CLAIMS

What is claimed is:

1. A method of purifying a charge-shielded fusion protein from a cell lysate or periplasmic releasate, wherein the charge-shielded fusion protein comprises a biologically active domain and a charge-shielding domain, and wherein the method comprises hydrophobic interaction chromatography as a first chromatography step.

2. A method for producing a charge-shielded fusion protein from a cell lysate or periplasmic releasate wherein the charge-shielded fusion protein comprises a biologically active domain and a charge-shielding domain, wherein the method comprises

i) culturing cells comprising a nucleic acid encoding the charge-shielded fusion protein; and

ii) purifying the charge-shielded fusion protein, wherein the charge-shielded protein is purified from the cell lysate or periplasmic releasate using hydrophobic interaction chromatography as a first chromatography step.

3. The method of claim 1 or 2, wherein

(a) the charge-shielded fusion protein is at least 45% pure after the first chromatography step;~~.~~

~~4. The method of any one of claims 1-3, wherein~~ (b) the method further comprises an anion exchange chromatography~~.~~; optionally wherein the anion exchange interaction chromatography is selected from the group consisting of a POROS 50HQ resin, a POROS XQ resin, and a Gigacap Q-650M resin; preferably wherein the anion exchange interaction chromatography is a Gigacap Q-650M resin;

~~5. The method of any one of claims 1-4, wherein~~ (c) the method further comprises a cation exchange chromatography~~.~~; optionally wherein

i) the cation exchange interaction chromatography is a strong cation exchanger;

ii) the cation exchange interaction chromatography is a mixed mode resin; or

iii) the cation exchange interaction chromatography is selected from the group consisting of a Capto MMC resin, a CMM Hypercel resin, a Capto SP impres resin, a Fracto gel SO_3- resin, a GigaCap S-650S resin, and a POROS XS resin; preferably wherein the cation exchange interaction chromatography is a POROS XS resin; and/or

~~6. The method of any one of claims 1-5 wherein~~ (d) the method comprises a sequence of chromatography steps comprising in order:

i) hydrophobic interaction chromatography;

ii) anion exchange chromatography; and

iii) cation exchange chromatography.

~~7~~4. The method of any one of any preceding claim~~s 1-6~~, wherein

(a) the biologically active domain is charged at pH of about 7.0, wherein the charge-shielding domain increases the hydrodynamic radius of the protein, and/or wherein the charge-shielding domain does not have a charge at pH of about 7.0~~.~~;

~~8. The method of any one of claims 1-7, wherein~~ (b) the molecular weight of the biologically active domain is less than the molecular weight of the charge-shielding domain~~.~~;

~~9. The method of any one of claims 1-8, wherein~~ (c) the molecular weight of the charge-shielding domain is between 10 kDa and 60 kDa~~.~~;

~~10. The method of any one of claims 1-9, wherein~~ (d) the molecular weight of the charge-shielding domain is between 10 kDa and 20 kDa~~.~~; and/or

~~11. The method of any one of claims 1-10, wherein~~ (e) the molecular weight of the biologically active domain is between 30 kDa and 40 kDa.

~~12~~5. The method of any one of any preceding claim~~s 1-11~~, wherein the molecular weight of the charge-shielding domain is sufficient to increase the *in vivo* half-life of the charge-shielded fusion protein or a multimer of the charge-shielded fusion protein~~.~~; optionally

~~13. The method of claim 12,~~ wherein the *in vivo* half-life of the charge-shielded fusion protein or a multimer of the charge-shielded fusion protein is increased compared to the half-

life of a protein comprising the biologically active domain without the charge-shielding domain or a multimeric protein comprising the biologically active domain without the charge-shielding domain.

~~14~~6. The method of any one of any preceding claim~~s 1-13~~, wherein

(a) the charge-shielding domain has a random coil or disordered structure~~.~~; and/or

~~15. The method of any one of claims 1-14, wherein~~ (b) the charge-shielding domain is a polypeptide consisting of one or more of alanine, serine and proline residues~~.~~; optionally

~~16. The method of claim 15,~~ wherein the charge-shielding domain is a polypeptide consisting of proline and alanine residues.

~~17~~7. A method for producing a PASylated biologically active fusion protein from a cell lysate or periplasmic releasate comprising

i) culturing cells comprising a nucleic acid encoding the PASylated biologically active protein; and

ii) purifying the PASylated biologically active protein,

wherein the PASylated biologically active protein is purified from the cell lysate or periplasmic releasate using hydrophobic interaction chromatography as a first chromatography step.

~~1~~8. A method for purifying a charge-shielded fusion protein comprising a biologically active domain and a charge-shielding domain from a cell lysate or periplasmic releasate, the method comprising the following steps in order

i) applying a load solution comprising the charge-shielded fusion protein to a hydrophobic interaction chromatography column;

ii) applying a wash solution to the hydrophobic interaction chromatography column;

iii) applying an elution solution to the hydrophobic interaction column to elute the charge-shielded protein;

iv) applying the eluted charge-shielded fusion protein in iii) as a load solution to an anion exchange chromatography column;

v) eluting the charge-shielded fusion protein from the anion exchange chromatography column;

vi) applying the eluted charge-shielded fusion protein in vi) as a load solution to a cation exchange chromatography column;

vii) applying a wash solution to the cation exchange chromatography column;

viii) applying an elution solution to the cation exchange chromatography column to elute the charge-shielded fusion protein.

~~1~~9. The method of claim ~~1~~8, wherein

(a) the load solution in step i) comprises 0.25 – 3 M Na_2SO_4 or 0.25- 0.6 M NH_4SO_4 and a pH of 5.5 to 6.5~~.~~;

~~20. The method of claim 18 or 19, wherein~~ (b) the elution solution in step iii) comprises 0.3-0.5 M NH_4SO_4 and has a pH of 5.5 to 6.5;~~.~~

~~21. The method of any one of claims 18-20, wherein~~ (c) the load solution in step iv) has a conductivity of 0.7- 4.0 mS/cm and a pH of 7.0 to 9.1~~.~~;

~~22. The method of any one of claims 18-21, wherein~~ (d) the load solution in step vi) has a pH of 5.9 to 7.0 and a conductivity of 0.7 to 2.5 mS/cm~~.~~; and/or

~~23. The method of any one of claims 18-22, wherein~~ (e) the elution solution in step viii) has a pH of 6.0 to 7.0 and a conductivity of 0.7 to 4.0 mS/cm.

~~24~~10. The method of any one of any preceding claim~~s 1-23~~, wherein the hydrophobic interaction chromatography is selected from the group consisting of a POROS Benzyl ultra resin, a Hexyl-650C resin, and a Phenyl-600M resin; preferably ~~.~~

~~25. The method of claim 24,~~ wherein the hydrophobic interaction chromatography is a Phenyl-600M resin.

~~26. The method of any one of claims 5-25, wherein the anion exchange interaction chromatography is selected from the group consisting of a POROS 50HQ resin, a POROS XQ resin, and a Gigacap Q-650M resin.~~

~~27. The method of claim 26, wherein the anion exchange interaction chromatography is a Gigacap Q-650M resin.~~

~~28. The method of any one of claims 6-27, wherein the cation exchange interaction chromatography is a strong cation exchanger.~~

~~29. The method of any one of claims 6-27, wherein the cation exchange interaction chromatography is a mixed mode resin.~~

~~30. The method of any one of claims 6-27, wherein the cation exchange interaction chromatography is selected from the group consisting of a Capto MMC resin, a CMM Hypercel resin, a Capto SP impres resin, a Fracto gel SO_3^- resin, a GigaCap S-650S resin, and a POROS XS resin.~~

~~31. The method of claim 30, wherein the cation exchange interaction chromatography is a POROS XS resin.~~

~~32~~11. The method any one of <u>any preceding</u> claim~~s 1-31~~, wherein the biologically active domain is an asparaginase subunit~~.~~<u>; optionally wherein</u>

~~33. The method of claim 32, wherein~~ <u>(a)</u> the asparaginase is selected from the group consisting of an *E. coli* asparaginase and an *Erwinia* asparaginase~~.~~<u>;</u>

~~34. The method of claim 32, wherein~~ <u>(b)</u> the asparaginase comprises the amino acid sequence set forth in SEQ ID NO:1, SEQ ID NO:5, or SEQ ID NO:7~~.~~<u>; or</u>

~~35. The method of claim 32, wherein~~ <u>(c)</u> the charge-shielded fusion protein comprises the amino acid sequence set forth in SEQ ID NO: 9 or SEQ ID NO:10.

~~36~~12. The method of claim 2 or claim ~~1~~7, wherein the cell is a bacterial cell~~.~~<u>; optionally</u>

~~37. The method of claim 36,~~ wherein the cell is an *E. coli* cell or a *Pseudomonas* cell.

~~38~~13. A charge-shielded protein produced by the method of any <u>preceding</u> ~~one of~~ claim~~s 1-37~~.

~~39~~14. A pharmaceutical composition comprising the charge-shielded protein of claim ~~38~~ 13 and a pharmaceutically acceptable carrier.

~~40~~15. The charge-shielded protein of claim 13 or the pharmaceutical composition of claim 14 for use as a medicament~~A method of treatment comprising administering a composition comprising the charge-shielded protein of claim 38 or the pharmaceutical composition of claim 39 to an individual in need thereof~~.

~~41~~16. A composition comprising a PASylated asparaginase, wherein the PASylated asparaginase is at least 45% pure.