Therefore, there is still a need for tailoring effector functions of recombinant IgG antibodies, especially for the provision of means for improving ADCC mediated by therapeutic antibodies.

In WO 2011/012297 a method for producing an immunoglobulin or immunoglobulin fragment with defined glycostructure comprising the steps of providing an affinity chromatography column eluate containing the immunoglobulin or immunoglobulin fragment, incubating the affinity chromatography column eluate with (a1,3)galactosidase of plant origin, e.g. from green coffee beans (EC 3.2.1.22), applying the incubated affinity chromatography column eluate to a protein A chromatography material and recovering the immunoglobulin or immunoglobulin fragment from the protein A chromatography material and thereby producing an immunoglobulin or immunoglobulin fragment with defined glycostructure is reported.

In WO 2015/123754 an enzymatic method is provided for restructuring an affinity ligand bound heterogeneous glycoform antibody sample to a substantially homogenous single desired glycoform antibody sample for therapeutic uses and kits for performing the methods. A method for enzymatically altering the Fc region of an affinity ligand bound antibody from a heterogeneous glycoform to a substantially homogenous single glycoform comprises: contacting the affinity ligand bound heterogeneous glycoform antibody with a reaction buffer designed for a particular glycoform modification for a time sufficient and under conditions to modify the glycoform of the Fc region to a substantially homogeneous single form; optionally adding one or more nucleotide sugars and/or cofactors; and releasing the substantially homogeneous single glycoform antibody sample from said affinity ligand. The invention also encompasses biopharmaceuticals comprising single glycoform mAbs and polyclonal antibodies enzymatically produced for the treatment of cancers and immune disorders as well as compositions comprising the single glycoform antibodies as a biopharmaceutical.

In WO 2016/037947 galactoengineered recombinant antibodies of IgG1 isotype, methods for the production of said antibodies and uses thereof are reported.

Summary

Herein is reported a method for the in vitro glycoengineering of antibodies, in one example of recombinantly produced monoclonal antibodies, wherein the modification of the glycosylation is performed while the antibody is bound to a

solid phase comprising an antibody light chain affinity ligand. The method as reported herein is, amongst other things, an improved method for the modification of immobilized antibodies.

In the method as reported herein a monoclonal antibody is bound to an affinity ligand, especially an antibody light chain affinity ligand, for enzymatic modification and subsequently released as a monoclonal antibody preparation with modified glycostructure. This modification can be at an N-glycosylation site in the Fab fragment or in the Fc-region. It was surprisingly found that an antibody light chain affinity ligand bound antibody can be effectively enzymatically modified as if the antibody would be in solution. The method as reported herein can be easily integrated into any antibody purification process thereby providing a novel, efficient and cost-effective process of in vitro antibody glycan modification.

The method as reported herein is useful for the modification of any monoclonal antibody without the need of modifications to the preceding up-stream production process steps. The method as reported herein can be integrated as a single in vitro modification and purification step into an existing process. Inherently no significant changes to existing antibody producing cell lines are required as the glycostructure modification is provided by the method as reported herein during down-stream processing.

With the method as reported herein the enzymes used for the modification of the glycosylation of the antibody can be removed from the antibody preparation resulting in an improved preparation.

As reported herein is a method for the enzymatic preparation/production of an antibody with a modified (substantially homogeneous) glycosylation at an N-glycosylation site wherein the antibody is bound to an antibody light chain affinity ligand during the enzymatic modification.

As reported herein is a method for the enzymatic modification of the glycosylation of an N-glycosylation site of an antibody (to a substantially homogeneous glycosylation) wherein the antibody is bound to an antibody light chain affinity ligand during the enzymatic modification.

In one example the method comprises the following step:

- incubating the antibody light chain affinity ligand-bound monoclonal antibody with a glycosylation at an N-glycosylation site with one or more enzymes for a time sufficient and under conditions suitable to modify the glycosylation of the N-glycosylation site to a defined (substantially homogeneous) form (homogeneous glycosylation).

In one example the method comprises prior to the incubation step the step of

- binding the monoclonal antibody with glycosylation at an N-glycosylation site to an antibody light chain affinity ligand,

and after the incubation step the step of

- releasing the antibody with a defined (substantially homogeneous) glycosylation at the N-glycosylation site from the antibody light chain affinity ligand.

One aspect as reported herein is a method for producing an antibody comprising the following steps:

- forming an antibody-antibody light chain affinity ligand complex, wherein the antibody light chain affinity ligand is immobilized on a solid phase, by applying a solution comprising the antibody to the immobilized antibody light chain affinity ligand,

- incubating the complex formed in the previous step with one or more enzymes to modify the glycosylation of the antibody,

and thereby producing the antibody.

In one example the method comprises the following steps in the following order

- applying a (buffered) solution comprising the antibody with glycosylation at an N-glycosylation site to an antibody light chain affinity ligand bound to a solid phase (antibody light chain affinity ligand chromatography material) whereby the antibody is bound to the ligand (resulting in a ligand-bound antibody),

- optionally washing the solid phase with a buffered solution,

- enzymatically modifying the glycosylation at the N-glycosylation site of the antibody by either

    - applying a first (buffered) solution comprising a first glycosylation modifying enzyme (and a first activated sugar residue) for a time sufficient and under conditions suitable for the enzymatic modification to the ligand-bound antibody, optionally washing the modified ligand-bound antibody, applying a second (buffered) solution comprising a second

glycosylation modifying enzyme (and a second activated sugar) for a time sufficient and under conditions suitable for the enzymatic modification to the modified ligand-bound antibody, optionally washing the two-times modified ligand-bound antibody,

or

- applying a first (buffered) solution comprising a first glycosylation modifying enzyme (and a first activated sugar) for a time sufficient and under conditions suitable for at least a partial enzymatic modification to the ligand-bound antibody, applying after a defined period of time a second (buffered) solution comprising a second glycosylation modifying enzyme (and a second activated sugar) for a time sufficient and under conditions suitable for the enzymatic modification to the modified ligand-bound antibody, optionally washing the two-times modified ligand-bound antibody,

or

- applying a (buffered) solution comprising a first and a second glycosylation modifying enzyme (and a first and second activated sugar) for a time sufficient and under conditions suitable for the enzymatic modification of the ligand-bound antibody, optionally washing the modified ligand-bound antibody,

- releasing the antibody with a defined glycosylation at the N-glycosylation site from the antibody light chain affinity ligand.

The antibodies as used in the methods as reported herein can be any antibody or antibody fragment, including Fab fragments, single chain antibodies, multispecific antibodies and antibody fusions, so long as it contains an N-glycosylation site.

In one example the N-glycosylation site is in the Fab or in the Fc-region.

Thus, in one example the antibody is selected from the group of antibodies consisting of an antibody Fab fragment, a full length antibody, a bivalent monospecific antibody, a bispecific antibody, a bivalent bispecific antibody, a trivalent bispecific antibody, a tetravalent bispecific antibody, a trivalent trispecific antibody, and a tetravalent tetraspecific antibody.

In one example the antibody is a bivalent monospecific antibody.

In one example the antibody is a bivalent or trivalent or tetravalent bispecific antibody.

In one example the antibody is a chimeric or humanized or human antibody.

In one example the antibody is a polyclonal antibody preparation.

In one example the antibody is a monoclonal antibody.

In one example of the antibody (preparation) is an antibody (preparation) of the human IgG class. In one example the antibody is an antibody of the human IgG1 or IgG4 subclass.

In one example the defined glycosylation is a glycosylation selected from the group consisting of G2 glycoform, G0 glycoform, M3 glycoform, S2 glycoform, A2B glycoform, A2BG2 glycoform and S1 glycoform.

In one example the defined glycosylation is a glycosylation selected from the group consisting of galactose as the terminal sugar, GlcNAc as the terminal sugar, mannose as the terminal sugar and sialic acid as the terminal sugar.

In one example the antibody is a recombinantly produced antibody.

As reported herein is an antibody produced with a method as reported herein.

As reported herein is a pharmaceutical formulation comprising an antibody with defined glycosylation produced by a method as reported herein.

There is also provided a method for the recombinant production of an antibody with defined glycosylation at an N-glycosylation site, comprising the steps of

a) recombinantly producing an antibody (of IgG1 isotype) in a (mammalian or CHO) cell, which comprises nucleic acids encoding the antibody, to obtain an antibody with glycosylation at an N-glycosylation site,

b) isolating (recovering and optionally purifying) the antibody with heterogeneous glycosylation at the N-glycosylation site,

c) enzymatically modifying the antibody with glycosylation at the N-glycosylation site with galactosyltransferase and/or a sialyl transferase to obtain an antibody with defined glycosylation at the N-glycosylation site, which comprises a relative amount of at least 70 % of bi-galactosylated antibodies (G2F glycoform) (wherein 100 % corresponds to the amount of G0F, G1F and G2F glycoforms) at the N-glycosylation site, and subsequent separation of the of the modified antibody from the enzyme(s),

d) optionally purifying the modified antibody by one or more chromatographic steps,

and thereby producing an antibody with defined glycosylation at the N-glycosylation site.

In one example the first glycosylation modifying enzyme is a galactosyltransferase.

In one example the first glycosylation modifying enzyme is a galactosyltransferase and the second glycosylation modifying enzyme is a sialyltransferase.

In one example the galactosyltransferase is **β**4GalT1.

In one example the sialyltransferase is ST6.

In one example the sialyltransferase is ST6Gal1 or ST6Gal2.

In one example the (first) buffered solution comprises UDP-Gal.

In one example the (second) buffered solution comprises CMP-NANA.

In one example the incubation is at room temperature (20 - 25 °C, preferably about 22 °C).

In one example the incubation is at 25 °C.

In one example the incubation is at 37 °C.

In one example the incubation is for 7 to 48 hours.

In one example the solution comprises a chromatographically purified antibody, the (first) glycosylation modifying enzyme is GalT1, and the incubation with the (first) glycosylation modifying enzyme is for 24 hours at 20-27 °C or 37 °C. In one example the incubation is at room temperature (about 22 °C).

In one example the solution comprises a chromatographically purified antibody, the (second) glycosylation modifying enzyme is ST6, and the incubation with the (second) glycosylation modifying enzyme is for 24 hours at 20-27°C or 37 °C. In one example the incubation is at room temperature (about 22 °C).

In one example the solution is a buffered, cell-free cultivation supernatant comprising the antibody, the first glycosylation modifying enzyme is GalT1, the second glycosylation modifying enzyme is ST6, which is added 6 to 24 hours, preferably 24 hours, after the first glycosylation modifying enzyme, the total incubation time is 24 hours to 48 hours, preferably 30 hours, at 20-27°C or 37 °C. In one example the incubation is at room temperature (about 22 °C).

As reported herein is a method for producing an antibody or fragment thereof comprising the following steps in the following order:

- providing a cell comprising a nucleic acid encoding the antibody or a fragment thereof comprising at least an antibody light chain,
- cultivating the cell under conditions suitable for the expression of the antibody or fragment thereof with glycosylation at an N-glycosylation site (the fragment comprises at least on light chain that can specifically be bound by the antibody light chain affinity chromatography material used in one of the next steps),

- recovering the antibody or fragment thereof from the cell or the cultivation medium,

- applying a solution comprising antibody or fragment thereof to an antibody light chain affinity chromatography column under conditions suitable for binding of the antibody or fragment thereof to the affinity chromatography material,

- modifying the glycosylation of the antibody or fragment thereof at the N-glycosylation site with a method as reported herein, and

- recovering the modified antibody or fragment thereof with a defined glycosylation at the N-glycosylation site from the affinity chromatography material,

and thereby producing an antibody.

In one example comprises the method the following step as final step:

- purifying the modified antibody or fragment thereof with one to three chromatography steps.

In one example the N-glycosylation site is in the Fab or in the Fc-region.

Detailed Description of the Invention

DEFINITIONS

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. Specifically the Kabat EU index numbering system (see pages 661-723) is used for the constant heavy chain domains (CH1, Hinge, CH2

Patent Claims

1. A method for producing an antibody comprising the following steps:

   - forming an antibody-antibody light chain affinity ligand complex, wherein the antibody light chain affinity ligand is immobilized on a solid phase, by applying a solution comprising the antibody to the immobilized antibody light chain affinity ligand,

   - incubating the complex formed in the previous step with one or more enzymes to modify the glycosylation of the antibody,

   and thereby producing the antibody.

2. The method according to claim 1, wherein the incubation step comprises

   - incubating the complex formed in the forming step with one or more enzymes and one or more activated sugar residues to modify the glycosylation of the antibody.

3. The method according to claim 1 or 2, comprising after the incubation step the step of

   - recovering the modified antibody from the antibody light chain affinity ligand.

4. The method according to any one of claims 1 to 3, comprising the following steps in the following order

   - applying the solution comprising the antibody to an antibody light chain affinity ligand bound to a solid phase, whereby the antibody is bound by the ligand,

   - optionally washing the solid phase with a buffered solution,

   - enzymatically modifying the glycosylation at the N-glycosylation site of the antibody by either

     - applying a first solution comprising a first glycosylation modifying enzyme for a time sufficient and under conditions suitable for the enzymatic modification of the ligand-bound antibody, optionally washing the modified ligand-bound

antibody, applying a second solution comprising a second glycosylation modifying enzyme for a time sufficient and under conditions suitable for the enzymatic modification of the modified ligand-bound antibody, optionally washing the two-times modified ligand-bound antibody,

or

- applying a first solution comprising a first glycosylation modifying enzyme for a time sufficient and under conditions suitable for at least a partial enzymatic modification of the ligand-bound antibody, applying after a defined period of time a second solution comprising a second glycosylation modifying enzyme for a time sufficient and under conditions suitable for the enzymatic modification of the modified ligand-bound antibody, optionally washing the two-times modified ligand-bound antibody,

or

- applying a solution comprising a first and a second glycosylation modifying enzyme for a time sufficient and under conditions suitable for the enzymatic modification of the ligand-bound antibody, optionally washing the modified ligand-bound antibody,

- releasing the antibody from the antibody light chain affinity ligand,

and thereby producing the antibody.

5. The method according to claim 4, wherein the solution comprising the antibody is a buffered solution.

6. The method according to claim 4 or 5, wherein the antibody has an N-glycosylation site.

7. The method according to any one of claims 4 to 6, wherein the binding by the ligand results in a ligand-bound antibody.

8. The method according to any one of claims 4 to 7, wherein the optional washing step does not desorb the antibody/disrupting the ligand antibody complex.

9. The method according to any one of claims 4 to 8, wherein in the enzymatically modifying step,

the first solution is a first buffered solution;

the first solution further comprises a first activated sugar residue;

the second solution is a second buffeded solution;

the second solution further comprises a second activated sugar;

the solution comprising the first and the second glycosylation modifying enzyme is a buffered solution; and

the solution comprising the first and the second glycosylation modifying enzyme further comprises a first and second activated sugar.

10. The method according to any one of claims 1 to 9, wherein the antibody is a glycosylation modified antibody.

11. The method according to any one of claims 1 to 10, wherein the antibody is selected from the group of antibodies consisting of a full length antibody, a bivalent monospecific antibody, a bispecific antibody, a bivalent bispecific antibody, a trivalent bispecific antibody, a tetravalent bispecific antibody, a trivalent trispecific antibody, an antibody Fab fragment, and a tetravalent tetraspecific antibody.

12. The method according to any one of claims 1 to 11, wherein the antibody is a bivalent or trivalent or tetravalent bispecific antibody or a Fab fragment.

13. The method according to any one of claims 1 to 12, wherein the antibody is a chimeric or humanized or human antibody.

14. The method according to any one of claims 4 to 13, wherein the first glycosylation modifying enzyme is a galactosyltransferase and the second glycosylation modifying enzyme is a sialyltransferase.

15. The method according to any one of claims 4 to 14, wherein the solution comprises a chromatographically purified antibody, the first glycosylation

modifying enzyme is GalT1, and the incubation with the first glycosylation modifying enzyme is for 24 hours at 37°C or room temperature.

16. The method according to any one of claims 4 to 15, wherein the solution comprises a chromatographically purified antibody, the second glycosylation modifying enzyme is ST6, and the incubation with the second glycosylation modifying enzyme is for 24 hours at 37 °C or room temperature.

17. The method according to any one of claims 4 to 16, wherein the solution is a buffered, cell-free cultivation supernatant comprising the antibody, the first glycosylation modifying enzyme is GalT1, the second glycosylation modifying enzyme is ST6, which is added 6 to 24 hours, preferably 24 hours, after the first glycosylation modifying enzyme, the total incubation time is 24 hours to 48 hours, preferably 30 hours, at 37 °C or room temperature.

18. The method according to any one of claims 1 to 17 comprising the steps of

a) recombinantly producing an antibody or a Fab fragment in a cell, which comprises nucleic acids encoding the antibody or Fab fragment, to obtain an antibody or Fab fragment with glycosylation at an N-glycosylation site,

b) isolating the antibody or Fab fragment produced in step a) with heterogeneous glycosylation at the N-glycosylation site,

c) enzymatically modifying the antibody or Fab fragment with heterogeneous glycosylation at an N-glycosylation site with a galactosyltransferase and/or a sialyl transferase to obtain a modified antibody, and subsequently separating the modified antibody or Fab fragment from the enzyme(s),

d) optionally purifying the modified antibody or Fab fragment by one or more chromatography steps,

wherein the produced antibody or Fab fragment comprises a relative amount of at least 70 % of bi-galactosylated antibody or Fab fragment at the N-glycosylation site.

19. The method according to claim 18 comprising the steps of

a) recombinantly producing an antibody of IgG1 isotype or a Fab fragment in a mammalian or CHO cell, which comprises nucleic acids encoding the antibody or Fab fragment, to obtain an antibody or Fab fragment with glycosylation at an N-glycosylation site,

b) isolating, recovering and optionally purifying the antibody or Fab fragment produced in step a) with heterogeneous glycosylation at the N-glycosylation site,

c) enzymatically modifying the antibody or Fab fragment with heterogeneous glycosylation at an N-glycosylation site with a galactosyltransferase and/or a sialyl transferase to obtain a modified antibody, and subsequently separating the modified antibody or Fab fragment from the enzyme(s),

d) optionally purifying the modified antibody or Fab fragment by one or more chromatography steps,

wherein the produced antibody or Fab fragment comprises a relative amount of at least 70 % of bi-galactosylated antibody or Fab fragment G2 glycoform, wherein 100 % corresponds to the amount of G0, G1 and G2 glycoforms, at the N-glycosylation site.

20. The method according to any one of claims 1 to 11, wherein the N-glycosylation site is a Fab region N-glycosylation site or the Fc-region N-glycosylation site at asparagine residue 297, where the numbering is according to Kabat.