CLAIMS

1. An in vitro method for generating a heterodimeric protein, said method comprising the following steps:

a) providing a first homodimeric protein comprising an Fc region of an immunoglobulin, said Fc region comprising a first CH3 region, wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409,

b) providing a second homodimeric protein comprising an Fc region of an immunoglobulin, said Fc region comprising a second CH3 region, wherein said second homodimeric protein has an amino-acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405 and 407

wherein the sequences of said first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions,

c) incubating said first protein together with said second protein under reducing conditions sufficient to allow the cysteines in the hinge region to undergo disulfide-bond isomerization, and

d) obtaining said heterodimeric protein.

2. The in vitro method according to claim 1, wherein said first homodimeric protein and said second homodimeric protein are selected from the group consisting of (i) an Fc region, (ii) an antibody, (iii) a fusion protein comprising an Fc region, and (iv) a Fc region conjugated to a prodrug, peptide, drug or a toxin.

3. The in vitro method according to claim 1, wherein said first homodimeric protein is a full-length antibody.

4. The in vitro method according to any one of the preceding claims, wherein said second homodimeric protein is a full-length antibody.

5. The in vitro method according to any one of the preceding claims, wherein said first and second homodimeric proteins are both antibodies and bind different epitopes.

6. The in vitro method according to any one of the preceding claims, wherein the Fc region of the first homodimeric protein is of an isotype selected from the group consisting of IgG1, IgG2, IgG3 and IgG4 and wherein the Fc region of the second homodimeric protein is of an isotype selected from the group consisting of IgG1, IgG2, IgG3 and IgG4.

7. The in vitro method according to any one of the preceding claims, wherein the Fc regions of both said first and said second homodimeric protein are of the IgG1 isotype.

8. The in vitro method according to any one of the preceding claims, wherein one of the Fc regions of said homodimeric proteins is of the IgG1 isotype and the other of the IgG4 isotype.

9. The in vitro method according to any one of the preceding claims, wherein the increased strength of the heterodimeric interaction as compared to each of the homodimeric interactions is due to CH3 modifications other than the introduction of covalent bonds, cysteine residues or charged residues.

10. The in vitro method according to any one of the preceding claims, wherein the heterodimeric interaction between said first and second proteins in the resulting heterodimeric protein is such that no Fab-arm exchange can occur at 0.5 mM glutathione under the conditions described in Example 13.

11. The in vitro method according to any one of the preceding claims, wherein the heterodimeric interaction between said first and second proteins in the resulting heterodimeric protein is such that no Fab-arm exchange occurs in vivo in mice under the conditions described in Example 14.

12. The in vitro method according to any one of the preceding claims, wherein the heterodimeric interaction between said first and second proteins in the resulting heterodimeric protein is more than two times stronger, such as more than three times stronger, e.g. more than five times stronger than the strongest of the two homodimeric interactions, e.g. when determined as described in Example 30.

13. The in vitro method according to any one of the preceding claims, wherein the sequences of said first and second CH3 regions are such that the dissociation constants of the heterodimeric interaction between said first and second proteins in the resulting heterodimeric protein is below 0.05 micromolar when assayed as described in Example 30.

14. The in vitro method according to any one of the preceding claims, wherein the sequences of said first and second CH3 regions are such that the dissociation constants of both homodimeric interactions are above 0.01 micromolar, such as above 0.05 micromolar, preferably between 0.01 and 10 micromolar, such as between 0.05 and 10 micromolar, more preferably between 0.01 and 5, such as between 0.05 and 5 micromolar, even more preferably between 0.01 and 1 micromolar, such as between 0.05 and 1 micromolar, between 0.01 and 0.5 or between 0.01 and 0.1 micromolar when assayed as described in Example 21.

15. The in vitro method according to any one of the preceding claims, wherein the sequences of said first and second CH3 regions contain amino acid substitutions at non-identical positions.

16. The in vitro method according to claim 15, wherein the amino acid substituents are natural amino acids or unnatural amino acids.

17. The in vitro method according to any one of the preceding claims, wherein said first homodimeric protein has no more than one amino acid substitution in the CH3 region, and the second homodimeric protein has no more than one amino acid substitution in the CH3 region relative to the wild-type CH3 regions.

18. The in vitro method according to any one of the preceding claims 1 to 16, wherein said second homodimeric protein has an amino acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405, and 407, and wherein said first homodimeric protein and said second homodimeric protein is not substituted in the same positions.

19. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has an amino acid other than Phe at position 405.

20. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has an amino acid other than Phe, Arg or Gly at position 405.

21. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein comprises a Phe at position 405 and an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein comprises an amino acid other than Phe at position 405 and a Lys at position 409.

22. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein comprises a Phe at position 405 and an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein comprises an amino acid other than Phe, Arg or Gly at position 405 and a Lys at position 409.

23. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein comprises a Phe at position 405 and an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein comprises a Leu at position 405 and a Lys at position 409.

24. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein comprises a Phe at position 405 and an Arg at position 409 and said second homodimeric protein comprises an amino acid other than Phe, Arg or Gly at position 405 and a Lys at position 409.

25. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein comprises Phe at position 405 and an Arg at position 409 and said second homodimeric protein comprises a Leu at position 405 and a Lys at position 409.

26. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein comprises an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein comprises a Lys at position 409, a Thr at position 370 and a Leu at position 405.

27. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein comprises an Arg at position 409 and said second homodimeric protein comprises a Lys at position 409, a Thr at position 370 and a Leu at position 405.

28. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein comprises a Lys at position 370, a Phe at position 405 and an Arg at position 409 and said second homodimeric protein comprises a Lys at position 409, a Thr at position 370 and a Leu at position 405.

29. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has an amino acid other than Tyr, Asp, Glu, Phe, Lys, Gln, Arg, Ser or Thr at position 407.

30. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has an Ala, Gly, His, Ile, Leu, Met, Asn, Val or Trp at position 407.

31. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has a Gly, Leu, Met, Asn or Trp at position 407.

32. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein has a Tyr at position 407 and an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has an amino acid other than Tyr, Asp, Glu, Phe, Lys, Gln, Arg, Ser or Thr at position 407 and a Lys at position 409.

33. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein has a Tyr at position 407 and an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has an Ala, Gly, His, Ile, Leu, Met, Asn, Val or Trp at position 407 and a Lys at position 409.

34. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein has a Tyr at position 407 and an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has a Gly, Leu, Met, Asn or Trp at position 407 and a Lys at position 409.

35. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein has a Tyr at position 407 and an Arg at position 409 and said second homodimeric protein has an amino acid other than Tyr, Asp, Glu, Phe, Lys, Gln, Arg, Ser or Thr at position 407 and a Lys at position 409.

36. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein has a Tyr at position 407 and an Arg at position 409 and said second homodimeric protein has an Ala, Gly, His, Ile, Leu, Met, Asn, Val or Trp at position 407 and a Lys at position 409.

37. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein has a Tyr at position 407 and an Arg at position 409 and said second homodimeric protein has a Gly, Leu, Met, Asn or Trp at position 407 and a Lys at position 409.

38. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409, and the second homodimeric protein has

(i) an amino acid other than Phe, Leu and Met at position 368, or

(ii) a Trp at position 370, or

(iii) an amino acid other than Asp, Cys, Pro, Glu or Gln at position 399.

39. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein has an Arg, Ala, His or Gly at position 409, and the second homodimeric protein has

(i) a Lys, Gln, Ala, Asp, Glu, Gly, His, Ile, Asn, Arg, Ser, Thr, Val, or Trp at position 368, or

(ii) a Trp at position 370, or

(iii) an Ala, Gly, Ile, Leu, Met, Asn, Ser, Thr, Trp, Phe, His, Lys, Arg or Tyr at position 399.

40. The in vitro method according to any one of the preceding claims 1 to 18, wherein said first homodimeric protein has an Arg at position 409, and the second homodimeric protein has

(i) an Asp, Glu, Gly, Asn, Arg, Ser, Thr, Val, or Trp at position 368, or

(ii) a Trp at position 370, or

(iii) a Phe, His, Lys, Arg or Tyr at position 399.

41. The method of any one of claims 1 to 18, wherein said first homodimeric protein comprises a Phe at position 405 and an Arg at position 409 and said second homodimeric protein comprises a Leu at position 405 and a Lys at position 409.

42. The in vitro method according to any one of the preceding claims, wherein said first and second CH3 regions, except for the specified mutations, comprise the sequence set forth in SEQ ID NO:1.

43. The in vitro method according to any one of the preceding claims, wherein neither said first nor said second homodimeric protein comprises a Cys-Pro-Ser-Cys sequence in the hinge region.

44. The in vitro method according to any one of the preceding claims, wherein both said first and said second homodimeric protein comprise a Cys-Pro-Pro-Cys sequence in the hinge region.

45. The in vitro method according to any one of the preceding claims, wherein said first and second homodimeric proteins, except for any specified mutations, are human antibodies.

46. The in vitro method according to any one of the preceding claims, wherein said first and second homodimeric proteins are heavy-chain antibodies.

47. The in vitro method according to any one of the preceding claims 1 to 46, wherein both said first and said second homodimeric proteins further comprise a light chain.

48. The in vitro method according to claim 47, wherein said light chains are different.

49. The in vitro method according to any one of the preceding claims, wherein said first and/or said second homodimeric protein comprises a mutation removing the acceptor site for Asn-linked glycosylation.

50. The in vitro method according to any one of the preceding claims, wherein said first and second homodimeric proteins provided in step a) and b) are purified.

51. The in vitro method according to any one of the preceding claims, wherein said first and/or second homodimeric protein is conjugated to a drug, a prodrug or a toxin or contains an acceptor group for the same.

52. The in vitro method according to any one of the preceding claims 4 to 51, wherein said first and/or said second epitope is located on a tumor cell.

53. The in vitro method according to any one of the preceding claims 4 to 52, wherein said first or said second epitope is located on a tumor cell and the other epitope is located on an effector cell.

54. The in vitro method according to any one of the preceding claims 4 to 53, wherein the epitope is located on a T cell, such as on CD3 expressed on a T cell.

55. The in vitro method according to any one of the preceding claims 4 to 52, wherein said first or said second epitope is located on a tumor cell and the other epitope is located on a radioisotope, a toxin, a drug or a prodrug, which may optionally be coupled or linked to a peptide or hapten.

56. The in vitro method according to any one of the preceding claims 4 to 52, wherein said first or said second epitope is located on a tumor cell and the other epitope is located on an electron dense vesicle or minicell.

57. The in vitro method according to any one of the preceding claims 1 to 52, wherein the homodimeric proteins are both antibodies, and wherein the first antibody and the second antibody binds to different epitopes on the same tumor cell.

58. The in vitro method according to any one of the preceding claims 1 to 52, wherein the homodimeric proteins are both antibodies, and wherein the first antibody binds to an epitope on a tumor cell, and the other antibody is an irrelevant or inactive antibody without any relevant in vivo binding activity for the application intended.

59. The in vitro method according to any one of the preceding claims, wherein the reducing conditions in step c) comprise the addition of a reducing agent, e.g. a reducing agent selected from the group consisting of: 2-mercaptoethylamine, dithiothreitol and tris(2-carboxyethyl)phosphine or chemical derivatives thereof.

60. The in vitro method according to any one of the preceding claims, wherein step c) is performed under reducing conditions with a redox potential between -150 and -600 mV, such as between -250 and -400 mV.

61. The in vitro method according to any one of the preceding claims, wherein step c) comprises incubation for at least 90 min at a temperature of at least 20°C in the presence of at least 25 mM 2-mercaptoethylamine or in the presence of at least 0.5 mM dithiothreitol.

62. The in vitro method according to any one of the preceding claims, wherein step d) comprises removal of a reducing agent, e.g. by desalting.

63. A method for the selection of a bispecific antibody having a desired property, said method comprising the steps of:

a) providing a first set of homodimeric antibodies comprising antibodies with different variable regions, wherein said antibodies of said first set comprise identical first CH3

regions, and wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409,

b) providing a second set of homodimeric antibodies comprising antibodies with different variable regions or identical variable regions, wherein said antibodies of said second set comprise identical second CH3 regions, and wherein each of said second homodimeric antibodies has an amino-acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405 and 407

wherein the sequences of said first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions,

c) incubating combinations of antibodies of said first set and of said second set under reducing conditions sufficient to allow the cysteines in the hinge region to undergo disulfide-bond isomerization, thus generating a set of bispecific antibodies,

d) optionally restoring the conditions to non-reducing,

e) assaying the resulting set of bispecific antibodies for a given desired property, and

f) selecting a bispecific antibody having the desired property.

64. A method of claim 63, wherein the homodimeric antibodies of the second set have different variable regions.

65. A method of claim 63, wherein the homodimeric antibodies of the second set have identical variable regions, but have different amino acid or structural variations outside of the antigen binding region.

66. A method for producing a heterodimeric protein, said method comprising the following steps:

a) providing a first nucleic-acid construct encoding a first polypeptide comprising a first Fc region of an immunoglobulin, said first Fc region comprising a first CH3 region,

b) providing a second nucleic-acid construct encoding a second polypeptide comprising a second Fc region of an immunoglobulin, said second Fc region comprising a first CH3 region,

wherein the sequences of said first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions, and

wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has an amino-acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405 and 407.

and/or

wherein the sequences of said first and second CH3 regions are such that the dissociation constants of homodimeric interactions of each of the CH3 regions are between 0.01 and 10 micromolar, such as between 0.05 and 10 micromolar, more preferably between 0.01 and 5, such as between 0.05 and 5 micromolar, even more preferably between 0.01 and 1 micromolar, such as between 0.05 and 1 micromolar, between 0.01 and 0.5 or between 0.01 and 0.1 when assayed as described in Example 21.

c) co-expressing said first and second nucleic-acid constructs in a host cell, and

d) obtaining said heterodimeric protein from the cell culture.

67. The method according to claim 66, wherein

said first CH3 region has an amino acid other than Lys, Leu or Met at position 409 and said second CH3 region has an amino acid other than Phe at position 405, such as other than Phe, Arg or Gly at position 405

or

said first CH3 region has an amino acid other than Lys, Leu or Met at position 409 and said second CH3 region has an amino acid other than Tyr, Asp, Glu, Phe, Lys, Gln, Arg, Ser or Thr at position 407.

68. The method according to claim 66 or 67, wherein said first and second polypeptides are full-length heavy chains of two antibodies that bind different epitopes.

69. The method according to any one of preceding claims 66 to 68, wherein step c) further comprises co-expressing one or more nucleic-acid constructs encoding a light-chain in said host cell.

70. The method according to any of the preceding claims 66 to 69, further comprising the features of any one or more of claims 2 to 58.

71. An expression vector comprising the nucleic-acid constructs specified in any one of claims 66 to 70.

72. A host cell comprising the nucleic-acid constructs specified in any one of claims 66 to 70.

73. A heterodimeric protein obtained or obtainable by the method of any one of the preceding claims 1 to 72.

74. A heterodimeric protein comprising a first polypeptide comprising a first Fc region of an immunoglobulin, said first Fc region comprising a first CH3 region, and a second polypeptide comprising a second Fc region of an immunoglobulin, said second Fc region comprising a second CH3 region, wherein the sequences of said first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions, and

wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has an amino-acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405 and 407

optionally

wherein the sequences of said first and second CH3 regions are such that the dissociation constants of homodimeric interactions of each of the CH3 regions are between 0.01 and 10 micromolar, such as between 0.05 and 10 micromolar, more preferably between 0.01 and 5, such as between 0.05 and 5 micromolar, even more preferably between 0.01 and 1 micromolar, such as between 0.05 and 1 micromolar, between 0.01 and 0.5 or between 0.01 and 0.1 when assayed as described in Example 21.

75. The heterodimeric protein according to claim 73, wherein

said first CH3 region has an amino acid other than Lys, Leu or Met at position 409 and said second CH3 region has an amino acid other than Phe at position 405, such as other than Phe, Arg or Gly, at position 405

or

said first CH3 region has an amino acid other than Lys, Leu or Met at position 409 and said second CH3 region has an amino acid other than Tyr, Asp, Glu, Phe, Lys, Gln, Arg, Ser or Thr at position 407.

76. The heterodimeric protein according to claim 74 or 75, wherein said first and second polypeptides are full-length heavy chains of two antibodies that bind different epitopes.

77. The heterodimeric protein according to claim 76, further comprising two full-length light chains.

78. The heterodimeric protein according to any one of the preceding claims 73 to 77, further comprising the features of any one or more of claims 2 to 58.

79. A heteromeric protein comprising a first polypeptide comprising a first Fc region of an immunoglobulin, said first Fc region comprising a first CH3 region, and a second polypeptide comprising a second Fc region of an immunoglobulin, said second Fc region comprising a second CH3 region, wherein the sequences of said first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions, and wherein said first homodimeric protein comprises a Phe at position 405 and an Arg at position 409 and said second homodimeric protein comprises a Leu at position 405 and a Lys at position 409

80. The heterodimeric protein according to any one of claims 73 to 79, wherein said bispecific antibody has binding specificity for a first and second epitope,

wherein said first and second epitope are located on a tumor cell and wherein the bispecific antibody has binding specificity for a target selected from the group consisting of: erbB1 (EGFR), erbB2 (HER2), erbB3, erbB4, MUC-1, CD19, CD20, CD4, CD38, CD138, CXCR5, c-Met, HERV-envelop protein, periostin, Bigh3, SPARC, BCR, CD79, CD37, EGFrvIII, L1-CAM, AXL, Tissue Factor (TF), CD74, EpCAM and MRP3; or

81. The heterodimeric protein according to any one of claims 73 to 79, wherein said bispecific antibody has binding specificity for a first and second epitope,

wherein said first and second epitope are located on a tumor cell and wherein said bispecific antibody has binding specificity for a target combination selected from the group consisting of: erbB1 + erbB2, erbB2 + erbB3, erbB1 + erbB3, CD19 + CD20, CD38 + CD34, CD4 + CXCR5, CD38 + RANKL, CD38 + CXCR4, CD20 + CXCR4, CD20 + CCR7,

CD20 + CXCR5, CD20 + RANKL, erbB2 + AXL, erbB1 + cMet, erbB2 + c-Met, erbB2 + EpCAM, c-Met + AXL, c-Met + TF, CD38 + CD20, CD38 + CD138; or

82. The heterodimeric protein according to any one of claims 73 to 79, wherein said bispecific antibody has binding specificity for a first and second epitope,

wherein said first or second epitope is located on a tumor cell and the other epitope is located on an effector cell, wherein the bispecific antibody has binding specificity for a target selected from the group consisting of: FcgammaRI (CD64), FcgammaRIII (CD16), CD3, CD89, CD32a, FcεRI; or

83. The heterodimeric protein according to any one of claims 73 to 79, wherein said bispecific antibody has binding specificity for a first and second epitope,

wherein said bispecific antibody has binding specificity for a target combination selected from: CD3 + HER2, CD3 + CD20, IL-12 + IL18, IL-1a + IL-1b, VEGF + EGFR, EpCAM + CD3, GD2 + CD3, GD3 + CD3, HER2 + CD64, EGFR + CD64, CD30 + CD16, NG2 + CD28, HER2 + HER3, CD20 + CD28, HER2 + CD16, Bcl2 + CD3, CD19 + CD3, CEA + CD3, EGFR + CD3, IgE + CD3, EphA2 + CD3, CD33 + CD3, MCSP + CD3, PSMA + CD3, TF + CD3, CD19 + CD16, CD19 + CD16a, CD30 + CD16a, CEA + HSG, CD20 + HSG, MUC1 + HSG, CD20 + CD22, HLA-DR + CD79, PDGFR + VEGF, IL17a + IL23, CD32b + CD25, CD20 + CD38, HER2 + AXL, CD89 + HLA class II, CD38+CD138, TF + cMet, Her2 + EpCAM, HER2 + HER2, EGFR + EGFR, EGFR + c-Met, c-Met + non-binding arm and combinations of G-protein coupled receptors.

84. The heterodimeric protein according to any one of claims 73 to 83 for use as a medicament.

85. The heterodimeric protein according to claim 84 for use in the treatment of cancer.

86. A pharmaceutical composition comprising a heterodimeric protein according to any one of claims 73 to 83 and a pharmaceutically-acceptable carrier.