

binding domains in the final format. Major challenges with such formats have been (a) the relatively poor efficiency of specific hetero-dimerization resulting in suboptimal production yields, and (b) the necessity to use either non target binding proteins as hetero-dimerization domains or engineered hetero-dimer Fc effector domains that come with poor flexibility in tuning serum half-life and that limit the flexibility in adding novel functionalities without increasing the molecular weight.

Thus, the optimal hetero-dimeric multi-specific format would exclusively consist of target binding domains and would allow for adjusting the geometry of the molecule for example by freely changing the linker lengths between the different binding domains to accommodate the geometrical constraints defined by the interaction partners (targets). The solution to this problem, i.e. the modification of the order of the variable domains on the monomeric chains, has hitherto neither been shown nor suggested in the prior art.

SUMMARY

This disclosure relates to novel hetero-dimeric multi-specific format of multiple antibody variable domains comprising a core of two split variable domain pairs wherein both variable light domains and two cognate variable heavy domains are positioned in tandem on two separate protein chains, respectively, thereby driving hetero-dimerization of the two protein chains. Up to two additional binding domains, particularly antibody-based binding domains, such as scFv fragments, are fused to the amino- or the carboxyl-terminus of either protein chain, resulting in an up to hexa-specific hetero-dimeric protein.

Thus, in a first example the present disclosure relates to a hetero-dimeric protein comprising a first and a second single-chain protein,

wherein said first single-chain protein comprises a first amino acid sequence consisting of (from the N- to the C-terminus):

- (ia) a first VL domain;
- (iia) a first polypeptide linker and

(iiia) a second VL domain, and

wherein said second single-chain protein comprises a second amino acid sequence consisting of (from the N- to the C-terminus):

(ib) a first VH domain;

(iib) a second polypeptide linker and

(iiib) a second VH domain, and

wherein said first VL domain forms a first cognate pair of variable domains with specificity to a first target antigen with either said first or said second VH domain and said second VL domain forms a second cognate pair of variable domains with specificity to a second target antigen with the other of said VH domains, and wherein at least one of said first or said second single-chain protein further comprises

(iv) at least one additional domain as third functional domain that is fused via a third polypeptide linker to said first or said second amino acid sequence,

wherein, optionally, said hetero-dimeric protein does not comprise a cognate pair of a first and a second immunoglobulin constant domain, wherein said first immunoglobulin constant domain is comprised in said first single-chain protein and wherein said second immunoglobulin constant domain is comprised in said second single-chain protein. In one aspect, the present disclosure refers to a hetero-dimeric protein comprising a first and a second single-chain protein, wherein said first single-chain protein comprises a first amino acid sequence consisting of (from the N- to the C-terminus): (ia) a first VL domain; (iia) a first polypeptide linker and (iiia) a second VL domain, and wherein said second single-chain protein comprises a second amino acid sequence consisting of (from the N- to the C-terminus): (ib) a first VH domain; (iib) a second polypeptide linker and (iiib) a second VH domain, and wherein said first VL domain forms a first cognate pair of variable domains with specificity to a first target antigen with either said first or said second VH domain, and said second VL domain forms a second cognate pair of variable domains with specificity to a second target antigen with the other of said VH domains, and wherein at least one of said first or said second single-chain protein further comprises (iv) at least one additional domain as third functional domain that is

fused via a third polypeptide linker to said first or said second amino acid sequence; wherein said hetero-dimeric protein does neither comprise (i) a cognate pair of antibody domains, wherein the first of said antibody domains is comprised in said first single-chain protein, and the second of said antibody domains is comprised in said second single-chain protein, nor (ii) a pair of non-antibody heteroassociation domains, wherein the first of said non-antibody heteroassociation domains is comprised in said first single-chain protein, and the second of said non-antibody heteroassociation domains is comprised in said second single-chain protein.

In a second example, the present disclosure relates to one or two nucleic acid sequences encoding said first and a second single-chain proteins. In a another aspect, the present disclosure refers to a nucleic acid sequence or two nucleic acid sequences encoding the first and the second single-chain proteins of the hetero-dimeric protein disclosed herein.

In a third example, the present disclosure relates to one or two vectors comprising said one or two nucleic acid sequences. In a yet another aspect, the present disclosure refers to a vector or two vectors comprising the nucleic acid sequence or the two nucleic acid sequences disclosed herein.

In a fourth example, the present disclosure relates to a host cell or host cells comprising one or two vectors. In a yet another aspect, the present disclosure refers to a host cell or host cells comprising the vector or the two vectors disclosed herein.

In a fourth example, the present disclosure relates to a method for producing the first and second single-chain proteins, or the hetero-dimeric protein, of the present disclosure, comprising (i) providing a nucleic acid or nucleic acids according to the present disclosure, or a vector or vectors according to the present disclosure, expressing said

nucleic acid or nucleic acids or said vector or vectors and collecting said first and second single-chain proteins, or said hetero-dimeric protein, from the expression system, or (ii) providing a host cell or host cells of the present disclosure, culturing said host cell or host cells, and collecting said first and second single-chain proteins, or said hetero-dimeric protein, from the cell culture. In yet another aspect, the present disclosure refers to a method for producing the hetero-dimeric protein disclosed herein, or the first and the second single-chain proteins of said hetero-dimeric protein, comprising (i) providing the nucleic acid sequence or the two nucleic acid sequences disclosed herein, or the vector or the two vectors disclosed herein, expressing said nucleic acid sequence or nucleic acid sequences, or said vector or vectors, and collecting said hetero-dimeric protein from the expression system, or (ii) providing a host cell or host cells disclosed herein, culturing said host cell or said host cells; and collecting said first and second single-chain proteins, or said hetero-dimeric protein, from the cell culture.

In a fifth example, the present disclosure relates to a pharmaceutical composition comprising the hetero-dimeric protein of the present disclosure and a pharmaceutically acceptable carrier. In yet another aspect, the present disclosure refers to a pharmaceutical composition comprising the hetero-dimeric protein disclosed herein and a pharmaceutically acceptable carrier.

In a sixth example, the present disclosure relates to the hetero-dimeric protein of the present disclosure for use in the treatment of a disease, particularly a human disease, more particularly a human disease selected from cancer, an inflammatory and an autoimmune disease, wherein at least one of said cognate pairs of VL and VH domains, or of said third, fourth, fifth, or sixth functional domain is able to specifically interact with a target of therapeutic relevance in the corresponding disease.

In a seventh example the present disclosure relates to a method for treating a patient suffering from a disease, particularly a human disease, more particularly a human disease selected from cancer, an inflammatory and an autoimmune disease, comprising administering to a subject an effective amount of the hetero-dimeric

protein of the present disclosure, wherein at least one of said cognate pairs of VL and VH domains, or of said third, fourth, fifth, or sixth functional domain is able to specifically interact with a target of therapeutic relevance in the corresponding disease.

[0017] Particular embodiments of the present invention are set forth in the appended dependent claims.

FIGURES

Figure 1 shows a schematic representation of Assembly 1 (see Example 1).

Figure 2 shows a schematic representation of Assembly 3 (see Example 1).

CLAIMS

1. A hetero-dimeric protein comprising a first and a second single-chain protein,
wherein said first single-chain protein comprises a first amino acid sequence
consisting of (from the N- to the C-terminus):

- 5 (ia) a first VL domain;
 (iia) a first polypeptide linker and
 (iiia) a second VL domain, and

wherein said second single-chain protein comprises a second amino acid
sequence consisting of (from the N- to the C-terminus):

- 10 (ib) a first VH domain;
 (iib) a second polypeptide linker and
 (iiib) a second VH domain, and

wherein said first VL domain forms a first cognate pair of variable domains with
specificity to a first target antigen with either said first or said second VH domain,
15 and said second VL domain forms a second cognate pair of variable domains with
specificity to a second target antigen with the other of said VH domains, and
wherein at least one of said first or said second single-chain protein further
comprises

- 20 (iv) at least one additional domain as third functional domain that is fused via a
 third polypeptide linker to said first or said second amino acid sequence;

wherein said hetero-dimeric protein does neither comprise (i) a cognate pair of
antibody domains, wherein the first of said antibody domains is comprised in said
first single-chain protein, and the second of said antibody domains is comprised in
said second single-chain protein, nor (ii) a pair of non-antibody heteroassociation
25 domains, wherein the first of said non-antibody heteroassociation domains is
comprised in said first single-chain protein, and the second of said non-antibody
heteroassociation domains is comprised in said second single-chain protein.

2. The hetero-dimeric protein of claim 1, wherein said hetero-dimeric protein does not comprise a cognate pair of a first and a second proteinaceous interaction domain, other than said first and second cognate pairs of variable domains, wherein said first proteinaceous interaction domain is comprised in said first single-chain protein and wherein said second proteinaceous interaction domain is comprised in said second single-chain protein.
3. The hetero-dimeric protein of claim 1 or 2, further comprising
- (v) a fourth functional domain that is fused via a fourth polypeptide linker to said first or said second amino acid sequence;
 - (vi) a fourth and a fifth functional domain that are fused via a fourth and a fifth polypeptide linker, respectively, to said first and said second amino acid sequence; or
 - (vi) a fourth, a fifth and a sixth functional domain that are fused via a fourth, a fifth and a sixth polypeptide linker, respectively, to said first and said second amino acid sequence.
4. The hetero-dimeric protein of any one of claims 1 to 3, wherein said first polypeptide linker consists of from 5 to 20 amino acid residues, particularly from 6 to 15 amino acid residues.
5. The hetero-dimeric protein of any one of claims 1 to 4, wherein (a) said first VL domain (ia) and said first VH domain (ib) form a first cognate pair of variable domains with specificity to a first target antigen, and said second VL domain (iia) and said second VH domain (iib) form a second cognate pair of variable domains with specificity to a second target antigen; or (b) said first VL domain (ia) and said second VH domain (iib) form a first cognate pair of variable domains with specificity to a first target antigen, and said second VL domain (iia) and said first VH domain (ib) form a second cognate pair of variable domains with specificity to a second target antigen.

6. The hetero-dimeric protein of any one of claims 1 to 4, wherein said third, fourth, fifth and/or sixth functional domains are independently selected from the list of: binding domains, toxins, enzymes, hormones, signaling proteins, and albumins; particularly wherein said third, fourth, fifth and/or sixth functional domains are independently selected from binding domains; particularly wherein said binding domains are independently selected from the list of: antibody-based binding domains, particularly scFv fragments, Fab fragments and single antibody variable domains, and binding domains based on alternative scaffolds, particularly ankyrin-based domains, fynomers, avimers, anticalins and binding sites being built into constant regions of antibodies.
7. The hetero-dimeric protein of any one of claims 1 to 6, wherein at least one of said antibody variable domains comprises CDR regions derived from a parental rabbit antibody.
8. The hetero-dimeric protein of any one of claims 1 to 7, wherein at least one of said antibody variable domains comprises human framework regions.
9. The hetero-dimeric protein of any one of claims 1 to 8, wherein the cognate pair of one of said first and said second VL and VH domains is specific for an antigen selected from the list of: a cancer target; and a target present on immune effector cells, in particular CD3.
10. A nucleic acid sequence or two nucleic acid sequences encoding the first and the second single-chain proteins of the hetero-dimeric protein of any one of claims 1 to 9.
11. A vector or two vectors comprising the nucleic acid sequence or the two nucleic acid sequences of claim 10.
12. A host cell or host cells comprising the vector or the two vectors of claim 11.

13. A method for producing the hetero-dimeric protein of any one of claims 1 to 9, or the first and the second single-chain proteins of said hetero-dimeric protein, comprising (i) providing the nucleic acid sequence or the two nucleic acid sequences of claim 10, or the vector or the two vectors of claim 11, expressing said nucleic acid sequence or nucleic acid sequences, or said vector or vectors, and collecting said hetero-dimeric protein from the expression system, or (ii) providing a host cell or host cells according to claim 12, culturing said host cell or said host cells; and collecting said first and second single-chain proteins, or said hetero-dimeric protein, from the cell culture.
14. A pharmaceutical composition comprising the hetero-dimeric protein of any one of claims 1 to 9 and a pharmaceutically acceptable carrier.
15. The hetero-dimeric protein of any one of claims 1 to 9 for use in the treatment of a disease, particularly a human disease, more particularly a human disease selected from cancer, an inflammatory and an autoimmune disease, wherein at least one of said cognate pairs of VL and VH domains, or of said third, fourth, fifth, or sixth functional domain is able to specifically interact with a target of therapeutic relevance in the corresponding disease.