HIGH SALT LOAD CONDITIONING DURING CATION EXCHANGE CHROMATOGRAPHY TO REMOVE PRODUCT-RELATED IMPURITIES

This application claims the benefit of U.S. Provisional Application No. 62/931,863, filed November 7, 2019, which is hereby incorporated by reference.

FIELD OF DISCLOSURE

The present invention relates to the field of biopharmaceutical manufacturing. In particular, the invention relates to methods for removal of low isoelectric point product-related impurities during cation exchange purification operations.

BACKGROUND

Antibody products are the largest sector of the biopharmaceuticals market and could easily reach hundreds of billions in sales over the next decade. The commercial development of therapeutic monoclonal antibodies began in the 1980s with the approval of the first therapeutic monoclonal antibody and has continued to evolve and expand ever since. While monoclonal antibodies bind a target with high affinity and specificity and have been very successful therapeutic treatments for some indications, they also have limitations. Monoclonal antibodies bind a single target; however many diseases are multifactorial. In cancer immunotherapy, a single target treatment may not be sufficient to destroy or immobilize cancer cells. In addition, some patients receiving monoclonal antibody therapies may fail to respond to treatment or develop drug resistance.

New antibody-like structures such as antibody Fab fragments, Fc-fusion proteins, antibody-drug conjugates, glycol-engineered antibodies, and most especially, bispecific and other multispecific antibody-like structures have been developed to meet these challenges. These antibody-like structures, particularly bispecific antibodies, offer improvements over traditional monoclonal antibody therapeutics and are proving to be effective next-generation of biotherapeutics, with an enormous variety of formats that can be developed to meet even more challenging therapeutic indications.

Bispecific antibodies are the most diverse group of these antibody-like structures with an ever-increasing variety of frameworks to meet the needs of therapeutic indications. These structures combine the binding properties of antibodies with additional molecular properties engineered to suit desired disease indications. Bispecific antibodies are being developed for a variety of indication and uses, such as redirecting immune effector cells to tumor cells for immune response against cancer, blocking signaling pathways, targeting tumor angiogenesis, blocking cytokines, crossing the blood-brain barrier, diagnostic assays, treatment of pathogens, and as delivery agents. (Sedykh et al., Drug Design, Development and Therapy 18(12), 195-208, 2018; Walsh, Nature Biotechnology, 32(10), 992-1000, 2014; Ecker et al., mAbs 7(1), 9-14, 2015; Spiess et al., Mol Immunol 67, 95-106, 2015; Fan et al., J Hematol & Oncology 8:130-143, 2015; Williams et al., Process Design for Bispecific Antibodies,

Biopharmaceutical Processing, Development, Design and Implementation of Processes, Jagschies et al., editors, Elsevier Ltd, pages 837-855, 2018).

Development of these multispecific proteins brings new biomanufacturing challenges, particularly with regard to product instability and low expression yields. In particular, purification of multispecific proteins is complicated by the formation of product-related variants, such as homodimers, half-antibodies, aggregates, high and low molecular weight species and the like. These variants share similar structural and physical properties, such as charge, with the multispecific protein of interest, making them difficult to separate from during purification. These product-related impurities lower the yield and activity of the multispecific drug product and impact the robustness of manufacturing processes.

Product-related impurities having similar charge (isoelectric point) to a multispecific protein of interest may co-elute with the multispecific protein during cation exchange chromatography unit operations, complicating purification and lowering yield. It would be beneficial to separate the low pI product-related impurities prior to elution. The invention described herein meets this need by providing high salt load conditioning for removal of these low pI impurities during cation exchange chromatography.

BRIEF SUMMARY ~~OF THE INVENTION~~

In one aspect, the disclosure provides a method of performing cation exchange chromatography under high salt loading conditions to reduce product-related impurities, the method comprising equilibrating a cation exchange chromatography medium with an equilibration buffer; loading the composition on to the cation exchange medium in a load buffer; washing the column with a first and a second wash buffer; and eluting the multispecific protein from the cation exchange chromatography medium; wherein the equilibration, loading and first wash buffers comprise 94-105 mM Sodium chloride.

In another aspect, the disclosure provides a method of producing an isolated, purified, recombinant multispecific protein, the method comprising establishing a cell culture in a bioreactor with a host cell expressing the multispecific protein; culturing the host cells to express the multispecific protein; harvesting the recombinant multispecific protein; affinity purifying the harvested recombinant multispecific protein; inactivating virus at low pH in the eluate pool from the affinity purification and neutralizing the pool; equilibrating a cation exchange chromatography medium with an equilibration buffer comprising 94-105 mM Sodium chloride; loading the neutralized affinity purified recombinant multispecific protein on to the equilibrated cation exchange medium in a load buffer comprises 94-105 mM Sodium chloride; washing the cation exchange medium with a wash buffer comprising 94-105 mM Sodium chloride, followed by a second wash buffer comprising 0-26mM Sodium chloride; eluting the multispecific protein from the cation exchange chromatography medium; loading the cation exchange

chromatography eluate comprising the recombinant multispecific protein onto a second chromatography resin in flow through mode; and concentrating the purified recombinant multispecific protein in a formulation buffer.

In another aspect, the disclosure provides an isolated, purified, recombinant multispecific protein prepared by a method according to the disclosure.

In another aspect, the disclosure provides a pharmaceutical composition comprising the isolated, purified, recombinant multispecific protein prepared by a method according to the disclosure.

The ~~invention~~ disclosure provides a method of purifying a multispecific protein from a composition comprising the multispecific protein and at least one product-related impurity, the method comprising equilibrating a cation exchange chromatography medium with an equilibration buffer comprising 94-105 mM Sodium chloride; loading the composition on to the cation exchange medium in a load buffer comprises 94-105 mM Sodium chloride; washing the column with at least one wash buffer comprising 94-105 mM Sodium chloride; and eluting the multispecific protein from the cation exchange chromatography medium. In one ~~embodiment~~ example the load buffer comprises 94-96 mM Sodium chloride. In a related ~~embodiment~~example the load buffer comprises 96-105 mM Sodium chloride. In a related ~~embodiment~~example the load buffer comprises 94 mM sodium chloride. In a related ~~embodiment~~example the load buffer comprises 96 mM sodium chloride. In a related ~~embodiment~~example the load buffer comprises 105 mM sodium chloride. In one ~~embodiment~~example the load buffer comprises acetate. In a related ~~embodiment~~example the load buffer comprises acetate, pH 4.9-5.1. In a related ~~embodiment~~example the load buffer comprises acetate, pH 5.0 ± 0.05 to 5.0 ± 0.1. In a related ~~embodiment~~example the load buffer comprises 100 mM acetate. In one ~~embodiment~~example the load buffer comprises acetate and 94 mM–105 mM sodium chloride. In one ~~embodiment~~example at least one wash buffer comprises 94-96 mM sodium chloride. In a related ~~embodiment~~example at least one wash buffer comprises 96-105 mM sodium chloride. In a related ~~embodiment~~example at least one wash buffer comprises 94 mM sodium chloride. In a related ~~embodiment~~example at least one wash buffer comprises 96 mM sodium chloride. In a related ~~embodiment~~example at least one wash buffer comprises 105

mM sodium chloride. In one ~~embodiment~~example at least one wash buffer comprises acetate. In one ~~embodiment~~example at least one wash buffer comprises acetate, pH 4.9-5.1. In a related ~~embodiment~~example, at least one wash buffer comprises acetate, pH 5.0 ± 0.05 to 5.0 ± 0.1. In a related ~~embodiment~~example at least one wash buffer comprises 100 mM acetate. In one ~~embodiment~~example at least one wash buffer comprises acetate and 94 mM–105 mM sodium chloride. In one ~~embodiment~~example at least one additional wash buffer comprises 0-26 mM sodium chloride. In one ~~embodiment~~example at least one wash buffer comprises acetate and 94-96 mM sodium chloride, followed by at least one additional wash buffer comprising acetate and 25 mM sodium chloride. In one ~~embodiment~~example at least one wash buffer comprises acetate and 105 mM sodium chloride, followed by at least one additional wash buffer comprising acetate. In one ~~embodiment~~example at least one equilibration buffer comprises 94-96 mM sodium chloride. In a related ~~embodiment~~example at least one equilibration buffer comprises 96-105 mM sodium chloride. In a related ~~embodiment~~example at least one equilibration buffer comprises 94 mM sodium chloride. In a related ~~embodiment~~example at least one equilibration buffer comprises 96 mM sodium chloride. In a related ~~embodiment~~example at least one equilibration buffer comprises 105 mM sodium chloride. In one ~~embodiment~~example the equilibration buffer comprises acetate. In a related ~~embodiment~~example the equilibration buffer comprises acetate, pH 4.9-5.1. In a related ~~embodiment~~example the equilibration buffer comprises acetate, pH 5.0 ± 0.05 to 5.0 ± 0.1. In a related ~~embodiment~~example the equilibration buffer comprises 100 mM acetate. In one ~~embodiment~~example the equilibration buffer comprises acetate and 94 mM–105 mM sodium chloride. In one ~~embodiment~~example the composition is loaded at 10-27 g/L. In one ~~embodiment~~example the composition is loaded at 15-27 g/L. In one ~~embodiment~~example the multispecific protein is eluted from the cation exchange resin by a gradient. In a related ~~embodiment~~example the gradient is linear. In one ~~embodiment~~example the gradient is a salt gradient. In one ~~embodiment~~example the multispecific protein is a bispecific protein. In one ~~embodiment~~example the multispecific protein is a bispecific antibody. In one ~~embodiment~~example is provided a purified, multispecific protein prepared by the method described above. In one ~~embodiment~~example the cation exchange chromatography medium is a resin.

The ~~invention~~disclosure provides a method of reducing low pI impurities in the eluate from cation exchange chromatography, the method comprising equilibrating a cation exchange chromatography medium with an equilibration buffer comprising 94-105 mM Sodium chloride; loading the composition on to the cation exchange medium in a load buffer comprises 94-105 mM Sodium chloride; washing the column with at least one wash buffer comprising 94-105 mM Sodium chloride; and eluting the multispecific protein from the cation exchange chromatography medium; wherein the cation exchange chromatography eluate has reduced low pI impurities compared to the cation exchange chromatography eluate recovered in a corresponding method in which no sodium chloride is used in the equilibration, load, and wash steps. In one ~~embodiment~~example the low pI impurity is a product-related impurity. In one ~~embodiment~~example at least one product-related impurity is a half antibody or 2X, 3X, or 4X light chain-mis-assembly.

The ~~invention~~disclosure provides a method of performing cation exchange chromatography under high salt loading conditions to reduce product-related impurities, the method comprising equilibrating a cation exchange chromatography medium with an equilibration buffer; loading the composition on to the cation exchange medium in a load buffer; washing the column with a first and a second wash buffer; and eluting the multispecific protein from the cation exchange chromatography medium; wherein the equilibration, loading and first wash buffers comprise 94-105 mM Sodium chloride. In one ~~embodiment~~example the second wash buffer comprises 0–26 mM.

The ~~invention~~disclosure provides a method of producing an isolated, purified, recombinant multispecific protein, the method comprising establishing a cell culture in a bioreactor with a host cell expressing the multispecific protein; culturing the host cells to express the multispecific protein; harvesting the recombinant multispecific protein; affinity purifying the harvested recombinant multispecific protein; inactivating virus at low pH in the eluate pool from the affinity purification and neutralizing the pool; equilibrating a cation exchange chromatography medium with an equilibration buffer comprising 94-105 mM Sodium chloride; loading the neutralized affinity purified recombinant multispecific protein on to the equilibrated cation exchange medium in a load buffer comprises 94-105 mM Sodium chloride; washing the cation exchange medium with a wash buffer comprising 94-105 mM Sodium chloride, followed by a second wash buffer comprising 0-26 mM Sodium chloride; eluting the multispecific protein from the cation exchange chromatography medium; loading the cation exchange chromatography eluate comprising the recombinant multispecific protein onto a second chromatography resin in flow through mode; and concentrating the purified recombinant multispecific protein in a formulation buffer. In one ~~embodiment~~example the second chromatography resin is selected from an anion exchange chromatography resin, cation exchange chromatography resin, multi-modal chromatography resin, hydrophobic interaction chromatography resin, and hydroxyapatite chromatography resin. In a related ~~embodiment~~example is provided an isolated, purified, recombinant multispecific protein prepared by the method described above. In a related ~~embodiment~~example is provided a pharmaceutical composition comprising the isolated, purified, recombinant multispecific protein prepared by the method described above.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Shows that impurities (half antibodies and 2X LC) are eluting with the main product, Bi-specific #1.

Figure 2 Shows that following high salt load conditioning, the low pI impurities flowed through the column between the load and first wash steps. The second wash returned the UV baseline to zero before the elution. During the elution of Bi-specific #1, the elution peak was reduced from four peaks to one peak.

What is claimed is

~~1. A method of purifying a multispecific protein from a composition comprising the multispecific protein and at least one product-related impurity, the method comprising~~

~~equilibrating a cation exchange chromatography medium with an equilibration buffer comprising 94-105 mM sodium chloride;~~

~~loading the composition on to the cation exchange medium in a load buffer comprises 94-105 mM sodium chloride;~~

~~washing the column with at least one wash buffer comprising 94-105 mM sodium chloride; and~~

~~eluting the multispecific protein from the cation exchange chromatography medium.~~

~~2. The method according to claim 1, wherein the load buffer comprises 94-96 mM sodium chloride.~~

~~3. The method according to claim 2, wherein the load buffer comprises 96-105 mM sodium chloride.~~

~~4. The method according to claim 2, wherein the load buffer comprises 94 mM sodium chloride.~~

~~5. The method according to claim 2, wherein the load buffer comprises 96 mM sodium chloride.~~

~~6. The method according to claim 2, wherein the load buffer comprises 98 mM sodium chloride.~~

~~7. The method according to claim 2, wherein the load buffer comprises 105 mM sodium chloride.~~

~~8. The method according to claim 1, wherein the load buffer comprises acetate.~~

~~9. The method according to claim 8, wherein the load buffer comprises acetate, pH 4.9-5.1.~~

~~10. The method according to claim 8, wherein the load buffer comprises acetate, pH 5.0 ± 0.05 to 5.0 ± 0.1~~

~~11. The method according to claim 8, wherein the load buffer comprises 100 mM acetate.~~

~~12. The method according to claim 1, wherein the load buffer comprises acetate, 94-105 mM sodium chloride.~~

~~13. The method according to claim 1, wherein at least one wash buffer comprises 94-105 mM sodium chloride.~~

~~14. The method according to claim 13, wherein at least one wash buffer comprises 94-96 mM sodium chloride.~~

~~15. The method according to claim 13, wherein at least one wash buffer comprises 96-105 mM sodium chloride.~~

~~16. The method according to claim 13, wherein at least one wash buffer comprises 94 mM sodium chloride.~~

~~17. The method according to claim 13, wherein at least one wash buffer comprises 96 mM sodium chloride.~~

~~18. The method according to claim 13, wherein at least one wash buffer comprises 98 mM sodium chloride.~~

~~19. The method according to claim 13, wherein at least one wash buffer comprises 105 mM sodium chloride.~~

~~20. The method according to claim 1, wherein at least one wash buffer comprises acetate.~~

~~21. The method according to claim 20, wherein at least one wash buffer comprises acetate, pH 4.9-5.1.~~

~~22. The method according to claim 20, wherein at least one wash buffer comprises acetate, pH 5.0 ± 0.05 to 5.0 ± 0.1.~~

~~23. The method according to claim 20, wherein at least one wash buffer comprises 100 mM acetate.~~

~~24. The method according to claim 1, wherein at least one wash buffer comprises acetate, 94 mM-105 mM sodium chloride.~~

~~25. The method according to claim 1, wherein the method comprises at least one additional wash buffer.~~

~~26. The method according to claim 25, wherein at least one additional wash buffer is a second wash buffer.~~

~~27. The method according to claim 25, wherein at least one additional wash buffer comprises 0-26 mM sodium chloride.~~

~~28. The method according to claim 25, wherein at least one wash buffer comprises acetate, 94-105 mM sodium chloride, followed by at least one additional wash buffer comprising acetate, 0-25 mM sodium chloride.~~

~~29. The method according to claim 1, wherein at least one equilibration buffer comprises 94-105mM sodium chloride.~~

~~30. The method according to claim 29, wherein at least one equilibration buffer comprises 94-96 mM sodium chloride.~~

~~31. The method according to claim 29, wherein at least one equilibration buffer comprises 96-105 mM sodium chloride.~~

~~32. The method according to claim 29, wherein at least one equilibration buffer comprises 94 mM sodium chloride.~~

~~33. The method according to claim 29, wherein at least one equilibration buffer comprises 96 mM sodium chloride.~~

~~34. The method according to claim 29, wherein at least one equilibration buffer comprises 98 mM sodium chloride.~~

~~35. The method according to claim 29, wherein at least one equilibration buffer comprises 105 mM sodium chloride.~~

~~36. The method according to claim 1, wherein the equilibration buffer comprises acetate.~~

~~37. The method according to claim 36, wherein the equilibration buffer comprises acetate, pH 4.9-5.1.~~

~~38. The method according to claim 36, wherein the equilibration buffer comprises acetate, pH 5.0 ± 0.05 to 5.0 ± 0.1.~~

~~39. The method according to claim 36, wherein the equilibration buffer comprises 100 mM acetate.~~

~~40. The method according to claim 1, wherein the composition is loaded at 10-27 g/L.~~

~~41. The method according to claim 40, wherein the composition is loaded at 15-27 g/L~~

~~42. The method according to claim 1, wherein the multispecific protein is eluted from the cation exchange resin by a gradient.~~

~~43. The method according to claim 42, wherein the gradient is linear.~~

~~44. The method according to claim 42, wherein the gradient is a salt gradient.~~

~~45. The method according to claim 1, wherein the multispecific protein is a bispecific protein.~~

~~46. The method according to claim 1, wherein the multispecific protein is a bispecific antibody.~~

~~47. A purified, multispecific protein prepared by a method according to claim 1.~~

~~48. The method according to claim 1, wherein the cation exchange chromatography medium is a resin.~~

~~49. A method of reducing low pI impurities in the eluate from cation exchange chromatography, the method comprising~~

~~equilibrating a cation exchange chromatography medium with an equilibration buffer comprising 94-105 mM Sodium chloride;~~

~~loading the composition on to the cation exchange medium in a load buffer comprises 94-105 mM Sodium chloride;~~

~~washing the column with at least one wash buffer comprising 94-105 mM Sodium chloride; and~~

~~eluting the multispecific protein from the cation exchange chromatography medium;~~

~~wherein the cation exchange chromatography eluate has reduced low pI impurities compared to the cation exchange chromatography eluate recovered in a corresponding method in which no sodium chloride is used in the equilibration, load, and wash steps.~~

~~50. The method according to claim 49, wherein the low pI impurity is a product-related impurity.~~

~~51. The method according to claim 50, wherein at least one product-related impurity is a half antibody or 2X, 3X, or 4X light chain mis-assembly.~~

~~52~~1. A method of performing cation exchange chromatography under high salt loading conditions to reduce product-related impurities, the method comprising

equilibrating a cation exchange chromatography medium with an equilibration buffer;

loading the composition on to the cation exchange medium in a load buffer;

washing the column with a first and a second wash buffer; and

eluting the multispecific protein from the cation exchange chromatography medium;

wherein the equilibration, loading and first wash buffers comprise 94-105 mM Sodium chloride.

~~53~~2. The method according to claim ~~52~~1, wherein the second wash buffer comprises 0–26 mM Sodium chloride.

~~2~~3. The method according to claim 1, wherein the load buffer comprises 94-96 mM sodium chloride~~.~~; or

~~3. The method according to claim 2,~~ wherein the load buffer comprises 96-105 mM sodium chloride~~.~~; or

~~4. The method according to claim 2,~~ wherein the load buffer comprises 94 mM sodium chloride~~.~~; or

~~5. The method according to claim 2,~~ wherein the load buffer comprises 96 mM sodium chloride~~.~~; or

~~6. The method according to claim 2,~~ wherein the load buffer comprises 98 mM sodium chloride~~.~~ or

~~7. The method according to claim 2,~~ wherein the load buffer comprises 105 mM sodium chloride.

~~13~~4. The method according to claim 1, wherein ~~at least one~~the wash buffer comprises 94-105 mM sodium chloride~~.~~; or

~~14. The method according to claim 13,~~ wherein ~~at least one~~the wash buffer comprises 94-96 mM sodium chloride~~.~~; or

~~15. The method according to claim 13,~~ wherein ~~at least one~~the wash buffer comprises 96-105 mM sodium chloride~~.~~; or

~~16. The method according to claim 13,~~ wherein ~~at least one~~the wash buffer comprises 94 mM sodium chloride~~.~~; or

~~17. The method according to claim 13,~~ wherein ~~at least one~~the wash buffer comprises 96 mM sodium chloride~~.~~; or

~~18. The method according to claim 13,~~ wherein ~~at least one~~the wash buffer comprises 98 mM sodium chloride~~.~~; or

~~19. The method according to claim 13,~~ wherein ~~at least one~~the wash buffer comprises 105 mM sodium chloride.

~~29~~5. The method according to claim 1, wherein ~~at least one~~the equilibration buffer comprises 94-105mM sodium chloride~~.~~; or

~~30. The method according to claim 29,~~ wherein ~~at least one~~the equilibration buffer comprises 94-96 mM sodium chloride~~.~~; or

~~31. The method according to claim 29,~~ wherein ~~at least one~~the equilibration buffer comprises 96-105 mM sodium chloride~~.~~; or

~~32. The method according to claim 29,~~ wherein ~~at least one~~the equilibration buffer comprises 94 mM sodium chloride~~.~~; or

~~33. The method according to claim 29,~~ wherein ~~at least one~~the equilibration buffer comprises 96 mM sodium chloride~~.~~; or

34. The method according to claim 29, wherein at least onethe equilibration buffer comprises 98 mM sodium chloride.; or

35. The method according to claim 29, wherein at least onethe equilibration buffer comprises 105 mM sodium chloride.

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546. A method of producing an isolated, purified, recombinant multispecific protein, the method comprising

establishing a cell culture in a bioreactor with a host cell expressing the multispecific protein;

culturing the host cells to express the multispecific protein;

10 harvesting the recombinant multispecific protein;

affinity purifying the harvested recombinant multispecific protein;

inactivating virus at low pH in the eluate pool from the affinity purification and neutralizing the pool;

equilibrating a cation exchange chromatography medium with an equilibration buffer
15 comprising 94-105 mM Sodium chloride;

loading the neutralized affinity purified recombinant multispecific protein on to the equilibrated cation exchange medium in a load buffer comprises 94-105 mM Sodium chloride;

washing the cation exchange medium with a wash buffer comprising 94-105 mM Sodium chloride, followed by a second wash buffer comprising 0-26mM Sodium chloride;

20 eluting the multispecific protein from the cation exchange chromatography medium;

loading the cation exchange chromatography eluate comprising the recombinant multispecific protein onto a second chromatography resin in flow through mode; and

concentrating the purified recombinant multispecific protein in a formulation buffer.

7. The method according to claim 6, wherein the load buffer comprises 94-96 mM sodium
25 chloride.; or

3. The method according to claim 2, wherein the load buffer comprises 96-105 mM sodium chloride.; or

4. The method according to claim 2, wherein the load buffer comprises 94 mM sodium chloride.; or

~~5. The method according to claim 2,~~ wherein the load buffer comprises 96 mM sodium chloride~~.~~; or

~~6. The method according to claim 2,~~ wherein the load buffer comprises 98 mM sodium chloride~~.~~ or

~~7. The method according to claim 2,~~ wherein the load buffer comprises 105 mM sodium chloride.

~~13~~8. The method according to claim 6, wherein the wash buffer comprises 94-105 mM sodium chloride~~.~~; or

~~14. The method according to claim 13,~~ wherein the wash buffer comprises 94-96 mM sodium chloride~~.~~; or

~~15. The method according to claim 13,~~ wherein the wash buffer comprises 96-105 mM sodium chloride~~.~~; or

~~16. The method according to claim 13,~~ wherein the wash buffer comprises 94 mM sodium chloride~~.~~; or

~~17. The method according to claim 13,~~ wherein the wash buffer comprises 96 mM sodium chloride~~.~~; or

~~18. The method according to claim 13,~~ wherein the wash buffer comprises 98 mM sodium chloride~~.~~; or

~~19. The method according to claim 13,~~ wherein the wash buffer comprises 105 mM sodium chloride.

~~29~~9. The method according to claim 6, wherein the equilibration buffer comprises 94-105mM sodium chloride~~.~~; or

~~30. The method according to claim 29,~~ wherein the equilibration buffer comprises 94-96 mM sodium chloride~~.~~; or

~~31. The method according to claim 29,~~ wherein the equilibration buffer comprises 96-105 mM sodium chloride~~.~~; or

~~32. The method according to claim 29,~~ wherein the equilibration buffer comprises 94 mM sodium chloride~~.~~; or

~~33. The method according to claim 29,~~ wherein the equilibration buffer comprises 96 mM sodium chloride~~.~~; or

~~34. The method according to claim 29,~~ wherein the equilibration buffer comprises 98 mM sodium chloride~~.~~; or

~~35. The method according to claim 29,~~ wherein the equilibration buffer comprises 105 mM sodium chloride.

~~55~~10. The method according to claim ~~47~~6~~,~~ wherein the second chromatography resin is selected from an anion exchange chromatography resin, cation exchange chromatography resin, multi-modal chromatography resin, hydrophobic interaction chromatography resin, and hydroxyapatite chromatography resin.

~~56~~11. An isolated, purified, recombinant multispecific protein prepared by a method according to claim ~~55~~9.

~~57~~12. A pharmaceutical composition comprising the isolated, purified, recombinant multispecific protein prepared by a method according to claim ~~55~~10.