

[6] Despite their conceptual advantages, current multispecific antibodies are challenging to manufacture and develop as biologic drugs. As artificial constructs, multispecific antibodies cannot be produced by normal B-cells. Initial attempts to produce multispecific antibodies involved chemical conjugation of monospecific antibodies and fusion of mAb-expressing cells, but these approaches suffer from low efficiency and the necessity of purification from abundant side products. Advanced methods in protein engineering and molecular biology have enabled recombinant construction of a variety of new multispecific antibody formats. However, once adopted in these known engineered multispecific antibody formats, the individual components, such as scFvs and mAbs, lose their favorable biochemical and/or biophysical properties, serum half-life, and/or stability, resulting in poor efficacy, instability and high immunogenicity.

[7] Single-domain antibodies (sdAbs) are antibody fragments each having a single monomeric antibody variable domain. Despite their much smaller sizes than common monoclonal antibodies having two heavy chains and two light chains, sdAbs can bind antigens with similar affinity and specificity as mAbs. Used as building blocks, the sdAbs can be fused to IgG Fc domains to create IgG-like antibodies, including bivalent and bispecific antibodies (see, for example, Hmila I. *et al. Mol. Immunol.* 2008; 45: 3847-3856).

[8] The disclosures of all publications, patents, patent applications and published patent applications referred to herein are hereby incorporated herein by reference in their entirety.

SUMMARY

[9] The present application provides multispecific (such as trispecific) antigen binding proteins comprising two or more different single-domain antibodies (sdAbs) fused to a full-length four-chain antibody or an antigen binding fragment derived therefrom.

[10] Accordingly, one example of the present application provides a multispecific (such as trispecific) antigen binding protein ("MABP") comprising: (a) a first antigen binding portion comprising a heavy chain variable domain (V_H) and a light chain variable domain (V_L), wherein the V_H and V_L together form an antigen-binding site that specifically binds a first epitope, (b) a second antigen binding portion comprising a first single-domain antibody (sdAb) that specifically binds a second epitope, and (c) a third antigen binding portion comprising a second single-domain antibody (sdAb) that specifically binds a third epitope, wherein the first antigen binding portion, the second antigen binding portion, and the third antigen binding portion are fused to each other. In some examples, the first epitope, the second epitope and the third epitope are from the same antigen. In some examples, the first epitope, the second epitope and the third epitope are from different antigens. In some examples, the second epitope and the third epitope are from the same antigen, and the first epitope is from a different antigen. In some examples, the first epitope and the third epitope are from same antigen, the second epitope is from a different antigen.

[10a] In one aspect, the present disclosure refers to a multispecific antigen binding protein (MABP) comprising:

(a) a first antigen binding portion ~~that is a full-length antibody comprising two~~comprising a heavy chain variable domains (V_H) and ~~two~~a light chain variable domains (V_L), wherein the V_H and V_L together form an antigen-binding site that specifically binds a first epitope,

(b) a second antigen binding portion comprising a first single-domain antibody (sdAb) that specifically binds a second epitope, wherein C-terminus of the first sdAb is fused to N-terminus of the V_H of the first antigen binding portion, and

(c) a third antigen binding portion comprising a second single-domain antibody (sdAb) that specifically binds a third epitope, wherein the C-terminus of the second sdAb is fused to the N-terminus of the V_L of the first antigen binding portion

~~wherein the first antigen binding portion, the second antigen binding portion, and the third antigen binding portion are fused to each other.~~

about 25 °C at a concentration of at least about 50 mg/mL. In some examples, the MABP is stable for at least about one week at 37 °C at a concentration of at least about 50 mg/mL. In some examples, the MABP is stable after at least about 5 freeze-thaw cycles at a concentration of at least 50 mg/mL. In some examples, the MABP has a high long-term stability in human serum for at least about any one of 1 day, 3 days or 7 days at physiological temperature, *e.g.*, about 37 °C.

[22] In another aspect, the present disclosure refers to a pharmaceutical composition comprising the MABPs as disclosed herein and a pharmaceutically acceptable carrier. In some examples, the concentration of the MABP in the pharmaceutical composition is at least about 100 mg/mL, such as at least about 150 mg/mL, 200 mg/mL or higher.

[23] Further provided in one example of the present application is a method of treating a disease in an individual, comprising administering to the individual an effective amount of any one of the pharmaceutical compositions described above. In some examples, the disease is a cancer. In some examples, the cancer is selected from the group consisting of breast cancer, renal cancer, melanoma, lung cancer, glioblastoma, head and neck cancer, prostate cancer, ovarian carcinoma, bladder carcinoma, and lymphoma. In some examples, the disease is an inflammatory or autoimmune disease. In some examples, the inflammatory or autoimmune disease is selected from the group consisting of arthritis (such as rheumatoid arthritis, juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis, and arthritic ulcerative colitis), colitis, psoriasis, severe asthma, and moderate to severe Crohn's disease.

[023a] In another aspect, the present disclosure refers to use of the pharmaceutical composition as disclosed herein in the manufacture of a medicament for treating a disease in an individual, wherein the disease is a cancer or an inflammatory or autoimmune disease.

BRIEF DESCRIPTION OF THE DRAWINGS

[24] FIG. 1 depicts a schematic structure of an exemplary trispecific antigen binding protein (also referred herein as "TABP") comprising a monospecific full-length antibody having two identical heavy chains and two identical light chains, a first sdAb and a second sdAb, wherein the C-terminus of the first sdAb is fused to the N-terminus of the heavy chain via a first optional peptide linker and the C-terminus of the second sdAb is fused to the N-terminus of the light chain via a second optional peptide linker. The full-length antibody has two antigen binding sites that specifically bind the first epitope. The first sdAb specifically binds the second epitope. The second sdAb specially binds to the third epitope. For example, the TABP can consist of four polypeptide chains with structures from the N-terminus to the C-terminus as follows: (1) V_HH2-V_L-C_L; (2) V_HH1-V_H-C_H1-C_H2-C_H3; (3) V_HH1-V_H-C_H1-C_H2-C_H3; and (4) V_HH2-V_L-C_L.

[25] FIG. 2 depicts a schematic structure of an exemplary trispecific antigen binding protein comprising a monospecific full-length antibody having two identical heavy chains and two identical light chains, a first sdAb, and a second sdAb, wherein the C-terminus of the first sdAb is fused to the N-terminus of the second sdAb via a first optional peptide linker, and the C-terminus of the second sdAb is fused to the N-terminus of the heavy chain via a second optional peptide linker. The full-length antibody has two antigen binding sites that specifically bind the first epitope. The first sdAb

CLAIMS

What is claimed is:

1. A multispecific antigen binding protein (MABP) comprising:
 - (a) a first antigen binding portion ~~that is a full-length antibody comprising two comprising a~~ heavy chain variable domains (V_H) and ~~two~~ light chain variable domains (V_L), wherein the V_H and V_L together form an antigen-binding site that specifically binds a first epitope,
 - (b) a second antigen binding portion comprising a first single-domain antibody (sdAb) that specifically binds a second epitope, wherein C-terminus of the first sdAb is fused to N-terminus of the V_H of the first antigen binding portion, and
 - (c) a third antigen binding portion comprising a second single-domain antibody (sdAb) that specifically binds a third epitope, wherein the C-terminus of the second sdAb is fused to the N-terminus of the V_L of the first antigen binding portion.
~~wherein the first antigen binding portion, the second antigen binding portion, and the third antigen binding portion are fused to each other.~~
2. The MABP of claim 1, wherein the MABP is a trispecific antigen binding protein.
- ~~3. The MABP of claim 1 or 2, wherein:~~
 - ~~(a) the first antigen binding portion is a full length antibody consisting of two heavy chains and two light chains; and/or~~
 - ~~(b) the first antigen binding portion is an antibody fragment comprising a heavy chain comprising the V_H and a light chain comprising the V_L.~~
- ~~4.3.~~ The MABP of ~~any one of claims 1 or 2-3~~, wherein the first sdAb and/or the second sdAb is a V_HH.
- ~~5. The MABP of claim 4, wherein the MABP comprises:~~
 - ~~(a) — (1) a first polypeptide comprising from the N terminus to the C terminus: the first sdAb and a heavy chain of the first antigen binding portion; and~~
 - ~~(2) a second polypeptide comprising from the N terminus to the C terminus: the second sdAb and a light chain of the first antigen binding portion;~~
 - ~~(b) — (1) a first polypeptide comprising from the N terminus to the C terminus: a heavy chain of the first antigen binding portion and the first sdAb; and~~
 - ~~(2) a second polypeptide comprising from the N terminus to the C terminus: the second sdAb and a light chain of the first antigen binding portion;~~
 - ~~(c) — (1) a first polypeptide comprising from the N terminus to the C terminus: the first sdAb and a heavy chain of the first antigen binding portion; and~~
 - ~~(2) a second polypeptide comprising from the N terminus to the C terminus: a light chain of the first antigen binding portion and the second sdAb;~~

- (d) — ~~(1) a first polypeptide comprising from the N-terminus to the C-terminus: a heavy chain of the first antigen binding portion and the first sdAb; and~~
- ~~(2) a second polypeptide comprising from the N-terminus to the C-terminus: a light chain of the first antigen binding portion and the second sdAb;~~
- (e) — ~~(1) a first polypeptide comprising from the N-terminus to the C-terminus: the first sdAb, a heavy chain of the first antigen binding portion, and the second sdAb; and~~
- ~~(2) a second polypeptide comprising from the N-terminus to the C-terminus: a light chain of the first antigen binding portion;~~
- (f) — ~~(1) a first polypeptide comprising from the N-terminus to the C-terminus: a heavy chain of the first antigen binding portion; and~~
- ~~(2) a second polypeptide comprising from the N-terminus to the C-terminus: the first sdAb, a light chain of the first antigen binding portion and the second sdAb;~~
- (g) — ~~(1) a first polypeptide comprising from the N-terminus to the C-terminus: the first sdAb, the second sdAb, and a heavy chain of the first antigen binding portion; and~~
- ~~(2) a second polypeptide comprising from the N-terminus to the C-terminus: a light chain of the first antigen binding portion;~~
- (h) — ~~(1) a first polypeptide comprising from the N-terminus to the C-terminus: a heavy chain of the first antigen binding portion, the first sdAb, and the second sdAb; and~~
- ~~(2) a second polypeptide comprising from the N-terminus to the C-terminus: a light chain of the first antigen binding portion;~~
- (i) — ~~(1) a first polypeptide comprising from the N-terminus to the C-terminus: a heavy chain of the first antigen binding portion; and~~
- ~~(2) a second polypeptide comprising from the N-terminus to the C-terminus: the first sdAb, the second sdAb, and a light chain of the first antigen binding portion; or~~
- (j) — ~~(1) a first polypeptide comprising from the N-terminus to the C-terminus: a heavy chain of the first antigen binding portion; and~~
- ~~(2) a second polypeptide comprising from the N-terminus to the C-terminus: a light chain of the first antigen binding portion, the first sdAb, and the second sdAb;~~
- ~~optionally wherein the MABP comprises two chains of the first polypeptide and two chains of the second polypeptide.~~

4. The MABP of any one of claims 1-3, wherein the MABP comprises:

- (a) a first polypeptide comprising from the N-terminus to the C-terminus: VHH1-VH-CH1-CH2-CH3; and
- (b) a second polypeptide comprising from the N-terminus to the C-terminus: VHH2-VL-CL, wherein VHH1 is the first sdAb that specifically binds the second epitope, and VHH2 is the second sdAb that specifically binds the third epitope.

~~6.5.~~ The MABP of any one of claims 1-~~45~~, wherein the first epitope, the second epitope and/or the third epitope is from an immune checkpoint molecule, optionally wherein the immune checkpoint molecule is selected from the group consisting of PD-1, PD-L1, PD-L2, CTLA-4, B7-H3, TIM-3, LAG-3, TIGIT, VISTA, ICOS, 4-1BB, OX40, GITR, and CD40.

~~7.6.~~ The MABP of claim ~~56~~, wherein:

- (a) the first antigen binding portion is an anti-PD-1 antibody or antigen binding fragment thereof, optionally wherein the anti-PD-1 antibody is derived from pembrolizumab;
- (b) the second antigen binding portion comprises an anti-TIGIT sdAb, optionally wherein the anti-TIGIT sdAb comprises the amino acid sequence of SEQ ID NO: 31, or a variant thereof comprising up to about 3 amino acid substitutions; and/or
- (c) the third antigen binding portion comprises an anti-LAG-3 sdAb, optionally wherein the anti-LAG-3 sdAb comprises the amino acid sequence of SEQ ID NO: 32, or a variant thereof comprising up to about 3 amino acid substitutions.

~~8.7.~~ The MABP of claim ~~67~~, wherein the MABP comprises:

- (1) a first polypeptide comprising the amino acid sequence of ~~any one of~~ SEQ ID NOs: 16; ~~18, 20, 23, 25, 26, 28~~, or a variant thereof comprising up to about 5 amino acid substitutions; and
- (2) a second polypeptide comprising the amino acid sequence of ~~any one of~~ SEQ ID NOs: ~~15, 17, 19, 21, 22, 24, 27~~, or a variant thereof comprising up to about 5 amino acid substitutions.

~~9.8.~~ The MABP of any one of claims 1-~~45~~, wherein the first epitope, the second epitope and/or the third epitope is from a tumor antigen or a cell surface antigen of an immune effector cell, optionally wherein the first epitope, the second epitope and/or the third epitope is a tumor antigen selected from the group consisting of HER2, BRAF, EGFR, VEGFR2, CD20, RANKL, CD38, and CD52, optionally wherein:

- (a) the first antigen binding portion is an anti-HER2 antibody or antigen binding fragment thereof, optionally wherein the anti-HER2 antibody is derived from trastuzumab;
- (b) the second antigen binding portion comprises an anti-CD3 sdAb; and/or
- (c) the third antigen binding portion comprises an anti-EGFR sdAb.

~~10.9.~~ The MABP of any one of claims 1-~~45~~, wherein the first epitope, the second epitope, and/or the third epitope is from a pro-inflammatory molecule, optionally wherein the pro-inflammatory molecule is selected from the group consisting of IL-1 β , TNF- α , IL-5, IL-6, IL-6R, IL-17A, IL-17F and eotaxin-1, optionally wherein:

- (a) the first antigen binding portion is an anti-TNF- α antibody or antigen binding fragment thereof, optionally wherein the anti-TNF- α antibody is derived from adalimumab; and/or
- (b) the second antigen binding portion comprises an anti-IL-17A sdAb; and/or

(c) the third antigen binding portion comprises an anti-IL-17F sdAb.

~~11~~.10. The MABP of any one of claims 1-~~4~~5, wherein the first epitope, the second epitope and/or the third epitope is from an angiogenic factor, optionally wherein:

(a) the first antigen binding portion is an anti-Ang2 antibody or antigen binding fragment thereof, optionally wherein the anti-Ang2 antibody is derived from LC10;

(b) the second antigen binding portion comprises an anti-VEGF sdAb; and/or

(c) the third antigen binding portion comprises an anti-DLL4 sdAb.

~~12~~.11. The MABP of any one of claims 1-1~~0~~4, wherein the first antigen binding portion comprises a human, humanized or chimeric antibody or antigen binding fragment thereof.

~~13~~.12. The MABP of any one of claims 1-1~~1~~2, wherein the first antigen binding portion comprises an Fc region, optionally wherein the Fc region is an IgG1 Fc or the Fc region is an IgG4 Fc having an S228P mutation.

~~14~~.13. The MABP of any one of claims 1-1~~2~~3, wherein the first antigen binding portion, the second antigen binding portion and/or the third antigen binding portion are fused to each other via a peptide linker, optionally wherein the peptide linker is no more than about 30 amino acids long, optionally wherein the peptide linker comprises any one of the amino acid sequences of SEQ ID NOs: 1-7.

~~15~~.14. The MABP of any one of claims 1-1~~3~~4, wherein the first sdAb and/or the second sdAb is a camelid, humanized, or human sdAb.

~~16~~.15. The MABP of any one of claims 1-1~~4~~5, wherein the ~~multispecific antigen binding protein~~MABP:

(a) has an aggregation onset temperature of at least about 55°C;

(b) is stable for at least about one week at 25 °C at a concentration of at least about 50 mg/mL; and/or

(c) is stable after at least about 5 freeze-thaw cycles at a concentration of at least 50 mg/mL.

~~17~~.16. A pharmaceutical composition comprising the MABP of any one of claims 1-1~~5~~6 and a pharmaceutically acceptable carrier.

~~18~~.17. Use of the pharmaceutical composition of claim 1~~6~~7 in the manufacture of a medicament for treating a disease in an individual, wherein the disease is a cancer or an inflammatory or autoimmune disease.

~~19~~.18. The use of claim 1~~7~~8, wherein ~~the disease is a cancer, optionally wherein~~ the cancer is selected from the group consisting of breast cancer, renal cancer, melanoma, lung cancer, glioblastoma, head and neck cancer, prostate cancer, ovarian carcinoma, bladder carcinoma, and lymphoma.

~~20~~.19. The use of claim 1~~8~~9, ~~wherein the disease is an inflammatory or autoimmune disease,~~

~~optionally~~ wherein the inflammatory or autoimmune disease is selected from the group consisting of arthritis, colitis, psoriasis, severe asthma, and moderate to severe Crohn's disease.