## Sequence dependent aggregation

Herein is reported a method for reducing immunoglobulin aggregation in concentrated solutions by reverting one or two mutation(s) in the fourth framework region, i.e. in the J-element, of an immunoglobulin heavy chain to the hydrophilic or natural germline amino acid residue.

## Background of the Invention

Due to their chemical and physical properties, such as molecular weight and domain architecture including secondary modifications, the downstream processing of immunoglobulins is very complicated. Concentrated immunoglobulin solutions are required not only for formulated drugs but also for intermediates in downstream processing (DSP) to achieve low volumes for economic handling and application storage.

Also the final formulation of the immunoglobulin requires a highly concentrated solution. For example, for subcutaneous application immunoglobulin concentrations of more than 100 mg/ml, i.e. about 150 mg/ml, are required. But at least some immunoglobulins tend to aggregate at unphysiologically high concentrations of 100 mg/ml or more.

In WO 2009/000098 a sequence based engineering and optimization of single chain antibodies is reported.

## Summary ~~of the Invention~~

Herein is reported a method for reducing immunoglobulin aggregation in a concentrated solution. It has been found that two or even a single reverting mutation in the fourth framework region of an immunoglobulin heavy chain can reduce the formation of aggregates allowing the provision of a highly concentrated solution of the reverted immunoglobulin variant with reduced aggregate content. Therefore, herein are reported as individual aspects a method for reducing the aggregation of an immunoglobulin in solution, a method for modifying an immunoglobulin, a method for producing an immunoglobulin, and a method for humanizing an immunoglobulin.

All the methods as reported herein comprise the following steps:

a) aligning the amino acid sequence of the fourth heavy chain framework region of an immunoglobulin with a reference amino acid sequence to achieve maximal level of amino acid sequence identity,

b) identifying aligned amino acid sequence positions with different amino acid residues in the fourth heavy chain framework region amino acid sequence of the immunoglobulin and of the reference amino acid sequence,

c) modifying the immunoglobulin amino acid sequence by substituting an or at least one amino acid residue in the fourth heavy chain framework region amino acid sequence of the immunoglobulin at a position identified in b) for the respective threonine or serine residue as present in the reference amino acid sequence, whereby at the substituted position the amino acid residue is threonine or serine in the reference amino acid sequence, and whereby the amino acid residue is not threonine and not serine in the fourth heavy chain framework region amino acid sequence of the immunoglobulin,

d) optionally providing a nucleic acid sequence encoding the modified immunoglobulin amino acid sequence.

One aspect as reported herein is a method for providing a nucleic acid sequence encoding an immunoglobulin comprising the following steps

a) aligning the amino acid sequence of the fourth heavy chain framework region of an immunoglobulin with the reference sequence xxxxTxxTxxx (SEQ ID NO: 11) to achieve maximal level of amino acid sequence identity,

b) identifying aligned amino acid positions with a small hydrophobic or non-polar amino acid residue at position 5 or/and 8 in the fourth heavy chain framework region,

c) modifying the immunoglobulin by substituting an amino acid residue in the fourth heavy chain framework region at a position identified in b), which is threonine in the reference sequence and which is not threonine in the fourth heavy chain framework region, for the respective threonine residue as in the reference sequence,

d) providing a nucleic acid sequence encoding the immunoglobulin.

In one embodiment only the difference of threonine residues is determined and changed. In another embodiment the reference amino acid sequence to the fourth framework region of the immunoglobulin is the amino acid sequence xxxxTTxTxSS (SEQ ID NO: 01) with x denoting any amino acid residue except threonine and serine. In a further embodiment the reference amino acid sequence is the amino acid sequence xxxxTxxTxxx (SEQ ID NO: 11) with x at positions 1, 2, 3, 4, 7 and 9 denoting any amino acid residue except threonine and serine and x at position 6 denoting threonine and x at position 10 and 11 denoting serine, whereby the difference of threonine residues at position 5 and/or 8 in the reference amino acid sequence to the amino acid sequence of the fourth framework region of the immunoglobulin heavy chain is identified in step b) and modified in step c). In one embodiment the reference amino acid sequence is a human germline amino acid sequence. In one embodiment of all aspects as reported herein the methods comprise the following steps:

a) aligning the amino acid sequence of the fourth heavy chain framework region of the immunoglobulin with the amino acid sequence WGQGTLVTVSS (SEQ ID NO: 02), or the amino acid sequence WGRGTLVTVSS (SEQ ID NO: 03), or the amino acid sequence WGQGTMVTVSS (SEQ ID NO: 04), or the amino acid sequence WGQGTTVTVSS (SEQ ID NO: 05), or the amino acid sequence WGKGTTVTVSS (SEQ ID NO: 06) to achieve maximal level of amino acid sequence identity,

b) identifying aligned amino acid positions with different amino acid residues in the fourth heavy chain framework region amino acid sequence of the immunoglobulin and the reference amino acid sequence,

c) modifying the immunoglobulin by substituting an amino acid residue in the fourth heavy chain framework region amino acid sequence at a position identified in b) for the respective threonine or serine residue present in the reference amino acid sequence, whereby the amino acid residue at the position is threonine or serine in the reference amino acid sequence and whereby the amino acid residue at the position is not threonine and not serine in the fourth heavy chain framework region amino acid sequence of the immunoglobulin,

d) optionally providing a nucleic acid sequence encoding the modified immunoglobulin amino acid sequence.

In one embodiment of the aspects as reported herein the methods comprise as first step the step of providing or determining the amino acid sequence of the fourth framework region of the immunoglobulin heavy chain. In one embodiment the reference sequence is the amino acid sequence WGQGTLVTVSS (SEQ ID NO: 02). In another embodiment the reference amino acid sequence is xxxxTxxTxxx (SEQ ID NO: 11). In one embodiment the difference of the threonine residues at position 5 or/and 8 of the reference amino acid sequence is determined. In one embodiment only the difference of threonine residues is determined and changed.

Another aspect as reported herein is a method for producing an immunoglobulin comprising the following steps

a) aligning the amino acid sequence of the fourth heavy chain framework region of the immunoglobulin with the reference sequence xxxxTxxTxxx (SEQ ID NO: 11) to achieve maximal level of amino acid sequence identity,

b) identifying aligned amino acid positions with a small hydrophobic or non-polar amino acid residue at position 5 or/and 8 in the fourth heavy chain framework region,

c) modifying the immunoglobulin by substituting an amino acid residue in the fourth heavy chain framework region at a position identified in b), which is threonine in the reference sequence and which is not threonine in the fourth heavy chain framework region, for the respective threonine residue as in the reference sequence,

d) cultivating a mammalian cell comprising the nucleic acid encoding the modified immunoglobulin heavy chain amino acid sequence and a nucleic acid encoding a corresponding light chain amino acid sequence for the expression of the immunoglobulin heavy and light chain,

e) recovering the immunoglobulin from the mammalian cell or the cultivation medium and thereby producing an immunoglobulin.

In one embodiment~~aspect~~ as reported herein the method is a method for producing an immunoglobulin comprising the following steps:

reference amino acid sequence and which is not threonine in the fourth heavy chain framework region amino acid sequence, for the respective threonine residue as present in the reference amino acid sequence. In another embodiment the modifying is of at least one position identified. In one embodiment the reference amino acid sequence is the amino acid sequence WGQGTLVTVSS (SEQ ID NO: 02). In one embodiment the immunoglobulin is a human or humanized immunoglobulin. In another embodiment the mammalian cell is a CHO cell or a HEK cell. In another embodiment the difference of the threonine residues at position 5 and/or 8 of the reference amino acid sequence is determined.

Another embodimentaspect as reported herein is a method for humanizing an immunoglobulin comprising the following steps:

a) aligning the amino acid sequence of the fourth heavy chain framework region of the immunoglobulin with the reference amino acid sequence xxxxTTxTxSS (SEQ ID NO: 01) or xxxxTxxTxxx (SEQ ID NO: 11) to achieve maximal level of amino acid sequence identity,

b) identifying aligned amino acid sequence positions with different amino acid residues in the fourth heavy chain framework region amino acid sequence of the immunoglobulin and the reference amino acid sequence,

c) humanizing the immunoglobulin by substituting an amino acid residue in the fourth heavy chain framework region amino acid sequence at a position identified in b) for the respective threonine or serine residue as in the reference sequence, whereby the amino acid residue at the position is threonine or serine in the reference amino acid sequence, and whereby the amino acid residue at the position is not threonine and not serine in the fourth heavy chain framework region amino acid sequence of the immunoglobulin,

d) optionally providing a nucleic acid sequence encoding the humanized immunoglobulin.

## Detailed Description of the Invention

It has been found that the exchange of two or even a single threonine and/or serine amino acid residue to a small hydrophobic or non-polar amino acid residue, such as