

US 2014/0242079 describes a vector ratio of 1:2:1:1 for single expression cassette vectors for the transient expression in HEK cells.

WO 2015/052230 discloses multispecific domain exchanged common variable light chain antibodies.

5 WO 2012/023053 discloses methods for the generation of multispecific and multivalent antibodies.

WO 2005/072112 discloses methods for producing and identifying multispecific antibodies.

WO 02/079255 discloses recombinant antibodies coexpressed with GnTIII.

10 US 2002/06210 discloses method for making multispecific antibodies having heteromultimeric and common components.

US 2013/045888 discloses multi-copy strategy for high-titer and high-purity production of multi-subunit proteins such as antibodies in transformed microbes such as pichia pastoris.

15 Frenzel et al. reported about the expression of recombinant antibodies in Front. Immunol. 4 (2013) Article 217.

Wurm et al. reported about the production of recombinant protein therapeutics in cultivated mammalian cells (Nat. Biotechnol. 22 (2004) 1393-1398).

SUMMARY

20 It has been found that for the generation of cell lines for the production of heterodimeric, i.e. multispecific, antibodies it is advantageous to use an expression vector, which comprises as sole antibody chain expression cassette a light chain expression cassette, for the transfection. This vector can be used together with the other expression vectors in a co-transfection or separately in a second subsequent
25 transfection step. With this approach a production cell line can be obtained that produces the heterodimeric antibody with an improved product profile, i.e. with increased product and reduced product-related impurities.

As disclosed herein is a method for producing a multispecific antibody, which comprises/is composed of/contains at least three different polypeptides,
30 comprising the following steps:

- cultivating a mammalian cell in a cultivation medium (under conditions suitable for the expression of the multispecific antibody), whereby the mammalian cell has been generated by

5 a) transfecting a mammalian cell (not expressing an antibody) with a first expression vector and one, two or three further expression vectors,

10 wherein the first expression vector comprises exactly one nucleic acid sequence encoding a polypeptide of the multispecific antibody, and the one, two or three further expression vectors each comprise at least two nucleic acid sequences each encoding different polypeptide chains of the multispecific antibody,

 wherein the exactly one nucleic acid sequence of the first expression vector is a nucleic acid sequence encoding a light chain polypeptide of the multispecific antibody,

15 wherein the transfection with the first expression vector is either concomitant, before or after the transfection with the one, two or three further expression vectors, and

 b) selecting a cell (stably) transfected in step a) growing under selective cultivation conditions,

- 20 - recovering the multispecific antibody from the cell or the cultivation medium,

and thereby producing the multispecific antibody.

25 One aspect as disclosed herein is a method for producing a multispecific antibody, which comprises at least three different polypeptides, comprising the following steps:

- cultivating a mammalian cell in a cultivation medium, whereby the mammalian cell has been generated by

 a) transfecting a mammalian cell with a first expression vector and one, two or three further expression vectors,

5 wherein the first expression vector comprises exactly one nucleic acid sequence encoding a polypeptide of the multispecific antibody, and the one, two or three further expression vectors each comprise at least two nucleic acid sequences each encoding different polypeptide chains of the multispecific antibody,

wherein the exactly one nucleic acid sequence of the first expression vector is a nucleic acid sequence encoding a light chain polypeptide of the multispecific antibody,

10 - recovering the multispecific antibody from the cell or the cultivation medium,

and thereby producing the multispecific antibody.

As disclosed herein is a method for generating/producing/obtaining a mammalian cell (capable of (stably)) expressing a multispecific antibody, which comprises/is composed of at least three different polypeptides, comprising the following step:

15

a) transfecting a mammalian cell (not expressing an antibody) with a first expression vector and one, two or three further expression vectors,

wherein the first expression vector comprises exactly one nucleic acid sequence encoding a polypeptide of the multispecific antibody,

and the one, two or three further expression vectors each comprise at least two nucleic acid sequences each encoding different polypeptide chains of the multispecific antibody,

5 wherein the exactly one nucleic acid sequence of the first expression vector is a nucleic acid sequence encoding a light chain polypeptide of the multispecific antibody,

wherein the transfection with the first expression vector is either concomitant, before or after the transfection with the one, two or three further expression vectors, and

10 b) selecting a cell transfected in step a) growing under selective cultivation conditions,

and thereby generating/producing/obtaining a mammalian cell (stably) expressing a multispecific antibody.

15 In an example, two of the polypeptides of the multispecific antibody comprise/have a (cognate) domain exchange.

In an example, the exactly one nucleic acid of the first expression vector encodes a light chain polypeptide with a domain exchange of the multispecific antibody.

20 In an example, step a) comprises: co-transfecting a mammalian cell (not expressing an antibody) with a first expression vector and one, two or three further expression vectors.

25 In an example, step a) comprises the following steps: i) transfecting (simultaneously or sequentially) a mammalian cell with one, two or three further expression vectors, optionally ii) selecting a (stably) transfected cell, iii) transfecting the cell of i) or ii) with the first expression vector, and optionally iv) selecting a (stably) transfected cell.

In an example, the selecting is based on the expression yield and/or the amount of product-related side- products/impurities.

In an example, the selecting is of the (stably) transfected cell(s) that produce(s) the least amount (fraction) of product-related side-products/impurities.

5 In an example, the selecting is of the (stably) transfected cell(s) that produce(s) the least amount (fraction) of product-related side-products/impurities and that has the highest yield.

In an example, the mammalian cell stably expresses the multispecific antibody.

In an example, the mammalian cell is a CHO cell.

In an example, the domain exchange is a CH1-CL crossover or a VH-VL-crossover.

10 In an example, the multispecific antibody is a bivalent, bispecific antibody comprising

- a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen, and
- 15 b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the variable domains VL and VH of the second light chain and the second heavy chain are replaced by each other.

In an example, the multispecific antibody is a bivalent, bispecific antibody comprising

- 20 a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen, and
 - b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 of the second light chain and the second heavy chain are replaced by each other.
- 25

In an example, the multispecific antibody is a trivalent, bispecific antibody, comprising

- a) a first light chain and a first heavy chain of a full length antibody which specifically binds to a first antigen,
- b) a second heavy chain of a full length antibody which when paired with the first light chain, specifically binds to the first antigen, and
- 5 c) a Fab fragment, which specifically bind to a second antigen, fused via a peptidic linker to the C-terminus of one of the heavy chains of a) or b), wherein the constant domains CL and CH1 of the second light chain and the second heavy chain are replaced by each other.

10 As disclosed herein is a (stably transfected) mammalian cell obtained with the method as reported herein.

As disclosed herein is a method for producing a multispecific antibody comprising the following steps:

- 15 - cultivating a (stably transfected) cell as disclosed herein in a cultivation medium (under conditions suitable for the expression of the multispecific antibody),
- recovering the multispecific antibody from the cell or the cultivation medium,
- optionally purifying the recovered antibody with one or more chromatography steps,

20 and thereby producing the multispecific antibody.

As disclosed herein is a method for producing a multispecific antibody preparation with low/reduced product-related impurities comprising the following steps:

- 25 - obtaining/producing/generating a (stably transfected) mammalian cell (stably) expressing a multispecific antibody with a method as disclosed herein,
- cultivating the obtained/produced/generated mammalian cell in a cultivation medium,

- recovering the antibody preparation from the cell or the cultivation medium,
- optionally purifying the recovered antibody with one or more chromatography steps,

5 and thereby producing a multispecific antibody preparation with low/reduced product-related impurities.

As disclosed herein is the use of a method as reported herein for reducing product-related impurities in a multispecific antibody preparation.

10 Herein is reported a method for the production of a multispecific antibody which comprises at least one chain with a domain crossover in a recombinant mammalian cell. The method results in an improved process wherein the improvement is amongst other things a reduction of the product-related side-products and an increase of the amount of correctly folded/correctly assembled multispecific antibody.

15 As disclosed herein is a method for producing a multispecific antibody (comprising at least one polypeptide chain with a domain crossover) comprising the following steps:

- a) providing a (stably transfected) mammalian cell (stably) expressing the multispecific antibody,
- 20 b) transfecting the mammalian cell of step a) with an expression cassette encoding a polypeptide chain of the multispecific antibody that has a domain crossover,
- c) cultivating the cell of step b) and recovering the antibody from the cell or the cultivation medium and thereby producing the multispecific antibody,
- 25 d) optionally purifying the recovered antibody with one or more chromatography steps.

In an example, the mammalian cell expressing the multispecific antibody stably expresses the multispecific antibody.

30 In an example, the expression cassette of step b) is in an expression vector.

In an example, the polypeptide chain of the multispecific antibody that has a domain crossover is an antibody light chain.

In an example, the domain crossover is a CH1-CL crossover or a VH-VL-crossover.

5 In an example, the multispecific antibody is a bivalent bispecific antibody, or a trivalent bispecific antibody, or a tetravalent bispecific antibody.

10 In an example, the mammalian cell expressing the multispecific antibody is obtained by transfecting a mammalian cell with one or more nucleic acid molecules encoding the multispecific antibody and selecting a stably transfected cell.

In an example, the multispecific antibody is a bivalent, bispecific antibody comprising

a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen, and

15 b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the variable domains VL and VH of the second light chain and the second heavy chain are replaced by each other.

20 In an example, the multispecific antibody is a bivalent, bispecific antibody comprising

a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen, and

25 b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 of the second light chain and the second heavy chain are replaced by each other.

In an example, the multispecific antibody is a trispecific or tetraspecific antibody, comprising

- a) a first light chain and a first heavy chain of a full length antibody which specifically binds to a first antigen, and
- b) a second (modified) light chain and a second (modified) heavy chain of a full length antibody which specifically binds to a second antigen, wherein the variable domains VL and VH are replaced by each other, and/or wherein the constant domains CL and CH1 are replaced by each other, and
- c) wherein one to two antigen binding peptides which specifically bind to one or two further antigens (i.e. to a third and/or fourth antigen) are fused via a peptidic linker to the C- or N-terminus of the light chains or heavy chains of a) and/or b).

In an example, the multispecific antibody is a bispecific, tetravalent antibody comprising

- a) two light chains and two heavy chains of an antibody, which specifically bind to a first antigen (and comprise two Fab fragments),
- b) two additional Fab fragments of an antibody, which specifically bind to a second antigen, wherein said additional Fab fragments are fused both via a peptidic linker either to the C- or N-termini of the heavy chains of a),

and

wherein in the Fab fragments the following modifications were performed

- i) in both Fab fragments of a), or in both Fab fragments of b), the variable domains VL and VH are replaced by each other, and/or the constant domains CL and CH1 are replaced by each other,
 - or
 - ii) in both Fab fragments of a) the variable domains VL and VH are replaced by each other, and the constant domains CL and CH1 are replaced by each other,
- and

in both Fab fragments of b) the variable domains VL and VH are replaced by each other, or the constant domains CL and CH1 are replaced by each other,

or

- 5 iii) in both Fab fragments of a) the variable domains VL and VH are replaced by each other, or the constant domains CL and CH1 are replaced by each other,

and

- 10 in both Fab fragments of b) the variable domains VL and VH are replaced by each other, and the constant domains CL and CH1 are replaced by each other,

or

- 15 iv) in both Fab fragments of a) the variable domains VL and VH are replaced by each other, and in both Fab fragments of b) the constant domains CL and CH1 are replaced by each other,

or

- v) in both Fab fragments of a) the constant domains CL and CH1 are replaced by each other, and in both Fab fragments of b) the variable domains VL and VH are replaced by each other.

20 In an example, in the Fab fragments the following modifications are performed:

- i) in both Fab fragments of a), or in both Fab fragments of b), the variable domains VL and VH are replaced by each other,
and/or
the constant domains CL and CH1 are replaced by each other.

25 In an example, the multispecific antibody is a bispecific, tetravalent antibody comprising:

- a) a (modified) heavy chain of a first antibody, which specifically binds to a first antigen and comprises a first VH-CH1 domain pair, wherein to the C-

terminus of said heavy chain is fused to the N-terminus of a second VH-CH1 domain pair of said first antibody via a peptidic linker,

- b) two light chains of said first antibody of a),
- c) a (modified) heavy chain of a second antibody, which specifically binds to a second antigen and comprises a first VH-CL domain pair, wherein to the C-terminus of said heavy chain is fused to the N-terminus of a second VH-CL domain pair of said second antibody via a peptidic linker, and
- d) two (modified) light chains of said second antibody of c), each comprising a CL-CH1 domain pair.

In an example, the first light chain comprises a VL domain and a CL domain and the first heavy chain comprises a VH domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain.

In an example, the antibody as produced in the method as reported herein is a multispecific antibody, which requires heterodimerization of at least two heavy chain polypeptides.

In an example, the full length antibody is

- a) a full length antibody of the human subclass IgG1,
- b) a full length antibody of the human subclass IgG4,
- c) a full length antibody of the human subclass IgG1 with the mutations L234A, L235A and P329G,
- d) a full length antibody of the human subclass IgG4 with the mutations S228P, L235E and P329G,
- e) a full length antibody of the human subclass IgG1 with the mutations L234A, L235A and P329G in both heavy chains and the mutations T366W and S354C or Y349C in one heavy chain and the mutations T366S, L368A, Y407V and Y349C or S354C in the respective other heavy chain,
- f) a full length antibody of the human subclass IgG4 with the mutations S228P and P329G in both heavy chains and the mutations T366W and

S354C in one heavy chain and the mutations T366S, L368A, Y407V and Y349C in the respective other heavy chain,

5 g) a full length antibody of the human subclass IgG1 with the mutations L234A, L235A, P329G, I253A, H310A and H435A in both heavy chains and the mutations T366W and S354C in one heavy chain and the mutations T366S, L368A, Y407V and Y349C in the respective other heavy chain, or

10 h) a full length antibody of the human subclass IgG1 with the mutations L234A, L235A, P329G, M252Y, S254T and T256E in both heavy chains and the mutations T366W and S354C in one heavy chain and the mutations T366S, L368A, Y407V and Y349C in the respective other heavy chain.

As disclosed herein is a cell comprising a nucleic acid encoding the bispecific antibody obtained with a method as disclosed herein.

15 As disclosed herein is a method of producing a multispecific antibody as disclosed herein comprising the following steps:

a) culturing the cell as disclosed herein producing/expressing the multispecific antibody, and
b) recovering the multispecific antibody from the cell or the cultivation medium,
20

and thereby producing the multispecific antibody as reported herein.

As disclosed herein is the antibody produced with the method as reported herein.

As disclosed herein is a pharmaceutical formulation comprising the antibody produced with the method as disclosed herein and a pharmaceutically acceptable carrier.
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As disclosed herein is the antibody produced with the method as disclosed herein for use as a medicament.

As disclosed herein is the use of the bispecific antibody produced with the method as disclosed herein in the manufacture of a medicament.

In an example, the bispecific antibody is selected from the group of bispecific antibodies consisting of an anti-Abeta/transferrin receptor antibody, an anti-CD20/transferrin receptor antibody, an anti-PD1/Tim3 antibody, and an anti-FAP/DR5 antibody.

5 In an example, the multispecific antibody is a bispecific, tetravalent antibody comprising

a) two light chains and two heavy chains of an antibody, which specifically bind to a first antigen (and comprise two Fab fragments),

10 b) two additional Fab fragments of an antibody, which specifically bind to a second antigen, wherein each of said additional Fab fragments is fused via a peptidic linker to an individual C-terminus of one of the heavy chains of a),

and

15 wherein in the additional Fab fragments the following modifications were performed

in both additional Fab fragments of b), the variable domains VL and VH are replaced by each other, and/or the constant domains CL and CH1 are replaced by each other,

20 wherein i) the first antigen is DR5 and the second antigen is FAP, or ii) the first antigen is FAP and the second antigen is DR5,

wherein the two heavy chains of an antibody, which specifically bind to a first antigen are of the human subclass IgG1 with the mutations L234A, L235A and P329G.

25 In an example, the multispecific antibody is a bivalent, bispecific antibody comprising

a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen, and

b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the variable domains VL and VH of

the second light chain and the second heavy chain are replaced by each other.

wherein i) the first antigen is PD1 and the second antigen is Tim3, or ii) the first antigen is Tim3 and the second antigen is PD1,

5 wherein the first heavy chain and the second heavy are both of the human subclass IgG1 with the mutations L234A, L235A and P329G and with the mutation T366W and optionally S354C or Y349C in one heavy chain and the mutations T366S, L368A, Y407V and optionally Y349C or S354C in the
10 respective other heavy chain, whereby the terminal glycine or glycine-lysine dipeptide can be absent,

wherein the first light chain comprises in the constant light chain domain (CL) at position 123 the amino acid residue arginine (instead of the wild-type glutamic acid residue; E123R mutation) and at position 124 the amino acid
15 residue lysine (instead of the wild-type glutamine residue; Q124K mutation) (numbering according to Kabat),

wherein the first heavy chain comprises in the first constant heavy chain domain (CH1) at position 147 a glutamic acid residue (instead of the wild-type lysine residue; K147E mutation) and at position 213 a glutamic acid
20 residue (instead of the wild-type lysine amino acid residue; K213E mutation) (numbering according to Kabat EU index).

In an example, the multispecific antibody is a trivalent, bispecific antibody comprising

- a) two light chains and two heavy chains of an antibody, which specifically bind to a first antigen (and comprise two Fab fragments),
- 25 b) one additional Fab fragment of an antibody, which specifically bind to a second antigen, wherein said additional Fab fragment is fused via a peptidic linker to the C-terminus of one of the heavy chains of a),

and

wherein in the additional Fab fragment the following modifications were
30 performed

the variable domains VL and VH are replaced by each other, and/or the constant domains CL and CH1 are replaced by each other,

wherein i) the first antigen is Abeta and the second antigen is the transferrin receptor, or ii) the first antigen is CD20 and the second antigen is the transferrin receptor.

In an example, the multispecific antibody is a bispecific antibody comprising

- a) one full length antibody comprising two pairs each of a full length antibody light chain and a full length antibody heavy chain, wherein the binding sites formed by each of the pairs of the full length heavy chain and the full length light chain specifically bind to a first antigen, and
- b) one additional Fab fragment, wherein the additional Fab fragment is fused to the C-terminus of one heavy chain of the full length antibody, wherein the binding site of the additional Fab fragment specifically binds to a second antigen,

wherein each of the full length antibody light chains comprises in the constant light chain domain (CL) at position 123 the amino acid residue arginine (instead of the wild-type glutamic acid residue; E123R mutation) and at position 124 the amino acid residue lysine (instead of the wild-type glutamine residue; Q124K mutation) (numbering according to Kabat),

wherein each of the full length antibody heavy chains comprises in the first constant heavy chain domain (CH1) at position 147 a glutamic acid residue (instead of the wild-type lysine residue; K147E mutation) and at position 213 an glutamic acid residue (instead of the wild-type lysine amino acid residue; K213E mutation) (numbering according to Kabat EU index),

wherein the additional Fab fragment specifically binding to the second antigen comprises a domain crossover such that the constant light chain domain (CL) and the constant heavy chain domain 1 (CH1) are replaced by each other, and

wherein the first antigen is human A-beta protein and the second antigen is human transferrin receptor.

In an example, the multispecific antibody is a bispecific antibody comprising

- a) one full length antibody comprising two pairs each of a full length antibody light chain and a full length antibody heavy chain, wherein the binding sites formed by each of the pairs of the full length heavy chain and the full length light chain specifically bind to a first antigen, and
- b) one additional Fab fragment, wherein the additional Fab fragment is fused to the C-terminus of one heavy chain of the full length antibody, wherein the binding site of the additional Fab fragment specifically binds to a second antigen,

wherein each of the full length antibody light chains comprises in the constant light chain domain (CL) at position 123 the amino acid residue arginine (instead of the wild-type glutamic acid residue; E123R mutation) and at position 124 the amino acid residue lysine (instead of the wild-type glutamine residue; Q124K mutation) (numbering according to Kabat),

wherein each of the full length antibody heavy chains comprises in the first constant heavy chain domain (CH1) at position 147 a glutamic acid residue (instead of the wild-type lysine residue; K147E mutation) and at position 213 an glutamic acid residue (instead of the wild-type lysine amino acid residue; K213E mutation) (numbering according to Kabat EU index),

wherein the additional Fab fragment specifically binding to the second antigen comprises a domain crossover such that the constant light chain domain (CL) and the constant heavy chain domain 1 (CH1) are replaced by each other, and

wherein the first antigen is human CD20 and the second antigen is human transferrin receptor.

In an example, each polypeptide is within an expression cassette each comprising in 5'- to 3'-direction a promoter, a structural gene encoding the polypeptide, a polyadenylation sequence and optionally a terminator sequence. In an example, all expression cassettes have the same promoter, the same polyadenylation site and optionally the same terminator sequence. In an example, the promoter is the human CMV (cytomegalovirus)

promoter. In an example, the CMV promoter comprises an intron A. In an example, the polyadenylation site is the BGH (bovine growth hormone) polyadenylation site. In an example, the terminator is present and is the HGT (human growth hormone terminator). In an example, the promoter is the CMV promoter optionally comprising an intron A and the polyadenylation site is the BGH polyadenylation site. In an example, the promoter is the CMV promoter optionally comprising an intron A, the polyadenylation site is the BGH polyadenylation site and the terminator is the HGT.

In an example, the further expression vector comprises or each of the further expression vectors each comprises at least two nucleic acid sequences each encoding different polypeptide chains of the multispecific antibody, wherein each encoding nucleic acid is present/contained exactly once on the respective vector.

DETAILED DESCRIPTION OF THE INVENTION

The knobs into holes dimerization modules and their use in antibody engineering are described in Carter P.; Ridgway J.B.B.; Presta L.G.: Immunotechnology, Volume 2, Number 1, February 1996, pp. 73-73(1).

General information regarding the nucleotide sequences of human immunoglobulins light and heavy chains is given in: Kabat, E.A., et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991).

As used herein, the amino acid positions of all constant regions and domains of the heavy and light chain are numbered according to the Kabat numbering system described in Kabat, et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991) and is referred to as “numbering according to Kabat” herein. Specifically, the Kabat numbering system (see pages 647-660) of Kabat, et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991) is used for the light chain constant domain CL of kappa and lambda isotype, and the Kabat EU index numbering system (see pages 661-723) is used for the constant heavy chain domains (CH1, Hinge, CH2 and CH3, which is herein further clarified by referring to “numbering according to Kabat EU index” in this case).