pegylated material being somewhat lower, probably due to the instability of the molecule in the assay conditions. To summarize, the following results were obtained:

TABLE 2

Percent Composition of N-terminally pegylated G-CSF

	Non-Reduced SDS PAGE	SEC HPLC	N-Terminal Sequencing*
Mono-pegylated G-CSF	97.44	99.43	96.6
Unmodified G-CSF	2.56	0.57	3.4

*The N-terminal sequencing, as discussed infra is not here considered quantitative, as there may have been artifactual separation of the polyethylene glycol molecule from the N-terminus of the protein during the sequencing process.

TABLE 3

Percent Monopegylated for Three Species

	N-terminal PEG-GCSF (RI/UV = .96)*	LYS35 PEG-GCSF** (RI/UV = .72)	LYS41 PEG-GCSF (RI/UV = 1.12)
Non-reduced SDS-PAGE	97.44	77.41	100.00
SEC HPLC	99.43	93.38	99.96

*RI/UV refers to the Index of Refraction/Ultraviolet light absorbance ratio, and is used to estimate the number of polyethylene glycol molecules per molecule of protein. It is calculated from the SEC HPLC data using an Index of Refraction for polyethylene glycol and an ultraviolet absorbance for protein.
**Note that this species is unstable under the assay conditions used.

METHODS

1. SDS-PAGE.

SDS-PAGE was carried out in a non-reduced 4–20% ISS Daiichi Pure Chemicals, Co., Tokyo, Japan minigel using a Coomassie Brillant Blue R-250 stain. The gel was scanned using a molecular Dynamics Densitometer with Image Quant. Results: Results are presented in FIG. **1**B. Lane number 1 (from the left hand side) included molecular weight protein standards (Novex Mark 12 Molecular Weight Standards). Lane 2 contains 3 μg rh-G-CSF standard. Lane 3 contains the SCM-PEG-GCSF reaction mix, with 10 μg loaded. Lane 4 contains N-terminally monopegylated G-CSF, with 10 μg loaded. Lane 5 contains 10 μg of monopegylated G-CSF with the pegylation site at the lysine found at the 35th residue from the N-terminal methionine. Lane 6 contains 10 μg of monopegylated G-CSF with the pegylation site at the lysine found at the 41st residue from the N-terminal methionine. As can be seen, Lane 3, containing the N-terminally monopegylated material, shows a single band

2. Size Exclusion Chromatography-High Pressure Liquid Chromatography.

SEC-HPLC was carried out using a Waters HPLC system with a BIOSEP SEC 3000 column, using 100 mM sodium phosphate, pH 6.9, 1ml/min for 20 minutes. The signal was monitored at 280 nm. Results: As can be seen from FIG. **2**, line "C," containing the N-terminally monopegylated rh-G-CSF contains a single peak, as do lines "D" (Lys-35 monopegylated material) and "E" (Lys-41 monopegylated material). This indicates substantial purity among the separated fractions of monopegylated G-CSF.

3. Peptide mapping.

The following methods were used. Three samples, called "Mono-PEG 1", "Mono-PEG-2", and "Mono-PEG-3", were analyzed. (a) Reductive alkylation. 500 μg aliquots of mono-PEG G-CSF were speed vac dried and reconstituted to a concentration of 1 mg in 950 μl in 0.3M Tris-HCl containing 6M Guanidinum HCl and 1 mM EDTA pH 8.4. Samples were then S-carboxymethylated by adding iodoacetic acid and incubated at 37° C. for 20 minutes. Samples were then desalted using SEPHADEX G+25 Quick Spin Protein Columns and buffer exchanged. After desalting and buffer exchange, sample concentration was adjusted to 0.5 mg/ml using additional buffer. (b) Endoproteinase SV8 digestion. Samples were digested with SV8 (enzyme to substrate ratio of 1:25) at 25° C. for 26 hours.(c) HPLC peptide mapping. Protein digests were injected onto a Vydac C4 column (4.6×250 mm, 5 μ particle size, 300 Å pore size) and peptides were mapped by HPLC using a linear gradient of acetonitrile in 0.1% TFA. Peptides were manually collected and dried in a Speed Vac for sequence analysis. Results: As compared to a reference standard, (i) (FIG. **3**A) for "Mono-PEG-1", (the N-terminally mono-pegylated material), a peak at 57.3 minutes diminished and a new peak appeared at 77.5 minutes; (ii) (FIG. **3**B) for "Mono-PEG-2", (the lysine 35 pegylated material), there was a decrease in peak height for a peptide with a retention time of 30.3 minutes, and a new peak eluted at 66.3 minutes; (iii) (FIG. **3**C) for "Mono-PEG-3" (the lysine 41 pegylated material), the peak at retention time of 30.3 minutes was missing, and a new peak appeared at 66.4 minutes. These peptides were the only significant differences in the sample maps. There were some small incomplete cleavages seen on either side of the peptide at 86.1 minutes due to minor digestion differences. (d) N-terminal sequence analysis. Each of the "new" peptides in the above maps were N-terminally sequenced for identification. The dried peptides were reconstituted in 0.1% TFA and sequenced on an ABI protein sequencer. For "Mono-PEG-1" (the N-terminally pegylated material), 60% of the "new" peak (at 77.5 minutes) was sequenced for 10 cycles. The initial yield was less than 5%, indicating that the N-terminal methionyl residue is blocked by a polyethylene glycol molecule. It is noted that this initial peptide should have resulted in a zero initial yield, and the <5% yield observed may be from detachment of the polyethylene glycol from the N-terminal methionyl during sequence analysis. The sequence detected was that of the N-terminal peptide, M-T-P-L-G-P-A-S-S. For "Mono-PEG-2", (the lysine 35 pegylated material), 80% of the total peak volume was collected for the peak at 66.3 minutes, and was sequenced for 9 cycles. The recovery of lysine 35 was significantly low, indicating pegylation at position 35. The recovery of lysine 41 was consistent with the other residue, indicating no modification of this position. The peptide at 30.3 minutes decreased in peak height compared to the corresponding peak in the standard reference map. The peptide at 30.3 minutes is only 57.5% of the peak area of the corresponding peptide. The sequence detected for this species was K-L-C-A-T-Y-K-L. For "Mono-PEG-3", the lysine 41 material, 80% of the total peak volume collected for the peptide eluting at 66.4 minutes was sequenced for 9 cycles. The sequence detected was K-L-C-A-T-Y-K-L, and contained lysine residues 35 and 41. The recovery of lysine 35 was consistent with other residue recoveries. The recovery of lysine 41 was significantly lower indicating pegylation at position 41. Results: "Mono-PEG-1" is N-terminally monopegylated material; "Mono-PEG-2" is lysine 35 partially pegylated; and "Mono-PEG-3" is lysine 41 pegylated material. By comparing both the reference standard (non-pegylated G-CSF) and GCSF monopegylated 1, 2, and 3 peptide maps, it was found that both the "Mono-PEG-2" (lysine 35) and "Mono-PEG-3" (lysine 41) maps exhibit

slightly diminished peak heights for the N-terminal peptide. This indicates that the lysine 35 and lysine 41 material contains a small amount of N-terminally pegylated material or that the N-terminal methionine has a small percentage of pegylation.

4. In vitro activity.

The material was active. FIG. **4** illustrates the results of in vitro assays. As can be seen, the N-terminally monopegylated material had 68% of the activity of non-modified rhG-CSF. Methods: The G-CSF in vitro bioassay is a mitogenic assay utilizing a G-CSF dependent clone of murine 32D cells. Cells were maintained in Iscoves medium containing 5% FBS and 20 ng/ml rhG-CSF. Prior to sample addition, cells were prepared by rinsing twice with growth medium lacking rhG-CSF. An extended twelve point rhG-CSF standard curve was prepared, ranging from 48 to 0.5ng/ml (equivalent to 4800 to 50 IU/ml). Four dilutions, estimated to fall within the linear portion of the standard curve, (1000 to 3000 IU/ml), were prepared for each sample and run in triplicate. Because of their apparent lower activity in vitro, the pegylated rhG-CSF samples were diluted approximately 4–10 times less. A volume of 40 μl of each dilution of sample or standard is added to appropriate wells of a 96 well microtiter plate containing 10,000 cells/well. After forty-eight hours at 37° C. and 5.5% CO_2, 0.5μmCi of methyl-^{3}H-thymidine was added to each well. Eighteen hours later, the plates were then harvested and counted. A dose response curve (log rhG-CSF concentration vs. CPM-background) was generated and linear regression analysis of points which fall in the linear portion of the standard curve was performed. Concentrations of unknown test samples were determined using the resulting linear equation and correction for the dilution factor. Results: Results are presented in FIG. **4**. As can be seen, of the three monopegylated species, N-terminally monopegylated G-CSF demonstrates the highest in vitro biological activity.

5. In vivo activity.

In vivo testing confirmed the activity of the N-terminally pegylated material. The in vivo testing was carried out by dosing male golden hamsters with a 0.1 mg/kg of sample, using a single subcutaneous injection. Four animals were subjected to terminal bleeds per group per time point. Serum samples were subject to a complete blood count on the same day that the samples were collected. The average white blood cell counts were calculated. As can be seen in FIGS. **5**A and **5**B, the response from each material peaks after one day following a single subcutaneous injection of 0.1 mg/kg. Two of the monopegylated materials, (N-terminal and Lys-35) showed prolonged responses, while the response for the protein pegylated at lysine-41 showed no increase in in vivo activity over unmodified rhG-CSF (indeed it shows less, FIG. **5**B). These results illustrate that attaching a single polyethylene glycol molecule can dramatically alter the therapeutic profile of a protein and that the benefit of pegylating a protein can be dependent upon the site of modification. (The net average WBC area under the curve after the single subcutaneous injection (calculated according to CRC Standard Mathematical Tables, 26th Ed. (Beyer, W. H., Ed.) CRC Press Inc., Boca Raton, Fla. 1981. p. 125) was similar for the Lys-35 and N-terminal monopegylated species.)

E. Stability Studies

In addition, stability studies were performed on the N-terminal and Lys-35 monopegylated species as prepared above. (The Lys-41 material was not used as it demonstrated no additional activity beyond unmodified G-CSF). These studies demonstrate that the N-terminally pegylated G-CSF is unexpectedly more stable upon storage than the other form of monopegylated G-CSF, monopegylated lysine 35. Stability was assessed in terms of breakdown of product, as visualized using SEC-HPLC. Methods: N-terminally pegylated G-CSF and lysine-35 monopegylated G-CSF were studied in two pH levels, pH 4.0 and pH 6.0 at 4° C., each for up to 16 days. Elevating the pH to 6.0 provides an environment for accelerated stability assays. For the pH 6.0 samples, N-terminal monopegylated G-CSF and Lysine 35 monopegylated G-CSF as prepared above were placed in a buffer containing 20 mM sodium phosphate, 5 mM sodium acetate, 2.5 % mannitol, 0.005 % TWEEN-80, pH 6.0 at a final protein concentration of 0.25 mg/ml. One ml aliquots were stored in 3 ml sterile injection vials. Vials of each was stored at 4° C. and 29° C. for up to 16 days. Stability was assessed by SEC-HPLC tracings. If the later measurements stayed the same (as ascertained by visual inspection) as the initial (Time=0) measurements, the sample was considered to be stable for that length of time. Results: Results are illustrated in FIGS. **6**A–**6**C. (a) Comparison at pH 6.0 at 4° C. FIG. **6**A shows the 4° C. SEC-HPLC profiles for N-terminally monopegylated G-CSF at pH 6 over time and FIG. **6**B shows the 4° C. SEC-HPLC profiles for lysine-35 monopegylated G-CSF at pH 6 over time. One interpretation is that the Lys-35 material is breaking down to a material with a molecular weight similar to that of unmodified G-CSF. (b) Extended duration at pH 4.0 at 4° C. PH 4.0 and 4° C. provides something of a control illustrating relatively stable conditions in that the N-terminal species shows no degradation. For the Lys 35 species, the break down of the material is still occurring, but at a much slower rate. (c) Comparison at pH 6.0 at 4° C. FIGS. **6**C illustrates the SEC-HPLC profiles for the monopegylated G-CSF's under these conditions, under extended time periods. As can be seen, at pH 6.0 and 4° C., the lysine-35 material exhibits no increase in depegylation at day 16 or day 35 beyond what was seen for day 6 (FIG. **6**B). This indicates that depegylation (instability) does not change, under those conditions, beyond day 6.

EXAMPLE 2

This example demonstrates a method of preparing a substantially homogenous population of monopegylated G-CSF using reductive alkylation, and characterization of this population. Recombinant G-CSF as described in the above example was used. As can be seen, not only do the present methods provide advantages in terms of yield of N-terminally chemically modified material, but also, the amine linkages of the present reductive alkylation process produce substantially more stable products as demonstrated by a large difference in the degree of aggregation upon storage.

A. Preparation of the mono-methoxypolyethylene glycol-GCSF conjugates with the site of attachment at the N-terminal α-amino residue.

To a cooled (4° C),stirred solution ofrhG-CSF (1 ml, 5 mg/ml as described in the Example above) in 100 mM sodium phosphate, pH 5, containing 20 mM NaCNBH3, was added a 5-fold molar excess of methoxypolyethylene glycol aldehyde (MPEG)(average molecular weight, 6 kDa). The stirring of the reaction mixture was continued at the same temperature.

The extent of the protein modification during the course of the reaction was monitored by SEC HPLC using BIO-SIL SEC 250–5 column (BIO-RAD) eluted with 0.05M NaH_2PO_4,0.05M Na_2HPO_4,0.15M NaCl, 0.01M NaN_3, pH 6.8 at 1 ml/min.

After 10 hours the SEC HPLC analysis indicated that 92% of the protein has been converted to the mono-MPEG-GCSF derivative. This can be seen in FIG. **7**, which is a recording of the protein concentration (as determined by absorbance at A_{280}) and shows the peak eluting at 8.72 minutes as monopegylated G-CSF, and a minor peak of unreacted G-CSF eluting at 9.78 minutes.

As a comparison, FIG. **8** shows the peaks obtained when using N-hydroxysuccinimidyl ester of MPEG. The molecular weight was also 6 kDa. As can be seen, the mixture obtained from this reaction was: tri-MPEG-GCSF conjugated (shoulder at approximately 7.25 minutes), di-MPEG-GCSF conjugate (peak at 7.62 minutes), mono-MPEG-GCSF conjugate (peak at 8.43 minutes) and unreacted G-CSF (peak at 9.87 minutes).

At this 10 hour time point, where 92% of the protein had been converted to monopegylated material, the pH of the reaction mixture was adjusted to pH 4 with 100 mM HCl and the reaction mixture was diluted 5 times with 1 mM HCl.

The mono-MPEG-GCSF derivative was purified by ion exchange chromatography using HiLoad 16/10 S SEPHAROSE HP column (Pharmacia) equilibrated with 20 mM sodium acetate buffer, pH 4. The reaction mixture was loaded on the column at a flow rate of 1 ml/min and the unreacted MPEG aldehyde eluted with three column volumes of the same buffer. Then a linear 400 minute gradient from 0% to 45% 20 mM sodium acetate, pH 4, containing 1M NaCl was used to the elute the protein-polymer conjugate at 4° C.

Fractions containing the mono-MPEG-GCSF derivative were pooled, concentrated and sterile filtered.

Various mono-MPEG-GCSF conjugates obtained by modifying rh-G-CSF with MPEG aldehydes of different average molecular weight (12, 20 and 25 kDa) were prepared in a similar manner.

B. Analysis of Monopeaylated G-CSF

1. Molecular Weight

The molecular weight at the monopegylated conjugates was determined by SDS-PAGE, gel filtration, matrix assisted laser desorption mass spectrometry, and equilibrium centrifugation. These results are presented in Table 4, below.

TABLE 4

	Molecular Weights of N-terminally Alkylated Mono-MPEG-GCSF Conjugates			
Conjugate	MW estimated	MW gel filtration	MW mass spectometry	MW ultra-centrifugation
MPEG-(6kDa)-GCSF	24800	53024	24737	25548
MPEG-(12kDa)-GCSF	30800	124343	30703	29711
MPEG-(20kDa)-GCSF	38800	221876	38577	38196
MPEG-(25kDa)-GCSF	43800	333266	N/D	

The structure of the prepared N-terminal mono-MPEG-GCSF conjugates was confirmed using the methods of N-terminal protein sequencing and peptide mapping. Cyanogen bromide cleavage of the N-terminal methionyl residue resulted in removal of the polyethylene glycol.

2. Biological Activity

The in vitro biological activity of the pegylated MPEG-GCSF conjugates was determined by measuring the stimulated uptake of ^3H thymidine into mouse bone marrow cells.

The in vivo biological activity was determined by subcutaneous injection to hamsters MPEG-GCSF conjugates or rhG-CSF (at 100 mg/kg) and measuring total white blood cell count. Bioactivity as compared to non-derivatized G-CSF was calculated as the area under the WBC/time curve after subtracting the vehicle control curve. Relative bioactivity of the MPEG-GCSF derivatives was expressed as the percentage bioactivity compared to unmodified G-CSF.

This is illustrated in FIG. **9**, which is a graph illustrating the total white blood cell response to mono-N-terminal MPEG-GCSF conjugates prepared by reductive alkylation of rhG-CF with MPEG aldehydes of different molecular weights (6 kDa, 12 kDa, and 20 kDa). As can be seen, all monopegylated molecules elicited a response. The higher the molecular weight of the polyethylene glycol moiety used, the higher the white blood cell count achieved, except the 12 kDa achieved a slightly higher count than did the 20 kDa version at day 2.

3. Stability Studies N-terminally pegylated G-CSF's prepared by the two different chemistries (amide vs. amine of the reductive alkylation here) were compared for the degree of aggregation. Unexpectedly, N-terminally pegylated G-CSF using the amine chemistry was found to be substantially more stable than N-terminally pegylated G-CSF with an amide linkage (NHS chemistry as described in Example 1).

Methods: Both N-terminally pegylated G-CSF samples were in 10 mM NaOac pH4.0 with 5% sorbitol, at a concentration of 1 mg protein/ml. The G-CSF's were pegylated with PEG 6000 for each. The amide-linked conjugate was prepared as in Example 1, and the amine linked conjugate was prepared as in Example 2. Six samples of each were stored for eight weeks at 45° C. At the end of eight weeks, the degree of aggregation was determined using size exclusion chromatography and ion exchange chromatography.

Results: The results demonstrate that the present reductive alkylation methodis advantageous over aceylation because, surprisingly, it produces a material with far fewer aggregates after 8 weeks at elevated temperatures. The table below shows the percent of non-aggregated material ("main peak" material) for both materials using size exclusion chromatography (SEC) or ion exchange (IE):

TABLE 5

Sample:8 wkg, 45° C.	% Main Peak SEC/IE
Amine	82%/84%
Amide	37%/65%*

*This is relatively high because ion exchange does not allow for full analysis of the aggregation.

EXAMPLE 3

This example demonstrates chemically modified consensus interferon. More specifically, this example demonstrates a method of preparing a substantially homogenous population of monopegylated IFN-con_1, and characterization of this population.

It should be noted that while the present example uses IFN-con_1, any of the consensus interferons as set forth above may be chemically modified. Such chemical modification may be with any of the water soluble polymers as listed above, although PEG is used here. For pegylation, PEG 12000 is used here, although any water soluble PEG species may be used (PEG 12000 was selected for ease in handling

and convenience). Again, a variety of means for chemical modification are available (such as acetylation) but, for selective N-terminal chemical modification, such as N-terminal pegylation, the present reductive alkylation method as described in this example is preferred.

A. Preparation of Consensus Interferon

IFN-αcon$_1$ (here referred to as IFN-con$_1$) as described in FIG. **2** of U.S. Pat. No. 4,695,623, which is incorporated by reference in its entirety, was used for the preparation of monopegylated consensus interferon. The IFN-con$_1$ was produced by expression of exogenous DNA in bacteria, and contained a methionyl residue at the N-terminus.

B. Pegylation of Consensus Interferon

To a cooled (4° C.), stirred solution of IFN-con$_1$ (3.45 mg/ml, containing 35.25% of the N-terminally blocked form) in 100 mM sodium phosphate, pH 4.0, containing 20 mM $NaCNBH_3$ was added a 8-fold molar excess of methoxypolyethylene glycol aldehyde (MPEG)(average molecular weight 12 kDa).

The extent of the protein modification during the course of the reaction was monitored by reverse phase HPLC using a polymer-based poly(styrene/divinylbenzene) column, such as PLRP-S (PL Separation Sciences Polymer Laboratories).

After 10 hours the reverse phase HPLC analysis indicated that 80% of the protein with unblocked α-amino group at the N-terminus has been converted to the MPEG-IFN-con$_1$ derivative.

At the 10 hour time point, the reaction mixture was diluted 5 times with water and the mono-MPEG-IFN-Con$_1$ derivative was purified by ion exchange chromatography using HiLoad 16/10 S SEPHAROSE HP column (Pharmacia) equilibrated with 20 mM sodium acetate buffer, pH 4.0. The reaction mixture was loaded on the column at a flow rate of 1 ml/min and the unreacted MPEG aldehyde eluted with three column volumes of the same buffer. Then a linear 420 minute gradient from 0% to 75% of 20 mM sodium acetate, pH 4.0, containing 1M NaCl was used to the elute the protein-polymer conjugate at 4° C.

Fractions containing the mono-MPEG-IFN-Con$_1$ derivative were pooled, concentrated and sterile filtered.

C. Analysis of Monopeaylated Consensus Interferon

1. Homogeneity

The homogeneity of the purified mono-MPEG-IFN-Con$_1$ conjugates was determined by SDS-PAGE using 10–20% or 4–20% precast gradient gels (Integrated Separation Systems). The gels showed a main band at MW 35 kDa.

To characterize the effective size (hydrodynamic radius) of each mono-MPEG-IFN-con$_1$ species a SUPEROSE 6 HR 10/30 (Pharmacia) gel filtration column was used. Proteins were detected by UV absorbance at 280 nm. The BIO-RAD gel filtration standards served as globular protein molecular weight markers.

The structure of the purified N-terminal mono-MPEG-IFN-con$_1$ conjugates was confirmed using the methods of N-terminal protein sequencing and peptide mapping.

It is noted that this IFN-con$_1$ preparation contained some N-terminally blocked material, and this material was not pegylated. The material which was pegylated, however, was monopegylated at the N-terminus. Thus, in this type of situation, one may wish to use other means to separate the blocked from the unblocked material, such as ion exchange or size exclusion chromatography.

2. Biological Activity

The in vitro biological activity of the mono-MPEG-IFN Con$_1$ conjugates was determined by measuring their antiviral bioactivity. The in vitro biological activity of the mono-MPEG-IFN-Con$_1$ conjugates was determined by measuring their antiviral bioactivity in human (HeLa) cells.

It was found that the mono-MPEG (12 kDa)-IFN-Con$_1$ conjugate shows 20% in vitro bioactivity (in U/mg of protein) when compared to the unmodified species. As noted above for pegylated G-CSF, the in vitro assays, while useful to demonstrate biological activity, may show a rather low level of activity for chemically modified proteins because of characteristic sustained release. The in vivo biological activity may be higher than the in vitro biological activity.

D. Chemically modified consensus interferon with the N-terminally blocked molecules removed

The present reductive alkylation was also performed on the above IFN-con$_1$ which had the portion of N-terminally blocked molecules pre-removed. Both PEG 12000 and PEG 20000 were used in the reductive alkylation method as described above.

The molecular apparent molecular weights were as follow:

Conjugate	Apparent MW by Gel Filtration	Apparent MW by SDS-PAGE
monoMPEG(12kDa) IFN-con$_1$	104.0 kDa	35.6 kDa
monoMPEG(20kDa) IFN-con$_1$	175.1 kDa	55.4 kDa

Analysis of the IFN-con$_1$ 20 kDa PEG conjugate using FPLC ion exchange chromatography resulted in three peaks:

MonoMPEG-IFN-con$_1$: 66% of the total area (eluting at 265.93 ml)

Protein aggregate and oligo MPEG-IFN-con$_1$ conjugate: 24% of the total area (eluting at 238.42 ml); and

Unreacted IFN-con$_1$: 10% of the total area (eluting at 328.77 ml).

The conditions were not further optimized. One may further separate the monopegylated material using chromatographic or other methods.

While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 2

(2) INFORMATION FOR SEQ ID NO:1:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 531 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATGACTCCAT TAGGTCCTGC TTCTTCTCTG CCGCAAAGCT TTCTGCTGAA ATGTCTGGAA      60
CAGGTTCGTA AAATCCAGGG TGACGGTGCT GCACTGCAAG AAAAACTGTG CGCTACTTAC     120
AAACTGTGCC ATCCGGAAGA GCTGGTACTG CTGGGTCATT CTCTTGGGAT CCCGTGGGCT     180
CCGCTGTCTT CTTGTCCATC TCAAGCTCTT CAGCTGGCTG GTTGTCTGTC TCAACTGCAT     240
TCTGGTCTGT TCCTGTATCA GGGTCTTCTG CAAGCTCTGG AAGGTATCTC TCCGGAACTG     300
GGTCCGACTC TGGACACTCT GCAGCTAGAT GTAGCTGACT TTGCTACTAC TATTTGGCAA     360
CAGATGGAAG AGCTCGGTAT GGCACCAGCT CTGCAACCGA CTCAAGGTGC TATGCCGGCA     420
TTCGCTTCTG CATTCCAGCG TCGTGCAGGA GGTGTACTGG TTGCTTCTCA TCTGCAATCT     480
TTCCTGGAAG TATCTTACCG TGTTCTGCGT CATCTGGCTC AGCCGTAATA G              531
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 175 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu
1               5                  10                  15

Lys Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu
            20                  25                  30

Gln Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu
        35                  40                  45

Val Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser
    50                  55                  60

Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His
65                  70                  75                  80

Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile
                85                  90                  95

Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala
            100                 105                 110

Asp Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala
        115                 120                 125

Pro Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala
    130                 135                 140

Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser
145                 150                 155                 160

Phe Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro
                165                 170                 175
```

What is claimed is:

1. A substantially homogenous preparation of N-terminally PEGylated G-CSF or analog thereof, optionally in a pharmaceutically acceptable diluent, carrier or adjuvant, said preparation being essentially free of G-CSF or analog thereof PEGylated at sites other than the N-terminus.

2. A preparation of claim 1 wherein the polyethylene glycol used for conjugation has a molecular weight of between about 2 kDa and 100 kDa.

3. A preparation of claim 2 wherein said polyethylene glycol has a molecular weight of between about 6 kDa and 25 kDa.

4. A preparation of claim 1 wherein said preparation is comprised of at least 90% N-terminally monoPEGylated G-CSF or analog thereof and at most 10% unPEGylated G-CSF or analog thereof.

5. A preparation of claim 4 wherein said preparation is comprised of at least 95% N-terminally monoPEGylated G-CSF or analog thereof and at most 5% unPEGylated G-CSF or analog thereof.

6. A preparation of claim 1 whererein said G-CSF has the sequence identified in SEQ. ID No. 1.

7. A substantially homogenous preparation of N-terminally monoPEGylated G-CSF, optionally in a pharmaceutically acceptable diluent, carrier or adjuvant, wherein: (a) said G-CSF has the amino acid sequence identified in SEQ. ID No. 1; (b) said G-CSF is monoPEGylated with a polyethylene glycol moiety having a molecular weight of between about 6 kDa and about 25 kDa.

8. A pharmaceutical composition comprising: (a) a substantially homogenous preparation of monoPEGylated G-CSF, said monoPEGylated G-CSF consisting of a polyethylene glycol moiety having a molecular weight of about 6 kDa and about 25 kDa connected to a G-CSF moiety solely at the N-terminus thereof via an amine linkage; (b) fewer than 5% non-PEGylated G-CSF molecules; and (c) a pharmaceutially acceptable diluent, adjuvant or carrier.

9. A method of treating a hematopoietic disorder comprising administering a therapeutically effective dose of a preparation of any one of claims 1–8.

10. A method for attaching a polyethylene glycol molecule to a G-CSF molecule, wherein said polyethylene glycol molecule has a single reactive aldehyde group, said method comprising:

(a) reacting said G-CSF with said polyethylene glycol molecule under reducing alkylation conditions, at a pH sufficiently acidic to selectively activate the alpha-amino group at the amino terminus of said G-CSF; and

(b) obtaining the pegylated G-CSF and

(c) optionally, separating the PEGylated G-CSF from from non-PEGylated G-CSF.

11. A method of claim 10 wherein said polyethylene glycol molecule has a molecular weight of about 6 kDa to about 25 kDa.

12. The PEGylated G-CSF product produced by the process of claim 10.

* * * * *

JS 44 (Rev. 12/12) (Modified by FLSD - April 29, 2013)

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.)* **NOTICE: Attorneys MUST Indicate All Re-filed Cases Below.**

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

DEFENDANTS Apotex Inc. and Apotex Corp.

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

County of Residence of First Listed Defendant Ontario, Canada
(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)*
Hogan Lovells US LLP, 600 Brickell Ave., #2700, Miami, FL 33131 &
Paul, Weiss, et al., 1285 Ave. of the Americas, New York, NY 10019

Attorneys *(If Known)*
Unknown

(d) Check County Where Action Arose: ☐ MIAMI- DADE ☐ MONROE ☑ BROWARD ☐ PALM BEACH ☐ MARTIN ☐ ST. LUCIE ☐ INDIAN RIVER ☐ OKEECHOBEE ☐ HIGHLANDS

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)*

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 (Excl. 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 - Med. 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

Other:
- ☐ 530 General
- ☐ 535 Death Penalty
- ☐ 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/Mgmt. Relations
- ☐ 740 Railway Labor Act
- ☐ 751 Family and Medical Leave Act
- ☐ 790 Other Labor Litigation
- ☐ 791 Empl. Ret. Inc. 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
- ☐ 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
- ☐ 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 Re-filed (See VI below) ☐ 4 Reinstated or Reopened ☐ 5 Transferred from another district *(specify)* ☐ 6 Multidistrict Litigation ☐ 7 Appeal to District Judge from Magistrate Judgment ☐ 8 Remanded from Appellate Court

VI. RELATED/ RE-FILED CASE(S)

(See instructions): a) Re-filed Case ☐YES ☑NO b) Related Cases ☐YES ☑NO

JUDGE DOCKET NUMBER

VII. CAUSE OF ACTION

Cite the U.S. Civil Statute under which you are filing and Write a Brief Statement of Cause *(Do not cite jurisdictional statutes unless diversity)*:
35 USC § 271(e)(2)(C); 28 USC § 2201 - § 2202

LENGTH OF TRIAL via 6 days estimated (for both sides to try entire case)

VIII. REQUESTED IN COMPLAINT:

☐ CHECK IF THIS IS A CLASS ACTION UNDER F.R.C.P. 23

DEMAND $

CHECK YES only if demanded in complaint:
JURY DEMAND: ☐ Yes ☑ No

ABOVE INFORMATION IS TRUE & CORRECT TO THE BEST OF MY KNOWLEDGE

DATE: August 6, 2015

SIGNATURE OF ATTORNEY OF RECORD: Jason Sternberg

FOR OFFICE USE ONLY

RECEIPT # AMOUNT IFP JUDGE MAG JUDGE

INSTRUCTIONS FOR ATTORNEYS COMPLETING CIVIL COVER SHEET FORM JS 44

Authority For Civil Cover Sheet

The JS 44 civil cover sheet and the information contained herein neither replaces nor supplements the filings and service of pleading 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. Consequently, a civil cover sheet is submitted to the Clerk of Court for each civil complaint filed. The attorney filing a case should complete the form as follows:

I. (a) Plaintiffs-Defendants. Enter names (last, first, middle initial) of plaintiff and defendant. If the plaintiff or defendant is a government agency, use only the full name or standard abbreviations. If the plaintiff or defendant is an official within a government agency, identify first the agency and then the official, giving both name and title.

(b) County of Residence. For each civil case filed, except U.S. plaintiff cases, enter the name of the county where the first listed plaintiff resides at the time of filing. In U.S. plaintiff cases, enter the name of the county in which the first listed defendant resides at the time of filing. (NOTE: In land condemnation cases, the county of residence of the "defendant" is the location of the tract of land involved.)

(c) Attorneys. Enter the firm name, address, telephone number, and attorney of record. If there are several attorneys, list them on an attachment, noting in this section "(see attachment)".

II. Jurisdiction. The basis of jurisdiction is set forth under Rule 8(a), F.R.C.P., which requires that jurisdictions be shown in pleadings. Place an "X" in one of the boxes. If there is more than one basis of jurisdiction, precedence is given in the order shown below.

United States plaintiff. (1) Jurisdiction based on 28 U.S.C. 1345 and 1348. Suits by agencies and officers of the United States are included here.

United States defendant. (2) When the plaintiff is suing the United States, its officers or agencies, place an "X" in this box.

Federal question. (3) This refers to suits under 28 U.S.C. 1331, where jurisdiction arises under the Constitution of the United States, an amendment to the Constitution, an act of Congress or a treaty of the United States. In cases where the U.S. is a party, the U.S. plaintiff or defendant code takes precedence, and box 1 or 2 should be marked.

Diversity of citizenship. (4) This refers to suits under 28 U.S.C. 1332, where parties are citizens of different states. When Box 4 is checked, the citizenship of the different parties must be checked. (See Section III below; federal question actions take precedence over diversity cases.)

III. Residence (citizenship) of Principal Parties. This section of the JS 44 is to be completed if diversity of citizenship was indicated above. Mark this section for each principal party.

IV. Nature of Suit. Place an "X" in the appropriate box. If the nature of suit cannot be determined, be sure the cause of action, in Section VI below, is sufficient to enable the deputy clerk or the statistical clerks in the Administrative Office to determine the nature of suit. If the cause fits more than one nature of suit, select the most definitive.

V. Origin. Place an "X" in one of the seven boxes.

Original Proceedings. (1) Cases which originate in the United States district courts.

Removed from State Court. (2) Proceedings initiated in state courts may be removed to the district courts under Title 28 U.S.C., Section 1441. When the petition for removal is granted, check this box.

Refiled (3) Attach copy of Order for Dismissal of Previous case. Also complete VI.

Reinstated or Reopened. (4) Check this box for cases reinstated or reopened in the district court. Use the reopening date as the filing date.

Transferred from Another District. (5) For cases transferred under Title 28 U.S.C. Section 1404(a). Do not use this for within district transfers or multidistrict litigation transfers.

Multidistrict Litigation. (6) Check this box when a multidistrict case is transferred into the district under authority of Title 28 U.S.C. Section 1407. When this box is checked, do not check (5) above.

Appeal to District Judge from Magistrate Judgment. (7) Check this box for an appeal from a magistrate judge's decision.

Remanded from Appellate Court. (8) Check this box if remanded from Appellate Court.

VI. Related/Refiled Cases. This section of the JS 44 is used to reference related pending cases or re-filed cases. Insert the docket numbers and the corresponding judges name for such cases.

VII. Cause of Action. Report the civil statute directly related to the cause of action and give a brief description of the cause. **Do not cite jurisdictional statutes unless diversity.**
Example: U.S. Civil Statute: 47 USC 553
Brief Description: Unauthorized reception of cable service

VIII. Requested in Complaint. Class Action. Place an "X" in this box if you are filing a class action under Rule 23, F.R.Cv.P.

Demand. In this space enter the dollar amount (in thousands of dollars) being demanded or indicate other demand such as a preliminary injunction.

Jury Demand. Check the appropriate box to indicate whether or not a jury is being demanded.

Date and Attorney Signature. Date and sign the civil cover sheet.