

monomeric peaks suggested that all BiXAbs and Fab-Fabs were correctly assembled and represented by a single species.

Characterization of BiXAbs by Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) was used to test the thermal stability of BiXAb2b. A Microcal™ VP-Capillary DSC system (Malvern Instruments) was used to perform differential scanning calorimetry experiments.

Samples were centrifuged (20,000x g, 5 min, 4 °C), and their protein content was quantitated prior to the DSC analysis using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific) employing the IgG analysis program. For assay, samples were diluted in PBS to a final concentration of 1mg/mL

The pre-equilibration time was 3 min, and the resulting thermograms were acquired between 20 and 110 °C at a scan rate of 60 °C/h, a filtering period of 25 sec, and medium feedback. Prior to sample analysis, 5 buffer/buffer scans were measured to stabilize the instrument, and a buffer/buffer scan was performed between each protein/buffer scan. The data were fit to a non-2-state unfolding model, with the pre- and post- transition adjusted by subtraction of the baseline.

The DSC results demonstrated that the DSC profile of BiXAb2b exhibited two transitions. The smaller peak had a Cp max of 96 Kcal/mole/°C and a Tm1 of 71.5°C, corresponding to the unfolding of both CH2 and Fab domains, and the larger peak had a Cp max of 190 Kcal/mole/°C and a Tm2 of 80.5°C, corresponding to the unfolding of the CH3 domain.

Liquid Chromatography/Mass spectroscopy (LC-MS) analysis

LC-MS/MS data were acquired using a Dionex Ultimate 3000 system coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) and a Proswift RP-4H reverse phase column (250 mm ×1 mm; Thermo Fisher). The column oven temperature was set to 65 °C. Ten microliters were injected for LC separation. A gradient of mobile phases consisting of LC-MS-grade water with 0.1% formic acid (phase A) and acetonitrile with 0.1% formic acid (phase B) was delivered at a flow rate of 0.2 mL/min (total run time of 20 minutes). Eluted antibody species were introduced into the Q-Exactive instrument by electrospray ionization (ESI), which operated in positive ion mode using full-scan 15,000 resolution. Xcalibur 2.2 software (Thermo Fisher Scientific, Bremen, Germany) was used for instrument control and processing of the data files.

Figures 4A and 4B present the LC-MS analysis of Fab-Fab3a (containing a linker with SEQ ID NO: 6) and Fab-Fab3b (containing a linker with SEQ ID NO: 3), respectively. **Figure 4A** demonstrates that the LC-MS spectrum of Fab-Fab3a with a linker corresponding to SEQ ID

CLAIMS

5

1. A multispecific antigen-binding fragment comprising at least two Fab fragments with different CH1 and CL domains, wherein each Fab fragment recognizes a different epitope of interest, and said Fab fragments are tandemly arranged in any order, the C-terminal end of the CH1 domain of a first Fab fragment being linked to the N-terminal end of the VH domain of the following Fab fragment through a polypeptide linker,
 10 characterized in that the polypeptide linker sequence comprises or consists of amino acid sequence EPKX₁CDKX₂HX₃X₄PPX₅PAPELLGGPX₆X₇PPX₈PX₉PX₁₀GG (SEQ ID NO: 1), wherein X₁, X₂ and X₃, identical or different, are Threonine (T) or Serine (S); X₄ and X₅, identical or different, are any amino acid selected from the group consisting of Cysteine (C),
 15 Alanine (A), and Glycine (G); and X₆, X₇, X₈, X₉, X₁₀, identical or different, are selected from the group consisting of Ala (A), Gly (G), Val (V), Asn (N), Asp (D) and Ile (I), with the proviso that the linker sequence does not comprise nor consist of sequences EPKSCDKTHTCPPCPAPELLGGPSTPPTSPSGG (SEQ ID NO: 5).

- 20 2. A multispecific antibody having two identical antigen-binding arms, each consisting of a multispecific antigen-binding fragment of claim 1.

3. The multispecific antibody of claim 2, which has an immunoglobulin-like structure, comprising:
 25 - two identical antigen-binding arms each consisting of a multispecific antigen-binding fragment as defined in claim 1;
 - the dimerized CH2 and CH3 domains of an immunoglobulin;
 - the hinge region of an IgA, IgG, or IgD, linking the C-terminal ends of CH1 domains of the antigen-binding arms to the N-terminal ends of the CH2 domains.

- 30 4. A multispecific antibody, preferably a bispecific antibody, comprising two heavy chains and four light chains, wherein each heavy chain comprises
 - a. a Fc region of an immunoglobulin comprising Hinge-CH2-CH3 domains,
 - 35 b. which Fc region is linked to Fab heavy chain CH1-VH of antibody 1 (Ab1) by said Hinge domain,

c. which in turn is linked to Fab heavy chain CH1-VH of antibody 2 (Ab2), by a polypeptide linker sequence, wherein the polypeptide linker sequence links the N-terminus of said Fab heavy chain VH domain of Ab1 with the C-terminus of said CH1 domain of Ab2,

and the four light chains comprise light chains of Ab1 and light chains of Ab2 associated with their cognate heavy chain domains;

wherein the polypeptide linker sequence comprises or consists of amino acid sequence EPKX₁CDKX₂HX₃X₄PPX₅PAPELLGGPX₆X₇PPX₈PX₉PX₁₀GG (SEQ ID NO: 1),

wherein X₁, X₂ and X₃, identical or different, are Threonine (T) or Serine (S); X₄ and X₅, identical or different, are any amino acid selected from the group consisting of Cysteine (C), Alanine (A), and Glycine (G); and X₆, X₇, X₈, X₉, X₁₀, identical or different, are selected from the group consisting of Ala (A), Gly (G), Val (V), Asn (N), Asp (D) and Ile (I); with the proviso that the linker sequence does not comprise nor consist of sequences EPKSCDKTHTCPPCPAPELLGGPSTPPTPSPSGG (SEQ ID NO: 5).

5. The multispecific antigen-binding fragment, or multispecific antibody of any of claims 1 to 4, wherein X₄ is Cysteine (C).

6. The multispecific antigen-binding fragment, or multispecific antibody of any of claims 1 to 5, wherein X₅ is Alanine (A) or Cysteine (C).

7. The multispecific antigen-binding fragment, or multispecific antibody of any of claims 1 to 6, wherein X₆, X₇, X₈, X₉, X₁₀, identical or different, are selected from the group consisting of Ala (A) and Gly (G).

8. The multispecific antigen-binding fragment, or multispecific antibody of any of claims 1 to 7, wherein X₆ and X₇ are identical and are preferably selected from the group consisting of Ala (A) and Gly (G).

9. The multispecific antigen-binding fragment, or multispecific antibody of claim 7, wherein the polypeptide linker sequence comprises or consists of a sequence selected from the group consisting of

EPKSCDKTHTSPPAPPELLGGPAAPPAPAPAGG (SEQ ID NO: 3);

EPKSCDKTHTSPPAPPELLGGPGGPPGPGPGGG (SEQ ID NO: 2);

and

EPKSCDKTHTSPPAPPELLGGPAAPPGPAPGGG (SEQ ID NO: 4).

10. The multispecific antigen-binding fragment, or multispecific antibody of any of claims 1 to 9, which is a bispecific antigen-binding fragment, or bispecific antibody.
11. The multispecific antigen-binding fragment, or multispecific antibody of any of claims 1 to 10, which recognizes both EGFR and HER2/neu.
12. The multispecific antibody of claim 4, wherein Ab1 and Ab2, being different, independently are selected from the group consisting of cetuximab or a mutated derivative thereof, on the one hand, and trastuzumab, or a mutated derivative thereof, on the other hand.
13. The multispecific antigen-binding fragment, or multispecific antibody of any of claims 1 to 10, which recognizes both CD38 and PD-L1.
14. A polypeptide which comprises, preferably consists of, a heavy chain of the antigen-binding fragment or a heavy chain of the multispecific antibody as defined in any of claims 1 to 13.
15. A polynucleotide comprising a sequence encoding the polypeptide of claim 14.
16. A host cell transfected with an expression vector comprising the polynucleotide of claim 15.
17. The host cell of claim 16, further transformed with at least two polynucleotides encoding two different light chains: a first light chain pairing specifically with a first VH/CH1 region of said heavy chain; a second light chain pairing specifically with a second VH/CH1 region of said heavy chain.
18. The host cell of claim 17, which is additionally transformed with a third polynucleotide encoding a third light chain which is different from the first and second light chains, and which pairs specifically with a third VH/CH1 region of said heavy chain.
19. A method for preparing an antigen-binding fragment or a multispecific antibody as defined in any of claims 1 to 13, wherein said method comprising culturing a host cell of any of claims 16 to 18 and recovering said antigen-binding fragment or antibody from said culture.

20. The multispecific antigen-binding fragment or a multispecific antibody as defined in any of claims 1 to 13, for use as a medicament.

21. The multispecific antigen-binding fragment or a multispecific antibody as defined in any of claims 11 to 13, for use in treating a cancer.

22. A polypeptide which comprises or consists of amino acid sequence EPKX₁CDKX₂HX₃X₄PPX₅PAPELLGGPX₆X₇PPX₈PX₉PX₁₀GG (SEQ ID NO: 1), wherein X₁, X₂, X₃, X₄, X₅, X₆, X₇, X₈, X₉, X₁₀, identical or different, are any amino acid as defined in any of claims 1 to 6; with the proviso that the polypeptide does not comprise nor consist of sequences EPKSCDKTHTCPPCPAPELLGGPSTPPTPSPSGG (SEQ ID NO: 5).

23. The polypeptide of claim 22, which sequence comprises or consists of a sequence selected from the group consisting of EPKSCDKTHTSPPAPAEELLGGPAAPPAPAPAGG (SEQ ID NO: 3); EPKSCDKTHTSPPAPAEELLGGPGGPPGPGPGGG (SEQ ID NO: 2); and EPKSCDKTHTSPPAPAEELLGGPAAPPGPAPGGG (SEQ ID NO: 4).