applicability of the disclosure is limited to a specific molecule and / or format. As such, there remains a need for improved multispecific binding proteins and methods of developing the same, which enhances the development of multispecific binding proteins without altering stability or affinity and which is not attendant upon unacceptable immunogenicity.

Accordingly, the present disclosure addresses one or more of the above needs by providing improved multispecific binding proteins and methods of developing the same. Embodiments of the multispecific binding proteins and methods of the present disclosure provide for enhanced purification of the desired multispecific binding protein, preserve and / or enhance assembly of the molecule, decreased protein aggregation, improved physical stability and are not attendant upon increased immunogenicity risk, altered effector function and / or altered pharmacokinetic properties. Additionally, embodiments of the present disclosure preserve affinity of the multispecific binding protein and reduces or eliminates undesired binding of kappa light chain to purification reagent. Furthermore, embodiments of the present disclosure do not add time and / or cost to the purification process or development process as a whole.

SUMMARY

According to a first aspect, the present disclosure refers to a multispecific binding protein that binds a first antigen and a second antigen, the multispecific binding protein comprising:

a first antigen binding domain comprising a first light chain Fab region and a first heavy chain Fab region, wherein the first light chain Fab region is a kappa light chain comprising:

a lysine at amino acid residue 143 (EU numbering) and a lysine at amino acid residue 199 (EU numbering);

a lysine at amino acid residue 143 (EU numbering), a lysine at amino acid residue 199 (EU numbering), and an alanine at amino acid residue 109 (EU numbering);

a lysine at amino acid residue 143 (EU numbering), a lysine at amino acid residue 199 (EU numbering), and an aspartic acid at amino acid residue 110 (EU numbering);

a lysine at amino acid residue 143 (EU numbering), a lysine at amino acid residue 199 (EU numbering), an alanine at amino acid residue 109 (EU numbering), and an aspartic acid at amino acid residue 110 (EU numbering);
an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 143 (EU numbering);
an aspartic acid at amino acid residue 110 (EU numbering), a lysine at amino acid residue 143 (EU numbering) and an alanine at amino acid residue 109 (EU numbering);
an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 199 (EU numbering);
an aspartic acid at amino acid residue 110 (EU numbering), a lysine at amino acid residue 199 (EU numbering) and an alanine at amino acid residue 109 (EU numbering);
an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 143 (EU numbering);
an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 199 (EU numbering); or
an alanine at amino acid residue 109 (EU numbering) and an aspartic acid at amino acid residue 110 (EU numbering); and

a second antigen binding domain comprising a second light chain Fab region and a second heavy chain Fab region,

wherein the first antigen binding domain binds the first antigen and the second antigen binding domain binds the second antigen.

According to another aspect, the present disclosure refers to a method of purifying a multispecific binding protein comprising a first antigen binding domain comprising a light chain Fab region that binds a first antigen and a second antigen binding domain comprising a light chain Fab region that binds a second antigen, wherein the first light chain Fab region is a kappa light chain comprising:
a lysine at amino acid residue 143 (EU numbering) and a lysine at amino acid residue 199 (EU numbering);
an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 143 (EU numbering);

an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 199 (EU numbering);
an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 143 (EU numbering);
an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 199 (EU numbering); or
an alanine at amino acid residue 109 (EU numbering) and an aspartic acid at amino acid residue 110 (EU numbering);
the method comprising:

expressing the multispecific binding protein, wherein the first antigen binding domain assembles with the second antigen binding domain; and

subjecting the multispecific binding protein to an affinity chromatography column; and

recovering purified multispecific binding protein.

DESCRIPTION

Accordingly, in particular embodiments, the present disclosure provides a multispecific binding protein that binds a first antigen and a second antigen. According to some embodiments, multispecific binding proteins are provided that bind a first antigen and a second antigen, the multispecific binding protein comprising a first antigen binding domain comprising a first light chain Fab region and a first heavy chain Fab region, wherein the first light chain Fab region is a kappa light chain and comprises: a lysine at amino acid residue 143 (EU numbering) and a lysine at amino acid residue 199 (EU numbering); a lysine at amino acid residue 143 (EU numbering), a lysine at amino acid residue 199 (EU numbering), and an alanine at amino acid residue 109 (EU numbering); a lysine at amino acid residue 143 (EU numbering), a lysine at amino acid residue 199 (EU numbering), and an aspartic acid at amino acid residue 110 (EU numbering); a lysine at amino acid residue 143 (EU numbering), a lysine at amino acid residue 199 (EU numbering), an alanine at amino acid residue 109 (EU numbering), and an aspartic acid at amino acid residue 110 (EU numbering); an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 143 (EU numbering); an aspartic acid at amino acid residue 110 (EU numbering), a lysine at

CLAIMS

1. A multispecific binding protein that binds a first antigen and a second antigen, the multispecific binding protein comprising:

a first antigen binding domain comprising a first light chain Fab region and a first heavy chain Fab region, wherein the first light chain Fab region is a kappa light chain ~~and comprises~~comprising:

a lysine at amino acid residue 143 (EU numbering) and a lysine at amino acid residue 199 (EU numbering);

a lysine at amino acid residue 143 (EU numbering), a lysine at amino acid residue 199 (EU numbering), and an alanine at amino acid residue 109 (EU numbering);

a lysine at amino acid residue 143 (EU numbering), a lysine at amino acid residue 199 (EU numbering), and an aspartic acid at amino acid residue 110 (EU numbering);

a lysine at amino acid residue 143 (EU numbering), a lysine at amino acid residue 199 (EU numbering), an alanine at amino acid residue 109 (EU numbering), and an aspartic acid at amino acid residue 110 (EU numbering);

an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 143 (EU numbering);

an aspartic acid at amino acid residue 110 (EU numbering), a lysine at amino acid residue 143 (EU numbering) and an alanine at amino acid residue 109 (EU numbering);

an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 199 (EU numbering);

an aspartic acid at amino acid residue 110 (EU numbering), a lysine at amino acid residue 199 (EU numbering) and an alanine at amino acid residue 109 (EU numbering);

an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 143 (EU numbering);

an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 199 (EU numbering); or

an alanine at amino acid residue 109 (EU numbering) and an aspartic acid at amino acid residue 110 (EU numbering); and

a second antigen binding domain comprising a second light chain Fab region and a second heavy chain Fab region,

wherein the first antigen binding domain binds the first antigen and the second antigen binding domain binds the second antigen.

2. The multispecific binding protein of Cclaim 1, wherein:

if the first light chain Fab region comprises a lysine at amino acid residue 143 (EU numbering) and a lysine at amino acid residue 199 (EU numbering), then the second light chain Fab region does not comprise a lysine at amino acid residue 143 (EU numbering) and a lysine at amino acid residue 199 (EU numbering);

if the first light chain Fab region comprises a lysine at amino acid residue 143 (EU numbering), a lysine at amino acid residue 199 (EU numbering), and an alanine at amino acid residue 109 (EU numbering), then the second light chain Fab region does not comprise a lysine at amino acid residue 143 (EU numbering), a lysine at amino acid residue 199 (EU numbering), and an alanine at amino acid residue 109 (EU numbering);

if the first light chain Fab region comprises a lysine at amino acid residue 143 (EU numbering), a lysine at amino acid residue 199 (EU numbering), and an aspartic acid at amino acid residue 110 (EU numbering), then the second light chain Fab region does not comprise a lysine at amino acid residue 143 (EU numbering), a lysine at amino acid residue 199 (EU numbering), and an aspartic acid at amino acid residue 110 (EU numbering);

if the first light chain Fab region comprises a lysine at amino acid residue 143 (EU numbering), a lysine at amino acid residue 199 (EU numbering), an alanine at amino acid residue 109 (EU numbering), and an aspartic acid at amino acid residue 110 (EU numbering), then the second light chain Fab region does not comprise a lysine at amino acid residue 143 (EU numbering), a lysine at amino acid residue 199 (EU numbering), an alanine at amino acid residue 109 (EU numbering);

if the first light chain Fab region comprises an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 143 (EU numbering), then

the second light chain Fab region does not comprise an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 143 (EU numbering);

if the first light chain Fab region comprises an aspartic acid at amino acid residue 110 (EU numbering), a lysine at amino acid residue 143 (EU numbering) and an alanine at amino acid residue 109 (EU numbering), then the second light chain Fab region does not comprise an aspartic acid at amino acid residue 110 (EU numbering), a lysine at amino acid residue 143 (EU numbering) and an alanine at amino acid residue 109 (EU numbering);

if the first light chain Fab region comprises an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 199 (EU numbering), then the second light chain Fab region does not comprise an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 199 (EU numbering);

if the first light chain Fab region comprises an aspartic acid at amino acid residue 110 (EU numbering), a lysine at amino acid residue 199 (EU numbering) and an alanine at amino acid residue 109 (EU numbering), then the second light chain Fab region does not comprise an aspartic acid at amino acid residue 110 (EU numbering), a lysine at amino acid residue 199 (EU numbering) and an alanine at amino acid residue 109 (EU numbering);

if the first light chain Fab region comprises an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 143 (EU numbering), then the second light chain Fab region does not comprise an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 143 (EU numbering);

if the first light chain Fab region comprises an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 199 (EU numbering), then the second light chain Fab region does not comprise an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 199 (EU numbering); and

if the first light chain Fab region comprises an alanine at amino acid residue 109 (EU numbering) and an aspartic acid at amino acid residue 110 (EU numbering), then the second light chain Fab region does not comprise an alanine at amino acid residue 109 (EU numbering) and an aspartic acid at amino acid residue 110 (EU numbering).

3. TheA multispecific binding protein of Claim 1, ~~that binds a first antigen and a second antigen, the multispecific binding protein comprising:~~

~~a first antigen binding domain comprising a first light chain Fab region and a first heavy chain Fab region,~~ wherein the first light chain Fab region ~~is a kappa light chain and~~ comprises a lysine at amino acid residue 143 (EU numbering) and a lysine at amino acid residue 199 (EU numbering)~~; and~~

~~a second antigen binding domain comprising a second light chain Fab region and a second heavy chain Fab region,~~

~~wherein the first antigen binding domain binds the first antigen and the second antigen binding domain binds the second antigen~~.

4. The multispecific binding protein of Claim 3, wherein the first light chain Fab region further comprises an aspartic acid at amino acid residue 110 (EU numbering).

5. The multispecific binding protein of ~~any of~~ Claim~~s~~ 3 ~~or 4~~, wherein the first light chain Fab region further comprises an alanine at amino acid residue 109 (EU numbering).

6. TheA multispecific binding protein of Claim 1,~~that binds a first antigen and a second antigen, the multispecific binding protein comprising:~~

~~a first antigen binding domain comprising a first light chain Fab region and a first heavy chain Fab region,~~ wherein the first light chain Fab region ~~is a kappa light chain and~~ comprises an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 143 (EU numbering)~~; and~~

~~a second antigen binding domain comprising a second light chain Fab region and a second heavy chain Fab region,~~

~~wherein the first antigen binding domain binds the first antigen and the second antigen binding domain binds the second antigen~~.

7. The multispecific binding protein of Claim 6, wherein the first light chain Fab region further comprises an alanine at amino acid residue 109 (EU numbering).

8. TheA multispecific binding protein of Claim 1, ~~that binds a first antigen and a second antigen, the multispecific binding protein comprising:~~

~~a first antigen binding domain comprising a first light chain Fab region and a first heavy chain Fab region,~~ wherein the first light chain Fab region ~~is a kappa light chain and~~ comprises an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 199 (EU numbering)~~; and~~

~~a second antigen binding domain comprising a second light chain Fab region and a second heavy chain Fab region,~~

~~wherein the first antigen binding domain binds the first antigen and the second antigen binding domain binds the second antigen~~.

9. The multispecific binding protein of Claim 8, wherein the first light chain Fab region further comprises an alanine at amino acid residue 109 (EU numbering).

10. TheA multispecific binding protein of Claim 1,~~that binds a first antigen and a second antigen, the multispecific binding protein comprising:~~

~~a first antigen binding domain comprising a first light chain Fab region and a first heavy chain Fab region,~~ wherein the first light chain Fab region ~~is a kappa light chain and~~ comprises an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 143 (EU numbering)~~; and~~

~~a second antigen binding domain comprising a second light chain Fab region and a second heavy chain Fab region,~~

~~wherein the first antigen binding domain binds the first antigen and the second antigen binding domain binds the second antigen~~.

11. TheA multispecific binding protein of Claim 1, ~~that binds a first antigen and a second antigen, the multispecific binding protein comprising:~~

~~a first antigen binding domain comprising a first light chain Fab region and a first heavy chain Fab region,~~ wherein the first light chain Fab region ~~is a kappa light chain and~~ comprises an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 199 (EU numbering)~~; and~~

~~a second antigen binding domain comprising a second light chain Fab region and a second heavy chain Fab region,~~

~~wherein the first antigen binding domain binds the first antigen and the second antigen binding domain binds the second antigen~~.

12. The~~A~~ multispecific binding protein of Claim 1, ~~that binds a first antigen and a second antigen, the multispecific binding protein comprising:~~

~~a first antigen binding domain comprising a first light chain Fab region and a first heavy chain Fab region,~~ wherein the first light chain Fab region ~~is a kappa light chain and~~ comprises an alanine at amino acid residue 109 (EU numbering) and an aspartic acid at amino acid residue 110 (EU numbering)~~; and~~

~~a second antigen binding domain comprising a second light chain Fab region and a second heavy chain Fab region,~~

~~wherein the first antigen binding domain binds the first antigen and the second antigen binding domain binds the second antigen~~.

13. The multispecific binding protein of ~~any one of~~ Claim~~s~~ 1 ~~to 12~~, wherein the first antigen binding domain further comprises a first heavy chain Fc region.

14. The multispecific binding protein of Claim 1~~3~~, wherein the first heavy chain Fc region comprises a human IgG1, a human IgG2 or a human IgG4 constant region.

15. The multispecific binding protein of ~~any one of~~ Claim~~s~~ 1~~3~~ ~~to 14~~, wherein the second antigen binding domain further comprises a second heavy chain Fc region.

16. The multispecific binding protein of Claim 15, wherein the second heavy chain Fc region comprises a human IgG1, a human IgG2 or a human IgG4 constant region.

17. The multispecific binding protein of ~~any one of~~ Claim~~s~~ 13 ~~to 16~~, wherein the first heavy chain Fc region comprises an arginine at amino acid residue 311 (EU

numbering) and a glutamic acid at amino acid residue 317 (EU numbering).

18. The multispecific binding protein of ~~any one of~~ Claim~~s 13 to 17~~ 15, wherein the second heavy chain Fc region comprises an arginine at amino acid residue 311 (EU numbering) and a glutamic acid at amino acid residue 317 (EU numbering).

19. The multispecific binding protein of ~~any one of~~ Claim~~s 13 to 18~~ 15, wherein both the first and second heavy chain Fc regions comprise a human IgG1 constant region; both comprise a human IgG2 constant region; or both comprise a human IgG4 constant region.

20. The multispecific binding protein of ~~any one of~~ Claim~~s 13 to 19~~ 15, wherein both the first and second heavy chain Fc regions comprise an arginine at amino acid residue 311 (EU numbering) and a glutamic acid at amino acid residue 317 (EU numbering).

21. The multispecific binding protein of ~~any one of~~ Claim~~s~~ 1~~ to 20~~, wherein the second light chain Fab region does not comprise an alanine at amino acid residue 109; does not comprise an aspartic acid at amino acid residue 110; does not comprise a lysine at amino acid residue 143; or does not comprise a lysine at amino acid residue 199.

22. The multispecific binding protein of ~~any one of~~ Claim~~s~~ 1~~ to 21~~, wherein the second light chain Fab region is a Kappa light chain.

23. The multispecific binding protein of ~~any one of~~ Claim~~s~~ 1~~ to 22~~, wherein the second light chain Fab region is a Lambda light chain.

24. A method of purifying a multispecific binding protein comprising a first antigen binding domain comprising a light chain Fab region that binds a first antigen and a second antigen binding domain comprising a light chain Fab region that binds a second antigen,

wherein the first light chain Fab region is a kappa light chain comprising:
a lysine at amino acid residue 143 (EU numbering) and a lysine at amino acid residue 199 (EU numbering);
an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 143 (EU numbering);
an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 199 (EU numbering);
an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 143 (EU numbering);
an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 199 (EU numbering); or
an alanine at amino acid residue 109 (EU numbering) and an aspartic acid at amino acid residue 110 (EU numbering);

the method comprising:

~~introducing into the first antigen binding domain a first light chain Fab region comprising a lysine at amino acid residue 143 (EU numbering) and a lysine at amino acid residue 199 (EU numbering), wherein the first light chain Fab region is a kappa light chain;~~

expressing the multispecific binding protein, wherein the first antigen binding domain assembles with the second antigen binding domain; and

subjecting the multispecific binding protein to an affinity chromatography column; and

recovering purified multispecific binding protein.

25. The method of Claim 24, wherein the ~~step of introducing further comprises introducing into~~ first light chain Fab region is a kappa light chain comprising a lysine at amino acid residue 143 (EU numbering) and a lysine at amino acid residue 199 (EU numbering), and the first antigen binding domain further comprises an alanine at amino acid residue 109 (EU numbering).

26. The method of ~~any of~~ Claim~~s~~ 24 ~~or 25~~, wherein the ~~step of introducing further comprises introducing into~~ first light chain Fab region is a kappa light chain comprising a lysine at amino acid residue 143 (EU numbering) and a lysine at amino acid residue 199 (EU numbering), and the first antigen binding domain further comprises an aspartic acid at amino acid residue 110 (EU numbering).

~~27. A method of purifying a multispecific binding protein comprising a first antigen binding domain that binds a first antigen and a second antigen binding domain that binds a second antigen, the method comprising:~~

~~introducing into the first antigen binding domain a first light chain Fab region comprising an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 143 (EU numbering), wherein the first light chain Fab region is a kappa light chain;~~

~~expressing the multispecific binding protein, wherein the first antigen binding domain assembles with the second antigen binding domain; and~~

~~subjecting the multispecific binding protein to an affinity chromatography column; and~~

~~recovering purified multispecific binding protein.~~

~~28.~~27. The method of Claim 24~~27~~, wherein the ~~step of introducing further comprises introducing into~~ first light chain Fab region is a kappa light chain comprising an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 199 (EU numbering), and the first antigen binding domain further comprises an alanine at amino acid residue 109 (EU numbering).

~~29. A method of purifying a multispecific binding protein comprising a first antigen binding domain that binds a first antigen and a second antigen binding domain that binds a second antigen, the method comprising:~~

~~introducing into the first antigen binding domain a first light chain Fab region comprising an aspartic acid at amino acid residue 110 (EU numbering) and a lysine at amino acid residue 199 (EU numbering), wherein the first light chain Fab region~~

~~is a kappa light chain;~~

~~expressing the multispecific binding protein, wherein the first antigen binding domain assembles with the second antigen binding domain; and~~

~~subjecting the multispecific binding protein to an affinity chromatography column; and~~

~~recovering purified multispecific binding protein.~~

~~30. The method of Claim 29, wherein the step of introducing further comprises introducing into the first antigen binding domain an alanine at amino acid residue 109 (EU numbering).~~

~~31. A method of purifying a multispecific binding protein comprising a first antigen binding domain that binds a first antigen and a second antigen binding domain that binds a second antigen, the method comprising:~~

~~introducing into the first antigen binding domain a first light chain Fab region comprising an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 143 (EU numbering), wherein the first light chain Fab region is a kappa light chain;~~

~~expressing the multispecific binding protein, wherein the first antigen binding domain assembles with the second antigen binding domain; and~~

~~subjecting the multispecific binding protein to an affinity chromatography column; and~~

~~recovering purified multispecific binding protein.~~

~~32. A method of purifying a multispecific binding protein comprising a first antigen binding domain that binds a first antigen and a second antigen binding domain that binds a second antigen, the method comprising:~~

~~introducing into the first antigen binding domain a first light chain Fab region comprising an alanine at amino acid residue 109 (EU numbering) and a lysine at amino acid residue 199 (EU numbering), wherein the first light chain Fab region is a kappa light chain;~~

~~expressing the multispecific binding protein, wherein the first antigen binding domain assembles with the second antigen binding domain; and~~

~~subjecting the multispecific binding protein to an affinity chromatography column; and~~

~~recovering purified multispecific binding protein.~~

~~33. A method of purifying a multispecific binding protein comprising a first antigen binding domain that binds a first antigen and a second antigen binding domain that binds a second antigen, the method comprising:~~

~~introducing into the first antigen binding domain a first light chain Fab region comprising an alanine at amino acid residue 109 (EU numbering) and an aspartic acid at amino acid residue 110 (EU numbering), wherein the first light chain Fab region is a kappa light chain;~~

~~expressing the multispecific binding protein, wherein the first antigen binding domain assembles with the second antigen binding domain; and~~

~~subjecting the multispecific binding protein to an affinity chromatography column; and~~

~~recovering purified multispecific binding protein.~~

~~34.~~28. The method of ~~any one of~~ Claim~~s~~ 24 ~~to 33~~, wherein the ~~step of introducing further comprises introducing into the~~ first antigen binding domain comprises a first heavy chain Fc region.

~~35. The method of Claim 34, wherein the first heavy chain Fc region comprises a human IgG1, a human IgG2 or a human IgG4 constant region.~~

~~36.~~29. The method of ~~any one of~~ Claim~~s 24 to 35~~ 28, wherein the ~~step of introducing further comprises introducing into the~~ second antigen binding domain comprises a second heavy chain Fc region.

~~37. The method of Claim 36, wherein the second heavy chain Fc region comprises a human IgG1, a human IgG2 or a human IgG4 constant region.~~

~~38.~~30. The method of ~~any one of~~ Claim~~s 34 to 37~~ 28, wherein the ~~step of introducing further comprises introducing into the~~ first heavy chain Fc region comprises an arginine at amino acid residue 311 (EU numbering) and a glutamic acid at amino acid residue 317 (EU numbering).

~~39.~~31. The method of ~~any one of~~ Claim~~s 34 to 38~~ 28, wherein the ~~step of introducing further comprises introducing into the~~ second heavy chain Fc region comprises an arginine at amino acid residue 311 (EU numbering) and a glutamic acid at amino acid residue 317 (EU numbering).

~~40. The method of any one of Claims 24 to 39, wherein both the first heavy chain Fc region and the second heavy chain Fc region comprise a human IgG1 constant region; both comprise a human IgG2 constant region; or both comprise a human IgG4 constant region.~~

~~41.~~32. The method of ~~any one of~~ Claim~~s 24 to 40~~ 30, wherein ~~the step of introducing further comprises introducing, into~~ both the first heavy chain Fc region and the second heavy chain Fc region~~,~~ comprise arginine at amino acid residues 311 (EU numbering) and glutamic acid at amino acid residues 317 (EU numbering).

~~42.~~33. The method of ~~any one of~~ Claim~~s~~ 24 ~~to 41~~, wherein the second light chain Fab region does not comprise an alanine at amino acid residue 109; does not comprise an aspartic acid at amino acid residue 110; does not comprise a lysine at amino acid residue 143; or does not comprise a lysine at amino acid residue 199.

~~43.~~34. The method of ~~any one of~~ Claim~~s~~ 24 ~~to 41~~, wherein the second light chain Fab region does not comprise an alanine at amino acid residue 109; does not comprise an aspartic acid at amino acid residue 110; does

not comprise a lysine at amino acid residue 143; and does not comprise a lysine at amino acid residue 199.

~~44. The method of any one of Claims 24 to 43, wherein the second light chain Fab region is a Kappa light chain.~~

~~45. The method of any one of Claims 24 to 43, wherein the second light chain Fab region is a Lambda light chain~~

~~46.~~35. The method of ~~any one of~~ Claim~~s~~ 24 ~~to 45~~, wherein the affinity chromatography column comprises a kappa affinity ligand.

~~47.~~36. The method of ~~any one of~~ Claim~~s~~ 24 ~~to 46~~, wherein the affinity chromatography column comprises a lambda affinity ligand.

~~48.~~37. The method of ~~any one of~~ Claim~~s~~ 24 ~~to 47~~, wherein the affinity chromatography column comprises Protein A.

~~49.~~38. The method of ~~any one of~~ Claim~~s~~ 24 ~~to 48~~, wherein the second light chain Fab region binds to the affinity chromatography column with greater affinity than the first light chain Fab region.

~~50.~~39. The method of ~~any one of~~ Claim~~s~~ 24 ~~to 49~~, wherein the first light chain Fab region does not bind to the affinity chromatography column.

~~51.~~40. The method of ~~any one of~~ Claim~~s~~ 24 ~~to 50~~, further comprising ~~the steps of~~:

subjecting the purified multispecific binding protein to a second affinity chromatography column after the step of recovering purified multispecific binding protein; and

recovering purified multispecific binding protein after the step of subjecting the purified multispecific binding protein to a second affinity chromatography column.

~~52.~~41. The method of Claim 40~~51~~, wherein the second affinity chromatography column comprises a kappa affinity ligand.

~~53.~~42. The method of ~~any one of~~ Claim~~s 51 or 52~~ 40, wherein the second affinity chromatography column comprises a lambda affinity ligand.

~~54.~~43. The method of ~~any one of~~ Claim~~s 51 to 53~~ 40, wherein the second affinity chromatography column comprises Protein A.

~~55.~~44. The method of ~~any one of~~ Claim~~s 51 to 54~~ 40, wherein the second light chain Fab region binds to the second affinity chromatography column with greater affinity than the first light chain Fab region.

~~56.~~45. The method of ~~any one of~~ Claim~~s 51 to 55~~ 40, wherein the first light chain Fab region does not bind to the second affinity chromatography column.