ABSTRACT

Provided herein are glycovariant Fc fusion proteins having increased serum half lives. Also provided are methods for increasing the serum half life of an Fc fusion protein by introducing one or more non-endogenous glycosylation sites.

We Claim:

1. A method for preparing a modified nucleic acid encoding a modified Fc fusion protein that has an extended serum half-life relative to an initial Fc fusion protein, wherein the modified and initial Fc fusion proteins each comprise an Fc portion and a heterologous portion, the method comprising: modifying the nucleic acid encoding the heterologous portion of the initial Fc fusion protein to code for one or more additional N-linked glycosylation sites, wherein (a) the modified nucleic acid encodes a modified Fc fusion protein that, when expressed in a suitable cell culture, has a serum half-life that is at least 10% longer than the serum half-life of the initial Fc fusion protein as measured in a pharmacokinetic monkey assay, and (b) the modified Fc fusion protein has substantially the same or greater *in vivo* biological activity relative to the unmodified Fc fusion protein.

2. The method of claim 1, wherein the one or more additional N-linked glycosylation sites are introduced into one or more amino acid sequences of the heterologous portion that are surface exposed.

3. The method of claim 2, wherein the one or more additional N-linked glycosylation sites are introduced into one or more amino acid sequences of the heterologous portion that are surface exposed and not incorporated into a β-sheet or α-helix.

4. The method of any one of claims 1-3, wherein the heterologous portion comprises a ligand binding domain.

5. The method of claim 4, wherein the ligand binding domain is derived from the extracellular domain of a transmembrane receptor.

6. The method of claim 4 or 5, wherein the one or more additional N-linked glycosylation sites are introduced at positions in the heterologous portion such that any additional sugar moieties do not substantially interfere with the ligand binding domain.

7. The method of any one of claims 4-6, wherein the one or more additional N-linked glycosylation sites are introduced at positions such that the amino acid sequence of the ligand binding domain is not modified.

8. The method of any one of claims 4-7, wherein the one or more additional N-linked glycosylation sites are introduced at positions such that any sugar moieties attached to the additional N-linked glycosylation sites are predicted not to interfere substantially with the ligand binding interface.

9. The method of any one of claims 4-8, wherein the modified Fc fusion protein binds to a ligand with half maximal inhibitory concentration (IC_{50}) that is no more than two-fold less than that of the initial Fc fusion protein.

10. The method of any one of claims 1-9, wherein the initial Fc fusion protein is modified by addition or deletion of at least one amino acid residue to introduce at least one glycosylation site.

11. The method of any one of claims 1-10, wherein the initial Fc fusion protein is modified by substitution of at least one amino acid residue to introduce at least one glycosylation site.

12. The method of any one of claims 1-11, wherein the heterologous portion of the initial Fc fusion protein comprises at least two structurally distinct α-helix and/or β-sheet domains that are connected by an unstructured polypeptide region that is surface exposed.

13. The method of claim 12, wherein an additional N-linked glycosylation site is positioned within the unstructured peptide region.

14. The method of any one of claims 1-13, wherein the heterologous portion of the initial Fc fusion protein comprises fewer than one N-linked glycosylation site per each 90 amino acids.

15. The method of any one of claims 1-14, wherein the heterologous portion of the initial Fc fusion protein comprises fewer than one N-linked glycosylation site per each 125 amino acids.

16. The method of any one of claims 1-15, wherein the heterologous portion of the modified Fc fusion protein comprises at least one N-linked glycosylation site per each 90 amino acids.

17. The method of any one of claims 1-16, wherein the heterologous portion of the modified Fc fusion protein comprises at least one N-linked glycosylation site per each 65 amino acids.

18. The method of any one of claims 1-17, wherein each amino acid of the heterologous portion of the modified Fc fusion protein that is attached to an N-linked glycosylation is separated by at least 20 amino acids from any other amino acid modified by an N-linked glycosylation.

19. The method of any one of claims 1-18, wherein the initial Fc fusion protein further comprises a polypeptide linker between the Fc portion and the heterologous portion that does not contain an N-linked glycosylation site.

20. The method of any one of claims 1-18, wherein the modified Fc fusion protein comprises an N-linked glycosylation site positioned within the linker.

21. The method of any one of claims 1-20, wherein the heterologous portion comprises the extracellular domain of a TNFR2 receptor that includes a ligand binding domain.

22. The method of claim 21, wherein the extracellular domain of the TNFR2 receptor comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 2.

23. A method for preparing a modified Fc fusion protein that has an extended serum half-life relative to an initial Fc fusion protein, the method comprising:

(a) expressing a modified nucleic acid prepared according to the method of any of claims 1 to 22 in a cell culture that provides mammalian or mammalian-like glycosylation; and

(b) recovering the modified Fc fusion protein from the cell culture.

24. The method of claim 23, further comprising a step of purifying the modified Fc fusion protein.

25. The method of claim 24, wherein a step of purifying the modified Fc fusion protein comprises exposing the modified Fc fusion protein to protein A and recovering the modified Fc fusion protein that is bound to the protein A.

26. The method of any one of claims 23-25, further comprising formulating the modified Fc fusion protein for administration to a patient.

27. The method of claim 23, wherein the modified nucleic acid is expressed by a mammalian cell line.

28. The method of claim 27, wherein the mammalian cell line is selected from the group consisting of: a CHO cell line, an NSO cell line, a COS cell line and an HEK236 cell line.

29. The method of any one of claims 23, 27 or 28, wherein the modified nucleic acid is expressed in a cell line that generates N-linked sugar moieties that comprise sialic acid.

30. The method of claim 23 or 29, wherein the modified nucleic acid is expressed by a non-mammalian cell that has been engineered to provide mammalian or mammalian-like glycosylation.

31. The method of claim 30, wherein the cell line derives from a fungal cell, an insect cell or a plant cell.

32. A cell line comprising a modified nucleic acid prepared according to a method of any of claims 1-22.

33. A modified Fc fusion protein prepared according to a method of any one of claim 23-32.

34. A fusion protein comprising an immunoglobulin Fc domain and at least one heterologous polypeptide domain, wherein the fusion protein is modified outside of the immunoglobulin Fc domain to introduce at least one non-endogenous N-linked glycosylation site, and wherein glycosylation at the one or more introduced glycosylation sites increases the serum half-life of the modified fusion protein by at least 10% relative to the serum half-life of the fusion protein lacking an introduced glycosylation site as measured in a pharmacokinetic monkey assay.

35. The fusion protein of claim 34, wherein the heterologous polypeptide domain comprises at least a portion of an extracellular domain of a receptor that includes a ligand binding domain.

36. The fusion protein of claim 35, wherein the glycosylation site is introduced in the extracellular domain outside of the ligand binding pocket.

37. The fusion protein of claim 35 or 36, wherein glycosylation at the one or more introduced glycosylation sites does not affect ligand binding activity of the receptor by more than 3-fold.

38. The fusion protein of any one of claims 34-37, wherein the fusion protein is modified by addition or deletion of at least one amino acid residue to introduce at least one glycosylation site.

39. The fusion protein of any one of claims 34-38, wherein the fusion protein is modified by substitution of at least one amino acid residue to introduce at least one glycosylation site.

40. The fusion protein of any one of claims 34-39, wherein the molecular weight of the heterologous polypeptide domain is at least 25 kDa.

41. The fusion protein of any one of claims 34-40, wherein glycosylation at one or more of the introduced glycosylation sites increase the serum half-life of the fusion protein by at least 20% relative to the serum half-life of the fusion protein lacking an introduced glycosylation site.

42. The fusion protein any one of claims 34-41, wherein the unmodified heterologous polypeptide domain comprises fewer than one N-linked glycosylation site per each 90 amino acids.

43. The fusion protein of any one of claims 34-41, wherein the unmodified heterologous polypeptide domain comprises fewer than one N-linked glycosylation site per each 125 amino acids.

44. The fusion protein of any one of claims 34-41, wherein the modified heterologous polypeptide comprises at least one N-linked glycosylation site per each 90 amino acids.

45. The fusion protein of any one of claims 34-41, wherein the modified heterologous polypeptide comprises at least one N-linked glycosylation site per each 65 amino acids.

46. The fusion protein of any one of claims 34-45, wherein each amino acid modified by an N-linked glycosylation is separated by at least 20 amino acids from any other amino acid modified by an N-linked glycosylation.

47. The fusion protein of any one of claims 34-46, wherein the fusion protein further comprises a polypeptide linker between the Fc domain and the heterologous polypeptide domain.

48. The fusion protein of claim 47, wherein at least one of the introduced glycosylation sites is located in the linker.

49. The fusion protein of any one of claims 34-48, wherein the heterologous portion of the Fc fusion protein at least two structurally distinct α-helix and/or β-sheet domains that are connected by an unstructured polypeptide region that is surface exposed.

50. The fusion protein of claim 49, wherein an introduced glycosylation site is positioned within the unstructured peptide region.

51. The fusion protein of claim 35-50, wherein the receptor is a member of the nerve growth factor/tumor necrosis factor receptor family.

52. The fusion protein of claim 51, wherein the receptor is a TNFR2 receptor.

53. The fusion protein of claim 52, wherein the extracellular domain of the TNFR2 receptor comprises an amino acid substitution at one or more amino acid residues selected from Q26, D25, A28, E133, or G231 of SEQ ID NO: 2.

54. The fusion protein of claim 53, wherein the extracellular domain of the TNFR2 receptor comprises a Q to N substitution at amino acid 26 of SEQ ID NO: 2 and an A to S substitution at amino acid 28 of SEQ ID NO: 2.

55. The fusion protein of claim 53, wherein the extracellular domain of the TNFR2 receptor comprises a D to N substitution at amino acid 25 of SEQ ID NO: 2 and an E to N substitution at amino acid 133 of SEQ ID NO: 2.

56. The fusion protein of claim 53, wherein the extracellular domain of the TNFR2 receptor comprises a D to N substitution at amino acid 25 of SEQ ID NO: 2 and an G to N substitution at amino acid 231 of SEQ ID NO: 2.

57. The fusion protein of any one of claim 53-56, wherein the extracellular domain of the TNFR2 receptor is at least 90% identical to the amino acid sequence of SEQ ID NO: 2.

58. The fusion protein of claim 57, wherein the extracellular domain of the TNFR2 receptor is at least 95% identical to the amino acid sequence of SEQ ID NO: 2.

59. The fusion protein of claim 58, wherein the extracellular domain of the TNFR2 receptor is at least 99% identical to the amino acid sequence of SEQ ID NO: 2.

60. The fusion protein of claim 53, wherein the fusion protein comprises a heterologous domain selected from:

a) a polypeptide comprising amino acids 13-257 of SEQ ID NO: 5;

b) a polypeptide comprising amino acids 13-257 of SEQ ID NO: 6;

c) a polypeptide comprising amino acids 13-257 of SEQ ID NO: 7; and

d) a polypeptide comprising amino acids 13-257 of SEQ ID NO: 8.

61. The fusion protein of claim 60, wherein the fusion protein comprises a linker and Fc portion having the amino acid sequence of SEQ ID NO: 18.

62. A pharmaceutical preparation comprising the fusion protein of any one of claims 34-61 and a pharmaceutically acceptable carrier, wherein the preparation is substantially free of pyrogenic materials so as to be suitable for administration to a mammal.

Dated this 12/06/2012

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