100 kDa. In this regard the skulled person considers that a standard IgG antibody is constituted of two heavy chains of about 50 kDa and two light chains of about 25 kDa, which are folded mainly separately and then joined together. In comparison, single chain antibody constructs such as a scFc BiTE® antibody construct is folded as only one, heavily engineered chain. So, the previous learnings on downstream processing need to be refined for heavily engineered single chain antibody constructs, even more for those of unnaturally high molecular weight.

SUMMARY

[11] Surprisingly, a downstream method could be provided which both ensures improved bispecific antibody construct product quality, hence increasing product yield, and can be integrated into existing downstream processing steps.

[12] Hence, in one aspect, the present disclosure refers to a method of purifying a bispecific single chain antibody construct, the construct comprising at least two domains: - a first domain having a pI of 5.0 to 9.5 which binds to a tumor target, - a second domain which binds to an extracellular epitope of the human and the Macaca CD3ε chain; wherein the first and the second domain have the format: the pairs of VH regions and VL regions are in the format of a single chain antibody (scFv), and - optionally a third domain which comprises two polypeptide monomers, each comprising a hinge, a CH2 domain and a CH3 domain, wherein said two polypeptide monomers are fused to each other via a peptide linker, wherein the construct comprises at least one open di-sulfide bridge, wherein one or more of the domains comprise a disulfide bond, the method comprising a step of refolding the construct, wherein the construct is contacted with a refolding buffer to refold the construct to its native form, the buffer comprising (i) a redox and/or oxidizing agent having a concentration of at least 0.1 mM, wherein the redox agent is selected from the group consisting of glutathione-reduced/glutathione-oxidized, cysteine/cystine, cysteine/cysteamine, cysteine/cystamine, dithiothreitol (DTT), beta-mercaptoethanol and glutathione, and wherein the oxidizing agent is selected from the group consisting of copper (II), dissolved oxygen and (L)-dehydroascorbic acid (DHA), and (ii) a chaotropic agent having a concentration in the range of above 1 to 2.5 M, wherein the chaotropic agent is selected from the group consisting of ~~urea,~~ dimethyl urea, methylurea, ethylurea, organic solvents and guanidinium, and wherein the pH of the refolding buffer corresponds to +/- 4.5 of the pI value of the first domain or +/- 4.0 of the pI value of the entire construct. In one example, it is envisaged in the context of the present invention to provide a downstream method of purifying a bispecific antibody construct, the construct comprising:

- a first domain having a pI of 5.0 to 9.5 which binds to a target, preferably a tumor target;
- a second domain which binds to an extracellular epitope of the human and the Macaca CD3ε chain; and
- optionally a third domain which comprises two polypeptide monomers, each comprising a hinge, a CH2 domain and a CH3 domain, wherein said two polypeptide monomers are fused to each other via a peptide linker, wherein the construct comprising at least one open di-sulfide bridge, the method comprising a step of refolding the construct, wherein one or more of the domains comprise a disulfide bond, and wherein

the construct is contacted with a refolding buffer to refold the construct to its native form, the buffer comprising (i) a redox and/or oxidizing agent having a concentration of at least 0.1 mM and (ii) a chaotropic agent having a concentration > 1 M, and wherein the pH of the refolding buffer corresponds to +/- 4.5 of the pI value of the first domain or 4.0 of the pI value of the entire construct.

Claims

1. A method of purifying a bispecific single chain antibody construct, the construct comprising at least two domains:

- a first domain having a pI of 5.0 to 9.5 which binds to a tumor target,
- a second domain which binds to an extracellular epitope of the human and the *Macaca* CD3ε chain; wherein the first and the second domain have the format: the pairs of VH regions and VL regions are in the format of a single chain antibody (scFv), and
- optionally a third domain which comprises two polypeptide monomers, each comprising a hinge, a CH2 domain and a CH3 domain, wherein said two polypeptide monomers are fused to each other via a peptide linker, wherein the construct comprises at least one open di-sulfide bridge, wherein one or more of the domains comprise a disulfide bond,

the method comprising a step of refolding the construct, wherein the construct is contacted with a refolding buffer to refold the construct to its native form,

the buffer comprising

(i) a redox and/or oxidizing agent having a concentration of at least 0.1 mM, wherein the redox agent is selected from the group consisting of glutathione-reduced/glutathione-oxidized, cysteine/cystine, cysteine/cysteamine, cysteine/cystamine, dithiothreitol (DTT), beta-mercaptoethanol and glutathione, and wherein the oxidizing agent is selected from the group consisting of copper (II), dissolved oxygen and (L)-dehydroascorbic acid (DHA), and

(ii) a chaotropic agent having a concentration in the range of above 1 to 2.5 M, wherein the chaotropic agent is selected from the group consisting of ~~urea,~~ dimethyl urea, methylurea, ethylurea, organic solvents and guanidinium,

and wherein the pH of the refolding buffer corresponds to +/- 4.5 of the pI value of the first domain or +/- 4.0 of the pI value of the entire construct.

2. The method of claim 1, wherein the oxidizing agent is copper (II).

3. The method of claim 1, wherein the oxidizing agent is copper (II) sulfate.

4. The method of claim 1, wherein the oxidizing agent is present at a concentration of 0.1 to 10 mM, preferably 0.1 to 1 mM.

5. The method of claim 1, wherein the redox agent is present at a concentration of 0.1 to 10 mM, preferably 0.1 to 1 mM.

6. The method of claim 1, wherein the refolding buffer comprises an oxidizing agent but no redox agent.

7. The method of claim 1, wherein the chaotropic agent is guanidinium.

8. The method of claim 1, wherein the chaotropic agent is present at a concentration of 1.2 to 2 M.

9. The method of claim 1, wherein the pH value is in a range of pH 5.0 to 9.5, more preferably 5.0 to 6.0 or 7.0 to 9.0, more preferably about 5.0 or 7.0 to 8.0.

10. The method of claim 1, wherein the first domain binds to CD33, CDH19, MSLN, FLT3, BCMA, CD19, MUC17, CDH3, CLDN18.2, CD70, EGFRviii, EpCAM, DLL3 and/or PSMA.

11. The method of claim 1, wherein the monomeric content of the construct determined e.g. by size exclusion chromatography (percental monomeric peak) after applying the refolding buffer is at least 60%, or at least 65%, or at least 70%, or at least 85%.

12. The method of claim 1, wherein the method is conducted at a temperature between 4 and 30°C, preferably at room temperature (25°C).

13. The method of claim 1, wherein the method has an incubation time of 1 to 24 h, preferably 4 h.

14. The method of claim 1, wherein contacting the construct with the refolding buffer takes place (i) in a process fluid (pool) in a downstream purification step or (ii) on a chromatography column in a downstream purification step.

15. The method of claim 1, wherein the construct concentration (i) in the liquid pool is 0.5 to 15 mg/ml ~~in a liquid pool~~, preferably 0.7 to 12 mg/ml or (ii) 5 to 15 mg/ml, preferably 10 to 15 mg/ml on the~~a~~ chromatography column.

16. The method of claim 14, wherein the liquid pool is selected from the group consisting of harvested cell culture fluid (HCCF), chromatography eluate pool, Protein A, Protein L, or Protein G affinity chromatography eluate pool, filtered viral inactivation pool, cation exchange chromatography (CEX) monomer pool and CEX high molecular weight post-peak pool.

17. The method of claim 1 comprising the steps of

(a) providing harvested cell culture fluid (HCCF) comprising the bispecific antibody construct secreted by mammalian cells;

(b) contacting the HCCF with a first separation matrix for chromatography purification under conditions suitable for the construct to associate with the separation matrix, wherein the first separation matrix is an affinity resin selected from the group consisting of Protein A, Protein G, Protein L and a synthetic mimetic affinity resin;

(c) washing the first separation matrix;

(d) eluting the construct from the first separation matrix;

(e) inactivate virus in the construct comprising eluate;

(f) contacting the construct comprising eluate with a second separation matrix for chromatographic polishing under conditions suitable for the construct to associate with the ~~second~~ separation matrix, wherein the ~~second~~ separation matrix is a cation exchange chromatography matrix;

(g) washing the second separation matrix;

(h) eluting the construct from the second separation matrix, wherein the construct is separated into two fractions of (i) monomer pool comprising construct monomers and (ii) high molecular weight pool comprising construct aggregates,

(i) separate virus by filtration from the eluate comprising fraction (i); and

(j) optionally subject eluate to a further ultrafiltration and/or diafiltration step,

wherein the construct

(1) in cell culture media before harvest or in the HCCF of step (a), and/or

(2) on the separation matrix (on column) in at least one of steps (b) or (f), and/or

(3) in a process fluid (pool) in at least one of steps (d), (e) or (h),

is contacted with the refolding buffer to refold the construct to its native form.

18. The method of claim 1, wherein the first binding domain of the construct comprises a VH region comprising CDR-H1, CDR-H2 and CDR-H3 and a VL region comprising CDR-L1, CDR-L2 and CDR-L3 selected from the group consisting of:

(a) CDR-H1 as depicted in SEQ ID NO: 4, CDR-H2 as depicted in SEQ ID NO: 5, CDR-H3 as depicted in SEQ ID NO: 6, CDR-L1 as depicted in SEQ ID NO: 1, CDR-L2 as depicted in SEQ ID NO: 2 and CDR-L3 as depicted in SEQ ID NO: 3,

(b) CDR-H1 as depicted in SEQ ID NO: 29, CDR-H2 as depicted in SEQ ID NO: 30, CDR-H3 as depicted in SEQ ID NO: 31, CDR-L1 as depicted in SEQ ID NO: 34, CDR-L2 as depicted in SEQ ID NO: 35 and CDR-L3 as depicted in SEQ ID NO: 36,

(c) CDR-H1 as depicted in SEQ ID NO: 42, CDR-H2 as depicted in SEQ ID NO: 43, CDR-H3 as depicted in SEQ ID NO: 44, CDR-L1 as depicted in SEQ ID NO: 45, CDR-L2 as depicted in SEQ ID NO: 46 and CDR-L3 as depicted in SEQ ID NO: 47,

(d) CDR-H1 as depicted in SEQ ID NO: 53, CDR-H2 as depicted in SEQ ID NO: 54, CDR-H3 as depicted in SEQ ID NO: 55, CDR-L1 as depicted in SEQ ID NO: 56, CDR-L2 as depicted in SEQ ID NO: 57 and CDR-L3 as depicted in SEQ ID NO: 58,

(e) CDR-H1 as depicted in SEQ ID NO: 65, CDR-H2 as depicted in SEQ ID NO: 66, CDR-H3 as depicted in SEQ ID NO: 67, CDR-L1 as depicted in SEQ ID NO: 68, CDR-L2 as depicted in SEQ ID NO: 69 and CDR-L3 as depicted in SEQ ID NO: 70,

(f) CDR-H1 as depicted in SEQ ID NO: 83, CDR-H2 as depicted in SEQ ID NO: 84, CDR-H3 as depicted in SEQ ID NO: 85, CDR-L1 as depicted in SEQ ID NO: 86, CDR-L2 as depicted in SEQ ID NO: 87 and CDR-L3 as depicted in SEQ ID NO: 88,

(g) CDR-H1 as depicted in SEQ ID NO: 94, CDR-H2 as depicted in SEQ ID NO: 95, CDR-H3 as depicted in SEQ ID NO: 96, CDR-L1 as depicted in SEQ ID NO: 97, CDR-L2 as depicted in SEQ ID NO: 98 and CDR-L3 as depicted in SEQ ID NO: 99,

(h) CDR-H1 as depicted in SEQ ID NO: 105, CDR-H2 as depicted in SEQ ID NO: 106, CDR-H3 as depicted in SEQ ID NO: 107, CDR-L1 as depicted in SEQ ID NO: 109, CDR-L2 as depicted in SEQ ID NO: 110 and CDR-L3 as depicted in SEQ ID NO: 111,

(i) CDR-H1 as depicted in SEQ ID NO: 115, CDR-H2 as depicted in SEQ ID NO: 116, CDR-H3 as depicted in SEQ ID NO: 117, CDR-L1 as depicted in SEQ ID NO: 118, CDR-L2 as depicted in SEQ ID NO: 119 and CDR-L3 as depicted in SEQ ID NO: 120,

(j) CDR-H1 as depicted in SEQ ID NO: 126, CDR-H2 as depicted in SEQ ID NO: 127, CDR-H3 as depicted in SEQ ID NO: 128, CDR-L1 as depicted in SEQ ID NO: 129, CDR-L2 as depicted in SEQ ID NO: 130 and CDR-L3 as depicted in SEQ ID NO: 131,

(k) CDR-H1 as depicted in SEQ ID NO: 137, CDR-H2 as depicted in SEQ ID NO: 138, CDR-H3 as depicted in SEQ ID NO: 139, CDR-L1 as depicted in SEQ ID NO: 140, CDR-L2 as depicted in SEQ ID NO: 141 and CDR-L3 as depicted in SEQ ID NO: 142,

(l) CDR-H1 as depicted in SEQ ID NO: 152, CDR-H2 as depicted in SEQ ID NO: 153, CDR-H3 as depicted in SEQ ID NO: 154, CDR-L1 as depicted in SEQ ID NO: 155, CDR-L2 as depicted in SEQ ID NO: 156 and CDR-L3 as depicted in SEQ ID NO: 157,

(m) CDR-H1 as depicted in SEQ ID NO: 167, CDR-H2 as depicted in SEQ ID NO: 168, CDR-H3 as depicted in SEQ ID NO: 169, CDR-L1 as depicted in SEQ ID NO: 170, CDR-L2 as depicted in SEQ ID NO: 171 and CDR-L3 as depicted in SEQ ID NO: 172,

(n) CDR-H1 as depicted in SEQ ID NO: 203, CDR-H2 as depicted in SEQ ID NO: 204, CDR-H3 as depicted in SEQ ID NO: 205, CDR-L1 as depicted in SEQ ID NO: 206, CDR-L2 as depicted in SEQ ID NO: 207 and CDR-L3 as depicted in SEQ ID NO: 208;

(o) CDR-H1 as depicted in SEQ ID NO: 214, CDR-H2 as depicted in SEQ ID NO: 215, CDR-H3 as depicted in SEQ ID NO: 216, CDR-L1 as depicted in SEQ ID NO: 217, CDR-L2 as depicted in SEQ ID NO: 218 and CDR-L3 as depicted in SEQ ID NO: 219;

(p) CDR-H1 as depicted in SEQ ID NO: 226, CDR-H2 as depicted in SEQ ID NO: 227, CDR-H3 as depicted in SEQ ID NO: 228, CDR-L1 as depicted in SEQ ID NO: 229, CDR-L2 as depicted in SEQ ID NO: 230 and CDR-L3 as depicted in SEQ ID NO: 231; and

(q) CDR-H1 as depicted in SEQ ID NO: 238, CDR-H2 as depicted in SEQ ID NO: 239, CDR-H3 as depicted in SEQ ID NO: 240, CDR-L1 as depicted in SEQ ID NO: 241, CDR-L2 as depicted in SEQ ID NO: 242 and CDR-L3 as depicted in SEQ ID NO: 243.

19. The method of claim 1, wherein the method is applied to a construct produced by an upstream continuous manufacturing method.

~~20. An apparatus to perform the method of claim 1.~~