Title: Methods and means for the production of Ig-like molecules

## FIELD

The invention relates to the fields of molecular biology, medicine and biological therapeutics. It particularly relates to the field of therapeutic antibodies for the treatment of various diseases.

## BACKGROUND

Many currently used biological therapeutics are isolated recombinant, human or humanized monoclonal antibodies that enhance the ability of the body's immune system to neutralize or eliminate cells and/or molecules involved in disease processes or to eradicate invading pathogens or infectious agents. Monoclonal antibodies bind to a single specific area, or epitope, of an antigen and, for use in therapy, are often selected for a desirable functional property such as for example killing of tumor cells, blocking of receptor-ligand interactions or virus neutralization. Nowadays, there are about 30 FDA approved monoclonal antibodies, which are typically produced at large quantities and their biophysical and biochemical characteristics can be analyzed in great detail to ensure batch-to-batch consistency, which facilitates regulatory acceptability. Despite these favorable characteristics, monoclonal antibodies have several disadvantages, some of which relate to their monospecific nature and the complexity of diseases. Diseases processes are often multifactorial in nature, and involve redundant or synergistic action of disease mediators or up-regulation of different receptors, including crosstalk between their signaling networks. Consequently, blockade of multiple, different factors and pathways involved in pathology may result in improved therapeutic efficacy. By nature of their monospecificity, monoclonal antibodies can only interfere with a single step within the complex disease processes which often does not have an optimal effect. In addition to not fully addressing multiple aspects of a disease process, it has become clear that targeting a single epitope on a single cellular or soluble protein or pathogen often will not suffice to efficiently treat disease because the target epitope may no longer be available for the monoclonal antibody to bind to and exert the desired effect. As an example, tumor cells often escape from monoclonal antibody therapy by down-

regulation, mutation or shielding of the target epitope present on a growth factor receptor. By activating alternative receptors and/or their ligands, tumor cells than may exploit a different path leading to continued growth and metastasis. Similarly, viruses and other pathogens frequently mutate and lose or shield the target epitope, thereby escaping monoclonal antibody treatment. Monoclonal antibodies that bind to a single epitope often do not recruit the full spectrum of effector mechanisms evoked by polyclonal antibodies, including, amongst other things, opsonization (enhancing phagocytosis of antigens), steric hindrance (antigens coated with antibodies are prevented from attaching to host cells or mucosal surfaces), toxin neutralization, agglutination or precipitation (antibodies binding several soluble antigens cause aggregation and subsequent clearance), activation of complement and antibody-dependent cellular cytotoxicity (antibodies enable the killing of target cells by natural killer cells and neutrophils).

Polyclonal antibodies for therapeutic applications may be obtained from pooled human serum. Such serum-derived therapeutic polyclonal antibodies may for example be used to treat or prevent infections caused by viruses such as the rabies virus, cytomegalovirus and respiratory syncytial virus, to neutralize toxins such as tetanus toxin and botulinum toxin or to prevent Rhesus D allograft immunization. A more widespread use of serum-derived polyclonal antibody preparations has been prevented by the fact that source plasma is only available for a limited range of targets such as infectious diseases and toxins. Moreover, the products are highly dependent on donor blood availability, both in terms of quantity and suitability, resulting in considerable variation between batches. In addition, screening technologies fail to keep up with constantly evolving viruses, thus, immunoglobulin products carry a potential risk of infectious disease transmission. Finally, the long process of blood collection, screening and immunoglobulin purification means plasma-derived immunoglobulins are expensive to produce.

Mixtures of monoclonal antibodies may improve the efficacy of monoclonal antibodies while avoiding the limitations associated with serum-derived polyclonal antibodies. In the art, combinations of two human or humanized monoclonal antibodies have been tested in preclinical models and in clinical trials (for example mixtures of 2

monoclonal antibodies against the HER2 receptor, mixtures of 2 antibodies against the EGFR receptor and , 2 monoclonal antibodies against the rabies virus).
In the art, it has been shown that combinations of 2 monoclonal antibodies may have additive or synergistic effects and recruit effector mechanisms that are not associated with either antibody alone. For example, mixtures of 2 monoclonal antibodies against the EGFR or HER2 were shown to more potently kill tumor cells based on a combination of activities including enhanced receptor internalization, improved blockade of signalling pathways downstream of the receptors as well as enhanced immune effector-mediated cytotoxicity. For combination therapies based on 2 monoclonal antibodies, the component antibodies may be produced separately and combined at the protein level. A drawback of this approach is the staggering cost of developing the 2 antibodies individually in clinical trials and (partially) repeating that process with the combination. This would lead to unacceptable cost of treatments based on antibody combinations. Alternatively, the 2 recombinant cell lines producing the component monoclonal antibodies may be mixed in a fermentor and the resultant mixture of antibodies may be purified as a single preparation (WO 2004/061104). A drawback of this approach is the poor control over the composition and hence reproducibility of the resulting recombinant polyclonal antibody preparation, especially when considering that such compositions may change over time as the cells are being cultured.

During the past decade, bispecific antibodies have emerged as an alternative to the use of combinations of 2 antibodies. Whereas a combination of 2 antibodies represents a mixture of 2 different immunoglobulin molecules that bind to different epitopes on the same or different targets, in a bispecific antibody this is achieved through a single immunoglobulin molecule. By binding to 2 epitopes on the same or different targets, bispecific antibodies may have similar effects as compared to a combination of 2 antibodies binding to the same epitopes. Furthermore, since bispecific antibodies of the IgG format combine 2 different monovalent binding regions in a single molecule and mixtures of 2 IgG antibodies combine 2 different bivalent binding molecules in a single preparation, different effects of these formats have been observed as well. From a technological and regulatory perspective, this makes development of a single bispecific antibody less complex because manufacturing,

preclinical and clinical testing involve a single, molecule. Thus, therapies based on a single bispecific antibody are facilitated by a less complicated and cost-effective drug development process while providing more efficacious antibody therapies.

Bispecific antibodies based on the IgG format, consisting of 2 heavy and two light chains have been produced by a variety of methods. For instance, bispecific antibodies may be produced by fusing two antibody-secreting cell lines to create a new cell line or by expressing two antibodies in a single cell using recombinant DNA technology. These approaches yield multiple antibody species as the respective heavy chains from each antibody may form monospecific dimers (also called homodimers), which contain two identical paired heavy chains with the same specificity, and bispecific dimers (also called heterodimers) which contain two different paired heavy chains with different specificity. In addition, light chains and heavy chains from each antibody may randomly pair to form inappropriate, non-functional combinations. This problem, known as heavy and light chain miss-pairings, can be solved by choosing antibodies that share a common light chain for expression as bispecific. But even when a common light chain is used, expression of two heavy chains and one common light chain in a single cell will result in 3 different antibody species, i.e. two monospecific 'parental' antibodies and the bispecific antibody so that the bispecific antibody of interest needs to be purified from the resulting antibody mixture. Although technologies have been employed to further increase the percentage of bispecific antibodies in the mixtures of parental and bispecific antibodies and to decrease the percentage of miss-paired heavy and light chains, there remains a need for bispecific formats that eliminate or minimize some of the disadvantages mentioned above.

Taken together, the art provides a variety of technologies and methods for generating monoclonal antibodies, bispecific antibodies, mixtures of monoclonal antibodies, or mixtures of monospecific and bispecific antibodies that can subsequently be used for therapeutic application in patients. However, as discussed above, each of these existing technologies and methods have their drawbacks and limitations.
There is thus a need for improved and/or alternative technologies for producing biological therapeutics in the form of mixtures or bispecific approaches for targeting multiple disease-modifying molecules

DESCRIPTION OF THE INVENTION

The invention provides methods and means for improved and/or alternative technologies for producing biological therapeutics in the form of mixtures or bispecific approaches for targeting multiple disease-modifying molecules, as well as products and uses resulting from these methods and means.

Various approaches are described in the art in order to promote the formation of a certain bispecific antibody of interest, thereby reducing the content of undesired antibodies in the resulting mixture.

For antibodies, it is well-known that the CH3-CH3 interaction is the primary driver for Fc dimerization (Ellerson JR., et al., J. Immunol 1976 (116) 510-517; Deisenhofer J. biochemistry 1981 (20) 2361-2370). It is furthermore well-known that when two CH3 domains interact with each other they meet in a protein-protein interface which comprises "contact" residues (also called contact amino acids, interface residues or interface amino acids). Contact amino acids of a first CH3 domain interact with one or more contact amino acids of a second CH3 domain. Contact amino acids are typically within 5.5 Å (preferably within 4.5 Å) of each other in the three-dimensional structure of an antibody. The interaction between contact residues from one CH3 domain and contact residues from a different CH3 domain may for instance be via Van der Waals forces, hydrogen bonds, water-mediated hydrogen bonds, salt bridges or other electrostatic forces, attractive interactions between aromatic side chains, disulfide bonds, or other forces known to one skilled in the art. It was previously shown that approximately one-third of the contact amino acid side chains at the human IgG1 CH3 domain interface can account for the majority of contributions to domain folding and association. It can further be envisaged that other (neighbouring) amino acid residues may affect the interactions in the protein-protein interface.

Approaches to interfere with the dimerization of antibody heavy chains have been employed in the art. Specific engineering in the CH3 domains was applied in order to favour heterodimerization over homodimerization. Examples of such engineering of the CH3-CH3 interface include the introduction of complementary protuberance and

cavity mutations, also known as 'knob-into-hole' approaches as described for instance in WO1998/050431, Ridgeway et al., 1996 and Merchant et al. 1998.

Generally, the method involves introducing a protuberance at the interface of a first polypeptide and a corresponding cavity in the interface of a second polypeptide, such that the protuberance can be positioned in the cavity so as to promote heteromultimer formation and hinder homomultimer formation. "Protuberances" or "knobs" are constructed by replacing small amino acid side chains from the interface of the first polypeptide with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" or "holes" of identical or similar size to the protuberances are created in the interface of the second polypeptide by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). The protuberance and cavity can be made by synthetic means such as altering the nucleic acid encoding the polypeptides or by peptide synthesis.

Using the knob-into-hole technology alone, the proportion of a bispecific antibody of interest is at best 87% of the mixture of the 2 parental and bispecific antibodies. Merchant et al., succeeded in raising the proportion of bispecific antibodies to 95% of the mixture by introduction of an additional disulfide bond between the two CH3 domains in the CH3-CH3 interface. Still, in order to use such bispecific antibody as a medicament, the bispecific antibody has to be purified (separated) from the homodimers and formulated into a pharmaceutically acceptable diluent or excipient. Purification of heterodimers from such mixtures poses a major challenge because of the similarity in physico-chemical properties of the homodimers and heterodimers. It is one object of the present invention to provide methods for producing a bispecific antibody in a single cell clone with a further improved proportion of the bispecific antibody in the mixture. According to the invention, knob-into-hole technology can thus be used as one of the means, alone or together with other means, to achieve said further improved bispecific proportion in a mixture.

Another example of such engineering of the CH3-CH3 interface is provided by a heterodimeric Fc technology that supports the design of bispecific and asymmetric fusion proteins by devising strand-exchange engineered domain (SEED) CH3 heterodimers. These SEED CH3 heterodimers are derivatives of human IgG and IgA CH3 domains that are composed of alternating segments of human IgA and IgG

CH3 sequences which results in pairs of complementary human SEED CH3 heterodimers, the so-called SEED-bodies (Davis JH. Et al., Protein Engineering, Design & Selection 2010(23)195-202; WO2007/110205).

Yet another approach for the production of a given bispecific antibody of interest is based on electrostatic engineering of contact residues within the CH3-CH3 interface that are naturally charged, as for example described in EP01870459 or US2010/0015133, WO2007/147901, WO2010/129304, Gunasekaran et al (2010) and WO 2009/089004. These publications describe mutations in the CH3 domains of heavy chains wherein naturally occurring charged amino acid contact residues are replaced by amino acid residues of opposite charge (i.e. a charge reversal strategy). This creates an altered charge polarity across the Fc dimer interface such that co-expression of electrostatically matched Fc chains support favorable attractive interactions thereby promoting desired Fc heterodimer formation, whereas unfavorable repulsive charge interactions suppress unwanted Fc homodimer formation.

It was described that within the CH3-CH3 interface four unique charges residue pairs are involved in the domain-domain interaction. These are D356/K439', E357/K370', K392/D399' and D399/K409' (numbering according to Kabat (1991) where residues in the first chain are separated from residues in the second chain by '/' and where the prime ( ' ) indicates the residue numbering in the second chain). As the CH3-CH3 interface displays a 2-fold symmetry, each unique charge pair is represented twice in intact IgG (i.e., also K439/D356', K370/E357', D399/K392' and K409/D399' charge interactions are present in the interface). Taking advantage of this two-fold symmetry, it was demonstrated that a single charge reversion, e.g. K409D in the first chain, or D399'K in the second chain resulted in diminished homodimer formation due to repulsion of identical charges. Combining different charge reversions further enhanced this repulsive effect. It was demonstrated that expression of different CH3 domains comprising different, complementary charge reversions, could drive heterodimerization, resulting in an increased proportion of the bispecific species in the mixture.

Using the approach described above, it is possible to produce a bispecific antibody in a single cell with proportions ranging between about 76% and about 96%. It is an object of the present invention to provide methods for producing a bispecific antibody in a

single cell with a further improved percentage of desired bispecific antibodies. According to the present invention, electrostatic engineering technology can be used as one of the means, alone or together with other means, e.g knob-into-hole approaches, to achieve said further improved percentages of desired (bispecific) antibodies.

In one aspect, the present invention provides a method for producing at least two different Ig-like molecules from a single host cell, wherein each of said two Ig-like molecules comprises two CH3 domains that are capable of forming an interface, said method comprising providing in said cell

a) a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain,

b) a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,

c) a third nucleic acid molecule encoding a 3rd CH3 domain-comprising polypeptide chain, and

d) a fourth nucleic acid molecule encoding a 4th CH3 domain-comprising polypeptide chain,

wherein at least two of said nucleic acid molecules are provided with means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides and said 3rd and 4th CH3-domain comprising polypeptides, said method further comprising the step of culturing said host cell and allowing for expression of said at least four nucleic acid molecules and harvesting said at least two different Ig-like molecules from the culture.

It is often desired to produce more than one (bispecific) antibody, for instance in order to more efficiently interfere with multiple biological pathways involved in a disease process or with the invasion, replication and/or spreading of a pathogen.

A mixture of more than one bispecific antibody is also particularly useful for the treatment of certain diseases. For example, tumor cells use many different strategies to develop resistance during treatment with antibodies or small molecule drugs. Resistance may involve multiple cell surface receptors and soluble molecules and it is

considered beneficial to develop antibody-based treatments for cancers that address multiple such disease- and escape-associated molecules simultaneously. In case more than 2 such disease- and escape-related target molecules or epitopes are involved, a mixture of bispecific antibodies provides an innovative and attractive therapeutic format. Preferably, such mixtures of bispecific antibodies are produced by a single cell to facilitate a drug development process that is less complicated from a regulatory point of view and cost-effective and feasible from a drug manufacturing and clinical development point of view. In a single cell-based approach, it is desirable to use methods that allow controlled and efficient production of the bispecific antibodies, thus reducing or even completely abrogating the need of separating the desired mixture of bispecific IgG molecules from non-desired monospecific IgG molecules. In the prior art, mixtures of monospecific and bispecific antibodies have been produced by a single cell (WO2004/009618), but these mixtures represent complex concoctions of several different bispecific and monospecific antibody species. It is a further object of the present invention to provide means and methods for producing defined mixtures of bispecific antibodies in single cells. Preferably, methods are provided which result in mixtures of (bispecific) antibodies with a proportion of at least 95%, at least 97% or even more than 99% of dimeric IgG molecules, irrespective of the amount of monomeric by-products, see herein below. Typically, in a cell where multiple intact IgG molecules are produced, half molecules (monomeric by-products) may be present that can be simply removed by size exclusion chromatography known in the art.

In one embodiment the present invention provides methods for producing a defined mixture of at least two different Ig-like molecules in single cells, instead of a single (bispecific) antibody of interest, wherein the formation of other, undesired dimeric antibody species is diminished or even absent. The resulting mixture is well defined and its composition is controlled by the design of CH3 domain mutants. Furthermore, regulation of expression levels and/or different transfection ratios used for expression affects the composition of the mixture. In a method according to the invention, a first nucleic acid molecule encodes a CH3 domain which preferentially pairs with a CH3 domain encoded by a second nucleic acid molecule, and a third nucleic acid molecules encodes a CH3 domain which preferentially pairs with a CH3 domain encoded by a

fourth nucleic acid molecule. The present invention also provides mixtures of at least two different Ig-like molecules obtainable by the methods of the invention.

As used herein, the term "preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides" means that essentially all the resulting dimers comprising the 1st CH3 domain-comprising polypeptide and/or the 2nd CH3 domain-comprising polypeptide will be dimers consisting of one 1st CH3 domain-comprising polypeptide paired with one 2nd CH3 domain-comprising polypeptide. Likewise, the term "preferential pairing of said 3rd and 4th CH3 domain-comprising polypeptides" means that essentially all of the resulting dimers comprising the 3rd CH3 domain-comprising polypeptide and/or the 4th CH3 domain-comprising polypeptide will be dimers consisting of one 3rd CH3 domain-comprising polypeptide paired with one 4th CH3 domain-comprising polypeptide. As a result, when nucleic acid molecules encoding four different (A, B, C, D) CH3 domain-comprising polypeptides are introduced in a single cell, instead of a mixture of 10 different Ig-like dimers (AA, AB, AC, AD, BB, BC, BD, CC, CD and DD), a mixture of predominantly two specific Ig-like molecules is produced.

As explained herein below in more detail, in a preferred embodiment said first CH3-domain comprising polypeptide chain comprises the amino acid substitution T366K, and said second CH3-domain comprising polypeptide chain comprises the amino acid substitution L351D. These amino acid changes are preferred means for preferential pairing of said first and second CH3-domain comprising polypeptide chains. Said first CH3-domain comprising polypeptide chain preferably further comprises the amino acid substitution L351K. Moreover, said second CH3-domain comprising polypeptide chain preferably further comprises an amino acid substitution selected from the group consisting of Y349E, Y349D and L368E, most preferably L368E. In yet another preferred embodiment, said third CH3-domain comprising polypeptide chain comprises the amino acid substitutions E356K and D399K, and said fourth CH3-domain comprising polypeptide chain comprises the amino acid substitutions K392D and K409D.

In a method according to the present invention, each of the CH3-domain comprising polypeptide chains preferably further comprises a variable region recognizing a target epitope. The variable regions that are part of the CH3-domain comprising polypeptide

chains preferably share a common light chain. In that case only the VHs of the variable regions differ whereas the VL in all variable regions is essentially the same. Hence, in a preferred aspect a method according to the invention is provided, which further comprises providing said host cell with a nucleic acid molecule encoding a common light chain. In one particularly preferred embodiment, each of said 4 variable regions of the 4 CH3-domain comprising polypeptide chains recognizes a different target epitope. For instance, if the first nucleic acid molecule encodes a heavy chain that further contains a variable domain with specificity for antigen A, the second nucleic acid molecule encodes a heavy chain that further contains a variable domain with specificity for antigen B, the third nucleic acid molecule encodes a heavy chain that further contains a variable domain with specificity for antigen C, and the fourth nucleic acid molecule encodes a heavy chain that further contains a variable domain with specificity for antigen D, a mixture will then be produced containing bispecific Ig-like molecules that are specific for AB and bispecific Ig-like molecules that are specific for CD. The formation of monospecific antibodies (with AA, BB, CC or DD specificity) or bispecific antibodies with specificity for AC, AD, BC or BD is lowered or even absent due to the means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides and said 3rd and 4th CH3 domain-comprising polypeptides. It is, of course, possible to use further nucleic acid molecules, for instance encoding a 5th and a 6th CH3 domain-comprising polypeptide, in order to produce defined mixtures comprising more than two different Ig-like molecules.

Of note, the ratio of the nucleic acids used in a method according to the invention does not need to be 1:1:1:1 and the ratio of the resulting Ig-like molecules that are expressed does not need to be 1:1. It is possible to use means known in the art to produce mixtures of antibodies with optimized ratios. For instance, expression levels of nucleic acid molecules and hence the ratios of the resulting Ig-like molecules produced may be regulated by using different genetic elements such as promoters, enhancers and repressors or by controlling the genomic integration site of copy number of the DNA constructs encoding antibodies.

Said means for preferential pairing preferably may comprise engineered complementary knob-into-hole mutations, disulfide bridges, charge mutations including charge reversal mutations, or combinations thereof. The skilled person will appreciate that said means for preferential pairing may be chosen within a certain

type of mutations, i.e. all at least 4 nucleic acid molecules encoding CH3-domain comprising polypeptide chains may for example comprise charge mutations as means for preferential pairing. Additionally, also non-engineered wildtype CH3 may in certain instances be used for preferential pairing of two wildtype CH3-domain comprising polypeptide chains. In a particularly preferred embodiment, said means for preferential pairing comprise at least one CH3 mutation selected from Table B, as explained elsewhere in this application. One preferred embodiment thus provides a method according to the present invention, wherein all 4 of said nucleic acid molecules are provided with means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides and said 3rd and 4th CH3-domain comprising polypeptides, wherein said means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides are different from those means for preferential pairing of said 3rd and 4th CH3-domain comprising polypeptides.

One aspect of the present invention provides a method according to the invention, wherein said means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides are different from said means for preferential pairing of said 3rd and 4th CH3-domain comprising polypeptides. By 'different' it is meant that the means for preferential pairing of said 1st and 2nd CH3 domain comprising polypeptides are designed such that preferential pairing of the 1st and 2nd chain is favoured. The design is such that essentially no interaction between the 1st and the 3rd and/or 4th CH3 domain comprising polypeptide chain will take place. In other words, dimerization between said 1st CH3 domain comprising polypeptide and said 3rd or 4th polypeptide is reduced to essentially zero and so forth. The 3rd and the 4th CH3 domain-comprising polypeptides may either be wildtype or may comprise means for preferential pairing that are different from the means for preferential pairing of the 1st and 2nd CH3 domains. Current studies have focused on the production of a single bispecific antibody, using for instance the knob-into-hole technology or mutations (reversions) of charged contact amino acids present in CH3 domains. Production of defined mixtures of at least two (bispecific) Ig-like molecules, without significant co-production of other dimeric by-products, has, however, not been realized prior to the present invention.

The present invention provides methods for the efficient and controlled production of a well-defined mixture of Ig-like molecules, with a high proportion of bispecifics in the mixture. Even a proportion of (two) bispecifics of at least 95%, at least 97% or more is obtained in a system where two bispecifics are desired. This means that only at most 5%, at most 3% or less monospecific bivalent by-products are obtained. Of note, the amount of monomeric by-products, i.e. half molecules, is less important since these half-molecules are easily separated from dimers using their size difference.

In another preferred embodiment, the variable regions of the 1st and the 2nd CH3-domain comprising polypeptide chains recognize different target epitopes, whereas the variable regions of the 3rd and the 4th CH3-domain comprising polypeptide chains recognize the same target epitopes. This will result in the predominant production of one kind of bispecific Ig-like molecule and one kind of monospecific Ig-like molecule. For instance, if the variable regions of the 1st and the 2nd CH3-domain comprising polypeptide chains recognize different target epitopes and if the variable regions of the 3rd and the 4th CH3-domain comprising polypeptide chains both recognize the same target epitope which is different from the target epitopes recognized by the 1st and the 2nd CH3-domains, a mixture of Ig-like molecules having specificity for AB or CC will be formed. Further provided is therefore a method according to the invention, wherein the target epitope recognized by the variable regions of the 3rd and 4th CH3 domain comprising polypeptide chain is the same, but different from the target epitope recognized by the variable region of the 1st or the 2nd CH3-domain comprising polypeptide chain.

Alternatively, when the variable regions of the 1st and the 2nd CH3-domain comprising polypeptide chains recognize different target epitopes and when the variable regions of the 3rd and the 4th CH3-domain comprising polypeptide chains both recognize the same epitope as the 1st or the 2nd CH3-domain comprising polypeptide chains, a mixture of Ig-like molecules having specificity for AB and AA, or AB and BB will be formed. A method according to the invention, wherein the target epitope recognized by the variable regions of the 3rd and 4th CH3 domain comprising polypeptide chain is the same as the target epitope recognized by the variable region of the 1st or the 2nd CH3-domain comprising polypeptide chain is therefore also herewith provided.

It is another object of the present invention to provide means and methods for producing defined mixtures of bispecific antibodies and monospecific antibodies in a single cell culture. A non-limiting example of such well-defined mixture is a mixture of bispecific antibodies with specificity AB and monospecific antibodies with specificity AA. Another example is a mixture of bispecific antibodies with specificity AB and monospecific antibodies with specificity BB. Yet another example is a mixture of bispecific antibodies with specificity AB and monospecific antibodies with specificity CC. Again, preferably means and methods are provided which yield mixtures of antibodies of interest with at least 90%, more preferably at least 95% and most preferably at least 97% or even more than 99% of desired antibodies.

In yet another embodiment, a method according to the invention is provided wherein the variable regions of the 1st and the 2nd CH3-domain comprising polypeptide chains recognize the same target epitope, whereas the variable regions of the 3rd and the 4th CH3-domain comprising polypeptide chains recognize a second target epitope which differs from the target epitope recognized by said 1st and 2nd variable regions. This will result in the predominant production of monospecific Ig-like molecules having either specificity for AA or specificity for BB. The formation of bispecific Ig-like molecules is diminished or even avoided. In several embodiments it is preferred to produce mixtures of monospecific antibodies in a single cell, rather than mixtures of bispecific antibodies. For instance when cross-linking of two identical target molecules is desired, or when two targets are located too far away from each other so that they cannot be bound by a single bispecific antibody. It can also be advantageous to produce mixtures of monospecific antibodies in a single cell as the mixture can be regarded as a single therapeutic product. In the art, the therapeutic efficacy and safety of various monospecific antibodies has already been proven and market authorisation has been obtained. Production of mixtures of monospecific antibodies in a single cell will thus facilitate the testing for efficacy and safety of several of such mixtures and will reduce the efforts and costs for regulatory approval and manufacturing. There are, however, currently no methods available for producing specific mixtures of monospecific antibodies in a single cell wherein the formation of bispecific by-products is reduced to below 5%. It is another object of the present invention to provide means and methods for producing such well-defined homodimeric

antibody mixtures in single cells wherein the formation of bispecific antibodies is reduced to below 5%.

Hence, a method according to the present invention is suitable for the production of any desired mixture of bispecific and/or monospecific Ig-like molecules. Again, it is possible to use further nucleic acid molecules, for instance encoding a 5th and a 6th (and 7th and 8th and so forth) CH3 domain-comprising polypeptide, in order to produce defined mixtures comprising more than two different Ig-like molecules.

Preferably, in a method according to the present invention at least two CH3 domains are used that comprise at least one combination of mutations provided by the present invention. Through these mutations novel specific interactions are formed between two CH3 domains. These mutations according to the present invention are discussed below in more detail.

The term 'Ig-like molecule' as used herein means a proteinaceous molecule that possesses at least one immunoglobulin (Ig) domain. Said Ig-like molecule comprises a sequence comprising the function of at least an immunoglobulin CH3 domain, preferably the sequence comprises an IgG1 CH3 domain. Proteinaceous molecules that possess at least a CH3 domain can be further equipped with specific binding moieties. The CH3 domains of the present invention, containing means for preferential pairing, can thus be used for preferential pairing of two CH3-domain comprising proteinaceous molecules to design desired heterodimeric binding molecules or mixtures of binding molecules. Binding moieties that can be engineered to the CH3-domain comprising proteinaceous molecules can be any binding agent, including, but not limited to, single chain Fvs, single chain or Tandem diabodies (TandAb®), VHHs, Anticalins®, Nanobodies®, a BiTE®, a Fab, ankyrin repeat proteins or DARPINs®, Avimers®, a DART, a TCR-like antibody, Adnectins®, Affilins®, Trans-bodies®, Affibodies®, a TrimerX®, MicroProteins, Fynomers®, Centyrins® or a KALBITOR®. In a preferred embodiment, the binding moieties are antibody variable regions (i.e. VH/VL combinations). Variable regions that are part of the CH3-domain comprising polypeptide chains preferably share a common light

chain. In that case, only the VHs of the variable regions differ whereas the VL in all variable regions is essentially the same.

Alternatively, or in addition, other molecules can be engineered to the CH3 domains of the present invention, including cytokines, hormones, soluble ligands, receptors and/or peptides.

In a more preferred embodiment, said Ig-like molecule comprises a full length Fc backbone. In a most preferred embodiment, the Ig-like molecules are antibodies. The variable regions of these antibodies preferably share a common light chain, but they may differ in their VH regions. The term 'antibody' as used herein means a proteinaceous molecule belonging to the immunoglobulin class of proteins, containing one or more domains that bind an epitope on an antigen, where such domains are derived from or share sequence homology with the variable region of an antibody. Antibodies are known in the art and include several isotypes, such as IgG1, IgG2, IgG3, IgG4, IgA, IgD, IgE, and IgM. An antibody according to the invention may be any of these isotypes, or a functional derivative and/or fragment of these. In a preferred embodiment, Ig-like molecules are produced that are antibodies of the IgG isotype because IgG antibodies e.g. have a longer half life as compared to antibodies of other isotypes.

Antibodies produced with methods according to the present invention can have sequences of any origin, including murine and human sequences. Antibodies can consist of sequences from one origin only, such as fully human antibodies, or they can have sequences of more than one origin, resulting for instance in chimeric or humanized antibodies. Antibodies for therapeutic use are preferably as close to natural antibodies of the subject to be treated as possible (for instance human antibodies for human subjects). Antibody binding can be expressed in terms of specificity and affinity. The specificity determines which antigen or epitope thereof is bound by the binding domain. The affinity is a measure for the strength of binding to a particular antigen or epitope. Specific binding is defined as binding with affinities ($K_D$) of at least $1x10^{-5}$ M, more preferably $1x10^{-7}$ M, more preferably higher than $1x10^{-9}$M. Typically, monoclonal antibodies for therapeutic applications have affinities of up to $1x10^{-10}$ M or even higher. The term 'antigen' as used herein means a substance or molecule that, when introduced into the body, triggers the production of an antibody by the immune system. An antigen, among others, may be derived from pathogenic

organisms, tumor cells or other aberrant cells, from haptens, or even from self structures. At the molecular level, an antigen is characterized by its ability to be bound by the antigen-binding site of an antibody. Also mixtures of antigens can be regarded as 'antigen', i.e. the skilled person would appreciate that sometimes a lysate of tumor cells, or viral particles may be indicated as 'antigen' whereas such tumor cell lysate or viral particle preparation exists of many antigenic determinants. An antigen comprises at least one, but often more, epitopes. The term 'epitope' as used herein means a part of an antigen that is recognized by the immune system, specifically by antibodies, B cells, or T cells. Although epitopes are usually thought to be derived from non-self proteins, sequences derived from the host that can be recognized are also classified as epitopes.

The term 'CH3 domain' is well known in the art. The IgG structure has four chains, two light and two heavy chains; each light chain has two domains, the variable and the constant light chain (VL and CL) and each heavy chain has four domains, the variable heavy chain (VH) and three constant heavy chain domains (CH1, CH2, CH3). The CH2 and CH3 domain region of the heavy chain is called Fc (Fragment crystallizable) portion, Fc fragment, Fc backbone or simply Fc. The IgG molecule is a heterotetramer having two heavy chains that are held together by disulfide bonds (-S-S-) at the hinge region and two light chains. The heavy chains dimerize through interactions at the CH3-CH3 domain interface and through interactions at the hinge region. The number of hinge disulfide bonds varies among the immunoglobulin subclasses (Papadea and Check 1989). The Fc fragment of an immunoglobulin molecule is a dimer of the two C-terminal constant regions, i.e. CH2 and CH3 domains, of the heavy chain. Among its physiological functions are interactions with the complement system and with specific receptors on the surface of a variety of cells. Interactions between the CH3 domains of two individual heavy chains are known to play an important role in driving heavy chain dimerization. Thus, CH3 domains direct the association of antibody heavy chains, and it is known that the interface between CH3 domains contains more than 20 contact residues from each chain that play a role in the CH3-CH3 interaction (Deisenhofer J., Biochemistry 1981(20)2361-2370; Miller S., J. Mol. Biol. 1990(216)965-973; Padlan, Advances in Protein Chemistry 1996 (49) 57-133). The CH3 variants of the present invention can thus be used in association

with other antibody domains to generate full length antibodies that are either bispecific or monospecific. The specificity of the antibody as defined by the VH/VL combinations typically does not affect the heavy chain dimerization behaviour that is driven by the CH3 domains.

The terms 'contact residue', 'contact amino acid', 'interface residue' and 'interface amino acid' as used herein typically refers to any amino acid residue present in the CH3 domain that can be involved in interdomain contacts, as can be calculated by technologies known in the art, including calculating solvent accessible surface area (ASA) of the CH3 domain residues in the presence and absence of the second chain (Lee and Richards J. Mol. Biol.1971(55)379) where residues that show difference (> lÅ$^2$) in ASA between the two calculations are identified as contact residues. Contact residues that have been identified include residues at positions 347, 349, 350, 351, 352, 353, 354, 355, 356, 357, 360, 364, 366, 368, 370, 390, 392, 394, 395, 397, 399, 400, 405, 407, 409, 439 according to the EU numbering system (Table A).

Table A: List of CH3 domain interface residues

| **Interface residue in chain A** | **Contacting residues in chain B** |
|---|---|
| Q347 | K360 |
| Y349 | S354, D356, E357, K360 |
| T350 | S354, R355 |
| L351 | L351, P352, P353, S354, T366 |
| S354 | Y349, T350, L351 |
| R355 | T350 |
| D356 | Y349, K439 |
| E357 | Y349, K370 |
| K360 | Q347, Y349 |
| S364 | L368, K370 |
| T366 | L351, Y407 |
| L368 | S364, K409 |
| K370 | E357, S364 |
| N390 | S400 |
| K392 | L398, D399, S400, F405 |
| T394 | T394, V397, F405, Y407 |
| P395 | V397 |
| V397 | T394, P395 |
| D399 | K392, K409 |
| S400 | N390, K392 |
| F405 | K392, T394, K409 |
| Y407 | T366, T394, Y407, K409 |
| K409 | L368, D399, F405, Y407 |
| K439 | D356 |

Contact residues within the CH3-CH3 interface can either be amino acids that are charged, or amino acid residues that are neutral. The term 'charged amino acid residue' or 'charged residue' as used herein means amino acid residues with electrically charged side chains. These can either be positively charged side chains, such as present in arginine (Arg, R), histidine (His, H) and lysine (Lys, K) or can be negatively charged side chains, such as present in aspartic acid (Asp, D) and glutamic acid (Glu, E). The term 'neutral amino acid residue' or neutral residue as used herein refers to all other amino acids that do not carry electrically charged side chains. These neutral residues include serine (Ser, S), threonine (Thr, T), asparagine (Asn, N), glutamine (GLu, Q), Cysteine (Cys, C), glycine (Gly, G), proline (Pro, P), alanine (Ala, A), valine (Val, V), isoleucine (Ile, I), leucine (Leu, L), methionine (Met, M), phenylalanine (Phe, F), tyrosine (Tyr, Y), and tryptophan (Trp, T).

The term 'CH3-CH3 domain interface', or 'CH3 interface', 'CH3-CH3 pairing', 'domain interface' or simply 'interface', as used herein, refers to the association between two

CH3 domains of separate CH3-domain comprising polypeptides that is a result of interacting amino acid residues, i.e. at least one interaction between an amino acid of a first CH3 domain and an amino acid of a second CH3 domain. Such interaction is for instance via Van der Waals forces, hydrogen bonds, water-mediated hydrogen bonds, salt bridges or other electrostatic forces, attractive interactions between aromatic side chains, the formation of disulfide bonds, or other forces known to one skilled in the art.

As used herein, said means for preferential pairing of the first and second CH3 domain-comprising polypeptides and said third and fourth CH3 domain-comprising polypeptide can be any means known in the art. In one embodiment, at least one nucleic acid molecule encodes a CH3 domain which contains at a contact residue position a large amino acid residue (i.e. a "knob" or "protuberance") such as for instance R, F, Y, W, I or L, whereas at least one other nucleic acid molecule encodes a CH3 domain which contains at a complementary contact residue position a small amino acid residue (i.e. a "hole" or "cavity") such as for instance G, A, S, T or V. The resulting CH3 domains will preferentially pair with each other due to the steric conformation of said contact amino acids. The knob-into-hole technology is described herein before in more detail. In a further embodiment of the present invention, at least one nucleic acid molecule encodes a CH3 domain which contains at a contact residue position that is naturally charged, i.e. a naturally occurring K, H, R, D or E, an amino acid that now carries the opposite charge as compared to wildtype, whereas at least one other nucleic acid molecule encodes a CH3 domain which contains at a complementary contact residue position that is naturally charged, an amino acid that now carries the opposite charge as compared to wildtype. The resulting engineered CH3 domains will preferentially pair with each other due to the opposite charges of said contact amino acids, whereas pairing of identical CH3 domains will be diminished due to electrostatic repulsion. In one embodiment, CH3 mutations as described in EP01870459, WO 2009/089004, Gunasekaran et al (2010), are used. In one embodiment, the means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides are "knob" and "hole" amino acid residues and the means for preferential pairing of said 3th and 4th CH3 domain-comprising polypeptides are charge-engineered amino acids. Preferably, both said means for preferential pairing of

said 1st and 2nd CH3 domain-comprising polypeptides and said 3th and 4th CH3 domain-comprising polypeptides are charge-engineered amino acids. In one embodiment, different amino acid residues are engineered for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides as compared to the amino acid residues that are engineered for preferential pairing of said 3th and 4th CH3 domain-comprising polypeptides. In a particularly preferred embodiment at least a first and a second nucleic acid molecule encode CH3 domains with novel mutations as provided by the present invention. As described herein below in more detail, the present invention provides novel CH3 mutations which enable the production of certain bispecific Ig-like molecules of interest without a significant amount of undesired (dimeric) by-products. The present invention also provides novel CH3 mutations which enable the production of certain monospecific Ig-like molecules of interest without a significant amount of undesired (dimeric) by-products. The use of at least one of these CH3 mutations according to the present invention is, therefore, preferred.

The term 'polypeptide', 'polypeptide molecule' or 'polypeptide chain' as used herein refers to a chain of amino acids that are covalently joined together through peptide bonds. Proteins are typically made up of one or more polypeptide molecules. One end of every polypeptide, called the amino terminal or N-terminal, has a free amino group. The other end, with its free carboxyl group, is called the carboxyl terminal or C-terminal. Polypeptides according to the present invention may have gone through post-translational modification processes and may e.g. be glycosylated. The CH3 domain-comprising polypeptide chains of the present invention thus refer to polypeptide chains that at least encompass an Ig CH3 domain and that may have gone through post-translational modification processes.

The term "nucleic acid molecule" as used herein is defined as a molecule comprising a chain of nucleotides, more preferably DNA and/or RNA. In one embodiment, double-stranded RNA is used. In other embodiments a nucleic acid molecule of the invention comprises other kinds of nucleic acid structures such as for instance a DNA/RNA helix, peptide nucleic acid (PNA), locked nucleic acid (LNA) and/or a ribozyme. Hence, the term "nucleic acid molecule" also encompasses a chain comprising non-natural

nucleotides, modified nucleotides and/or non-nucleotide building blocks which exhibit the same function as natural nucleotides.

The present invention further provides a method for making a host cell for production of at least two different Ig-like molecules, the method comprising the step of introducing into said host cell nucleic acid sequences encoding at least a first, a second, a third and a fourth CH3-domain comprising polypeptide chain, wherein at least two of said nucleic acid sequences are provided with means for preferential pairing of said first and second CH3-domain comprising polypeptides and said third and fourth CH3-domain comprising polypeptides, wherein said nucleic acid sequences are introduced consecutively or concomitantly.

It is a further aspect of the present invention to provide a method for making a host cell for production of a heterodimeric Ig-like molecule, the method comprising the step of introducing into said host cell nucleic acid sequences encoding at least a first and a second CH3-domain comprising polypeptide chain, wherein said first CH3 domain-comprising polypeptide chain comprises at least one substitution of a neutral amino acid residue by a positively charged amino acid residue and wherein said second CH3 domain-comprising polypeptide chain comprises at least one substitution of a neutral amino acid residue by a negatively charged amino acid residue, wherein said nucleic acid sequences are introduced consecutively or concomitantly. Said methods for making said host cells preferably further comprise the step of introducing into said host cell a nucleic acid sequence encoding a common light chain.

Also provided herein is a recombinant host cell comprising nucleic acid sequences encoding at least a first, a second, a third and a fourth CH3-domain comprising polypeptide chain, wherein at least two of said nucleic acid molecules are provided with means for preferential pairing of said first and second CH3-domain comprising polypeptides and said third and fourth CH3-domain comprising polypeptides.

The invention furthermore provides a recombinant host cell comprising nucleic acid sequences encoding at least a first and a second CH3-domain comprising polypeptide chain, wherein said first CH3 domain-comprising polypeptide chain comprises at least one substitution of a neutral amino acid residue by a positively charged amino acid residue and wherein said second CH3 domain-comprising polypeptide chain comprises

at least one substitution of a neutral amino acid residue by a negatively charged amino acid residue.

A recombinant host cell according to the invention preferably further comprises a nucleic acid sequence encoding a common light chain.

A "host cell" according to the invention may be any host cell capable of expressing recombinant DNA molecules, including bacteria such as for instance *Escherichia* (e.g. *E. coli*), *Enterobacter, Salmonalla, Bacillus, Pseudomonas, Streptomyces*, yeasts such as *S. cerevisiae, K. lactis, P. pastoris, Candida*, or *Yarrowia*, filamentous fungi such as *Neurospora, Aspergillus oryzae, Aspergillus nidulans* and *Aspergillus niger*, insect cells such as *Spodoptera frugiperda* SF-9 or SF-21 cells, and preferably mammalian cells such as Chinese hamster ovary (CHO) cells, BHK cells, mouse cells including SP2/0 cells and NS-0 myeloma cells, primate cells such as COS and Vero cells, MDCK cells, BRL 3A cells, hybridomas, tumor-cells, immortalized primary cells, human cells such as W138, HepG2, HeLa, HEK293, HT1080 or embryonic retina cells such as PER. C6, and the like. Often, the expression system of choice will involve a mammalian cell expression vector and host so that the antibodies can be appropriately glycosylated. A human cell line, preferably PER.C6, can advantageously be used to obtain antibodies with a completely human glycosylation pattern. The conditions for growing or multiplying cells (see e. g. Tissue Culture, Academic Press, Kruse and Paterson, editors (1973)) and the conditions for expression of the recombinant product may differ somewhat, and optimization of the process is usually performed to increase the product proportions and/or growth of the cells with respect to each other, according to methods generally known to the person skilled in the art. In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach (M. Butler, ed., IRL Press, 1991). Expression of antibodies in recombinant host cells has been extensively described in the art (see e.g. EP0120694; EP0314161; EP0481790; EP0523949; US patent 4,816,567; WO 00/63403). The nucleic acid molecules encoding the light and heavy chains may be present as extrachromosomal copies and/or stably integrated into the chromosome of the host cell, the latter is preferred.

It is a further aspect of the present invention to provide a culture of recombinant host cells according to the invention, or a culture of recombinant host cells obtainable or obtained by a method according to the invention, said culture either producing at least two different Ig-like molecules or a heterodimeric Ig-like molecule.

To obtain expression of nucleic acid sequences encoding the CH3 domain-comprising polypeptides, it is well known to those skilled in the art that sequences capable of driving such expression can be functionally linked to the nucleic acid sequences encoding the CH3 domain-comprising polypeptides. Functionally linked is meant to describe that the nucleic acid sequences encoding the CH3 domain-comprising polypeptides or precursors thereof is linked to the sequences capable of driving expression such that these sequences can drive expression of the CH3 domain-comprising polypeptides or precursors thereof. Useful expression vectors are available in the art, e.g. the pcDNA vector series of Invitrogen. Where the sequence encoding the polypeptide of interest is properly inserted with reference to sequences governing the transcription and translation of the encoded polypeptide, the resulting expression cassette is useful to produce the polypeptide of interest, referred to as expression. Sequences driving expression may include promoters, enhancers and the like, and combinations thereof. These should be capable of functioning in the host cell, thereby driving expression of the nucleic acid sequences that are functionally linked to them. Promoters can be constitutive or regulated, and can be obtained from various sources, including viruses, prokaryotic, or eukaryotic sources, or artificially designed. Expression of nucleic acids of interest may be from the natural promoter or derivative thereof or from an entirely heterologous promoter. Some well-known and much used promoters for expression in eukaryotic cells comprise promoters derived from viruses, such as adenovirus, e.g. the E1A promoter, promoters derived from cytomegalovirus (CMV), such as the CMV immediate early (IE) promoter, promoters derived from Simian Virus 40 (SV40), and the like. Suitable promoters can also be derived from eukaryotic cells, such as methallothionein (MT) promoters, elongation factor 1α (EF-1α) promoter, actin promoter, an immunoglobulin promoter, heat shock promoters, and the like. Any promoter or enhancer/promoter capable of driving expression of the sequence of interest in the host cell is suitable in the invention. In one embodiment the sequence capable of driving expression comprises a region from a CMV promoter,

preferably the region comprising nucleotides –735 to +95 of the CMV immediate early gene enhancer/promoter. The skilled artisan will be aware that the expression sequences used in the invention may suitably be combined with elements that can stabilize or enhance expression, such as insulators, matrix attachment regions, STAR elements (WO 03/004704), and the like. This may enhance the stability and/or levels of expression.

Protein production in recombinant host cells has been extensively described, e.g. in Current Protocols in Protein Science, 1995, Coligan JE, Dunn BM, Ploegh HL, Speicher DW, Wingfield PT, ISBN 0-471-11184-8; Bendig, 1988. Culturing a cell is done to enable it to metabolize, and/or grow and/or divide and/or produce recombinant proteins of interest. This can be accomplished by methods well known to persons skilled in the art, and includes but is not limited to providing nutrients for the cell. The methods comprise growth adhering to surfaces, growth in suspension, or combinations thereof. Several culturing conditions can be optimized by methods well known in the art to optimize protein production yields. Culturing can be done for instance in dishes, roller bottles or in bioreactors, using batch, fed-batch, continuous systems, hollow fiber, and the like. In order to achieve large scale (continuous) production of recombinant proteins through cell culture it is preferred in the art to have cells capable of growing in suspension, and it is preferred to have cells capable of being cultured in the absence of animal- or human-derived serum or animal- or human-derived serum components. Thus purification is easier and safety is enhanced due to the absence of additional animal or human proteins derived from the culture medium, while the system is also very reliable as synthetic media are the best in reproducibility.

Ig-like molecules are expressed in host cells and are harvested from the cells or, preferably, from the cell culture medium by methods that are generally known to the person skilled in the art. After harvesting, these Ig-like molecules may be purified by using methods known in the art. Such methods may include precipitation, centrifugation, filtration, size-exclusion chromatography, affinity chromatography, cation- and/or anion-exchange chromatography, hydrophobic interaction chromatography, and the like. For a mixture of antibodies comprising IgG molecules,

protein A or protein G affinity chromatography can be suitably used (see e.g. US patents 4,801,687 and 5,151,504).

Ig-like molecules, and/or mixtures thereof, produced with methods according to the present invention preferably have a common light chain. Further provided is, therefore, a method according to the invention, further comprising providing said host cell with a nucleic acid molecule encoding a common light chain. This is a light chain that is capable of pairing with at least two different heavy chains, thereby forming functional antigen binding domains. A functional antigen binding domain is capable of specifically binding to an antigen. Preferably, a common light chain is used that is capable of pairing with all heavy chains produced with a method according to the invention, thereby forming functional antigen binding domains, so that mispairing of unmatched heavy and light chains is avoided. In one aspect, only common light chains with one identical amino acid sequence are used. Alternatively, those of skill in the art will recognize that "common" also refers to functional equivalents of the light chain of which the amino acid sequence is not identical. Many variants of said light chain exist wherein mutations (deletions, substitutions, additions) are present that do not materially influence the formation of functional binding regions. Such variants are thus also capable of binding different heavy chains and forming functional antigen binding domains. The term 'common light chain' as used herein thus refers to light chains which may be identical or have some amino acid sequence differences while retaining the binding specificity of the resulting antibody after pairing with a heavy chain. It is for instance possible to prepare or find light chains that are not identical but still functionally equivalent, e.g. by introducing and testing conservative amino acid changes, and/or changes of amino acids in regions that do not or only partly contribute to binding specificity when paired with the heavy chain, and the like. A combination of a certain common light chain and such functionally equivalent variants is encompassed within the term "common light chain". Reference is made to WO 2004/009618 for a detailed description of the use of common light chains. Preferably, a common light chain is used in the present invention which is a germline-like light chain, more preferably a germline light chain, preferably a rearranged germline human kappa light chain, most preferably either the rearranged germline human kappa light chain IgVκ1-39/Jκ or IGVκ3-20/Jκ.

Alternatively, the skilled person may select, as an alternative to using a common light chain and to avoid mispairing of unmatched heavy and light chains, means for forced pairing of the heavy and light chain, such as for example described in WO2009/080251, WO2009/080252 and/or WO2009/080253.

The present invention provides novel engineered CH3 domains as well as novel combinations of CH3 mutations. Before the present invention, charged contact amino acids of CH3 domains that were known to be involved in CH3-CH3 pairing were substituted by amino acids of opposite charge (charge reversal), thereby influencing the CH3-CH3 pairing. The mutations according to the present invention are an inventive alternative to this approach, because now CH3 amino acids that are non-charged or neutral in wildtype CH3 are substituted with charged residues. The present invention in this embodiment does not exchange charged contact amino acids by amino acids of opposite charge but substitutes non-charged CH3 amino acids for charged ones. The approach of the present invention provides not only a method for efficiently steering the dimerization of CH3 domains but also has the advantage that at least one additional charge-charge interaction in the CH3 interface is created. In view of this additional charge-charge interaction on top of the existing charge-pairs in the CH3-CH3 interface, the dimers according to the invention are generally more stable as compared to the wild type dimers (the wild type dimer is defined as a bispecific IgG (AB) without CH3 engineering in contrast to its parental homodimers (AA or BB)). Moreover, it has surprisingly become possible to increase the proportion of one or more Ig-like molecules of interest in a mixture even further. As described herein before, methods known in the art for preferential production of a bispecific antibody typically involves the production of some undesired dimeric side products. For instance, the proportion of a bispecific antibody of interest using the knob-into-hole technology is at best 87%, whereas the electrostatic engineering approach wherein charged contact amino acids are substituted by amino acids of opposite charge, also results in proportions of up to 96% (see for instance Example 11). Quite surprisingly, the present inventors have succeeded in introducing mutations that further enhance the proportion of an Ig-like molecule of interest in a mixture. For instance, Example 17 discloses a method using mutations according to the present invention, wherein the proportion of a bispecific antibody of interest was raised to

such extent that no dimeric by-product was detectable in the resulting mixture at all. Unpaired half-molecules consisting of only a single heavy chain paired with a common light chain were present to some extent in the mixtures, but these are the result of unbalanced expression of the heavy chains and can be easily separated from the mixture by size exclusion chromatography. Hence, with such mutations according to the present invention, a bispecific Ig-like molecule can be produced in a single cell with a high proportion with essentially no contaminating dimeric by-products being present, which is particularly suitable for the production of a pharmaceutical composition.

One preferred embodiment of the present invention therefore provides a method for producing a heterodimeric Ig-like molecule from a single cell, wherein said Ig-like molecule comprises two CH3 domains that are capable of forming an interface, said method comprising providing in said cell

a. A first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain,

b. A second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,

wherein said first CH3 domain-comprising polypeptide chain comprises at least one substitution of a neutral amino acid residue by a positively charged amino acid residue and wherein said second CH3 domain-comprising polypeptide chain comprises at least one substitution of a neutral amino acid residue by a negatively charged amino acid residue, said method further comprising the step of culturing said host cell and allowing for expression of said two nucleic acid molecules and harvesting said heterodimeric Ig-like molecule from the culture.

Said method preferably further comprises the step of providing said host cell with a nucleic acid molecule encoding a common light chain, which has advantages as outlined herein before.

The amino acids at position 366 of one CH3 domain and position 351 of a second CH3 domain have been reported to be a pair of contact residues in the CH3-CH3 interface, meaning that they are located sufficiently close to each other in the three-dimensional conformation of the resulting Ig-like molecule in order to be capable of interacting

with each other. Hence, the first CH3 domain will preferentially pair with the second CH3 domain.

In one embodiment, threonine (T) at position 366 of a first CH3 domain is replaced by a first charged amino acid and leucine (L) at position 351 of a second CH3 domain is replaced by a second charged amino acid, wherein said first and second charged amino acids are of opposite charge. If the first CH3 domain-comprising polypeptide, that carries a charged residue at position 366, further comprises a variable domain which has specificity for antigen A, and if the second CH3 domain-comprising polypeptide, that carries an oppositely charged residue at position 351, further comprises a variable domain which has specificity for antigen B, bispecific Ig-like molecules with an AB specificity will be predominantly formed. Further provided is therefore a method according to the present invention, wherein said means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides or said means for preferential pairing of said 3rd and 4th CH3 domain-comprising polypeptides are a substitution of threonine at position 366 of said 1st or 3rd CH3 domain by a first charged amino acid and substitution of leucine at position 351 of said 2nd or 4th CH3 domain by a second charged amino acid, wherein said first and second charged amino acids are of opposite charge.

One preferred combination of mutations according to the present invention is the substitution of threonine (T) by lysine (K) at position 366 of a first CH3 domain-comprising polypeptide which further comprises a variable domain (for instance with specificity A) and the substitution of leucine (L) by aspartic acid (D) at position 351 of a second CH3 domain-comprising polypeptide which further comprises a variable domain (for instance with specificity B). This is denoted as a T366K/L351'D pair mutation. As explained before, the amino acids at position 366 of one CH3 domain and position 351 of a second CH3 domain have been reported to be a pair of contact residues in the CH3-CH3 interface. The lysine that is introduced at position 366 and the aspartic acid introduced at position 351 have opposite charges, so that these amino acids will electrostatically attract each other. Hence, the first CH3 domain will preferentially attract the second CH3 domain and Ig-like molecules comprising a first CH3 domain containing lysine at position 366 paired with a second CH3 domain containing aspartic acid at position 351 will be predominantly formed. If the first CH3

domain-comprising polypeptide has specificity for antigen A, and if the second CH3 domain-comprising polypeptide has specificity for antigen B, bispecific Ig-like molecules with 'AB' specificity will be predominantly formed. Nota bene, in some embodiments the specificity of the variable domains of both said first and second CH3-domain comprising polypeptide chains may be the same, which will result in the formation of monospecific Ig-like molecules (for instance with 'AA' specificity) . As mentioned above, one of the advantages of the mutations according to the present invention is the fact that a novel interaction between a newly introduced pair of charged amino acids is created, instead of replacing existing charged amino acid interactions. This was not previously disclosed or suggested. One aspect of the invention therefore provides a method according to the present invention for producing at least two different Ig-like molecules from a single host cell, wherein said 1st CH3 domain-comprising polypeptide chain comprises the amino acid substitution T366K, and said 2nd CH3 domain-comprising polypeptide chain comprises the amino acid substitution L351D. One embodiment provides a method for producing a heterodimeric Ig-like molecule from a single cell, wherein said Ig-like molecule comprises two CH3 domains that are capable of forming an interface, said method comprising providing in said cell:

- a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain, and
- a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,

wherein said first CH3 domain-comprising polypeptide chain comprises the amino acid substitution T366K and wherein said second CH3 domain comprising polypeptide chain comprises the amino acid substitution L351D, said method further comprising the step of culturing said host cell and allowing for expression of said two nucleic acid molecules and harvesting said heterodimeric Ig-like molecule from the culture.

Using the above mentioned amino acid substitutions according to the present invention, it has become possible to produce a heterodimeric Ig-like molecule from a single cell, whereby the presence of contaminating homodimers is less than 5%, preferably less than 2%, more preferably less than 1%, or, most preferably, whereby contaminating homodimers are essentially absent. One embodiment therefore

provides a method for producing a heterodimeric Ig-like molecule from a single cell, wherein said Ig-like molecule comprises two CH3 domains that are capable of forming an interface and wherein the presence of contaminating homodimers is less than 5%, preferably less than 2%, more preferably less than 1%, and most preferably contaminating homodimers are essentially absent, said method comprising providing in said cell:

- a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain, and
- a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,

wherein said first CH3 domain-comprising polypeptide chain comprises the amino acid substitution T366K and wherein said second CH3 domain comprising polypeptide chain comprises the amino acid substitution L351D, said method further comprising the step of culturing said host cell and allowing for expression of said at two nucleic acid molecules and harvesting said heterodimeric Ig-like molecule from the culture.

Preferably, a method according to the present invention for producing at least two different Ig-like molecules, or a method according to the invention for producing a heterodimeric Ig-like molecule, is provided wherein said first CH3-domain comprising polypeptide chain further comprises the amino acid substitution L351K. It is further preferred that said second CH3-domain comprising polypeptide chain further comprises an amino acid substitution selected from the group consisting of Y349E, Y349D and L368E. Most preferably said second CH3-domain comprising polypeptide chain further comprises the amino acid substitution L368E.

Thus, in a preferred embodiment the above mentioned T366K/L351'D mutations according to the present invention are further combined with the substitution of leucine (L) by glutamic acid (E) at position 368 of the second CH3 domain. This is, for example, denoted as a T366K/L351'D,L368'E mutation (but alternative ways of denoting are also possible, such as T336K/L351D-L368E or T366K/L351D,L368E or T366K - L351D,L368E). As shown in Example 17, introduction of this mutation according to the invention into a first CH3 domain-comprising polypeptide with specificity for antigen A, and a second CH3 domain-comprising polypeptide with

specificity for antigen B results in a particular good proportion of bispecific Ig-like molecules with dual AB specificity. With this mutational pair it has even become possible to obtain bispecific antibody without any detectable amount of homodimers formed. A particularly preferred embodiment therefore provides a method for producing a heterodimeric Ig-like molecule from a single cell, wherein said Ig-like molecule comprises two CH3 domains that are capable of forming an interface and wherein the presence of contaminating homodimers is less than 5%, preferably less than 2%, more preferably less than 1%, and most preferably contaminating homodimers are essentially absent, said method comprising providing in said cell:

- a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain, and
- a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,

wherein said first CH3 domain-comprising polypeptide chain comprises the amino acid substitution T366K and wherein said second CH3 domain comprising polypeptide chain comprises the amino acid substitutions L351D and L368E, said method further comprising the step of culturing said host cell and allowing for expression of said at two nucleic acid molecules and harvesting said heterodimeric Ig-like molecule from the culture.

In yet another preferred embodiment, threonine (T) is substituted by lysine (K) at position 366 of a first CH3 domain and leucine (L) is substituted by aspartic acid (D) at position 351 of a second CH3 domain and tyrosine (Y) is substituted by glutamic acid (E) at position 349 of said second CH3 domain. This is for example denoted as a T366K/L351'D,Y349'E mutation but other ways of denoting these mutations may include for example T366K – L351D:Y349E, or T366K/L351D,Y349E or simply T366K/L351DY349E. Residue Y349 is a neighboring residue of the residue at position 351 that may contribute to dimer interactions. According to in silico data, Y349E adds to the stability of the heterodimer (lower in silico scores) as well as to the destabilization of the monodimer (higher in silico scores) and glutamic acid (E) on position 349 is more favorable than aspartic acid (D). Thus, introduction of a second amino acid substitution in the second CH3 domain comprising polypeptide,

comprising already the amino acid substitution at position 351, favors heterodimerization further.

A particularly preferred embodiment therefore provides a method for producing a heterodimeric Ig-like molecule from a single cell, wherein said Ig-like molecule comprises two CH3 domains that are capable of forming an interface and wherein contaminating homodimers are less than 5%, more preferably less than 2%, even more preferably less than 1%, and most preferably essentially absent, said method comprising providing in said cell:

- a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain, and
- a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,

wherein said first CH3 domain-comprising polypeptide chain comprises the amino acid substitution T366K and wherein said second CH3 domain comprising polypeptide chain comprises the amino acid substitutions L351D and Y349E, said method further comprising the step of culturing said host cell and allowing for expression of said at two nucleic acid molecules and harvesting said heterodimeric Ig-like molecule from the culture.

In yet another preferred embodiment, threonine (T) is substituted by lysine (K) at position 366 of a first CH3 domain and leucine (L) is substituted by aspartic acid (D) at position 351 of a second CH3 domain and tyrosine (Y) is substituted by glutamic acid (E) at position 349 of said second CH3 domain and leucine (L) is substituted by glutamic acid (E) at position 368 of said second CH3 domain. This is denoted as a T366K/L351'D,Y349'E,L368'E mutation. The two residues Y349 and L368 are residues that may contribute to dimer interactions. According to the in silico data, Y349E and L368E add to the stability of the heterodimer (lower in silico scores) as well as to the destabilization of the BB dimer (higher in silico scores) and glutamic acids (E) on positions 349 and 368 are more favorable than aspartic acids (D). Thus, introduction of a second and third amino acid substitution in the B-chain, which already comprises the amino acid substitution at position 351, favors heterodimerization further. A particularly preferred embodiment therefore provides a method for producing a heterodimeric Ig-like molecule from a single cell, wherein said

Ig-like molecule comprises two CH3 domains that are capable of forming an interface and wherein contaminating homodimers are less than 5%, more preferably less than 2%, even more preferably less than 1%, and most preferably essentially absent, said method comprising providing in said cell:

- a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain, and
- a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,

wherein said first CH3 domain-comprising polypeptide chain comprises the amino acid substitution T366K and wherein said second CH3 domain comprising polypeptide chain comprises the amino acid substitutions L351D and Y349E and L368E, said method further comprising the step of culturing said host cell and allowing for expression of said at two nucleic acid molecules and harvesting said heterodimeric Ig-like molecule from the culture.

In yet another preferred embodiment, threonine (T) is substituted by lysine (K) at position 366 of a first CH3 domain and leucine (L) is substituted by lysine (K) at position 351 of said first CH3 domain and leucine (L) is substituted by aspartic acid (D) at position 351 of a second CH3 domain and leucine (L) is substituted by glutamic acid (E) at position 368 of said second CH3 domain. This is denoted as a T366K,L351K/L351'D,L368'E mutation. This mutation also enhances the proportion of the (bispecific) antibody of interest, as shown in the Examples. Also with this mutation it has become possible to obtain bispecific antibody without any detectable amount of homodimers formed. Further provided is therefore a method for producing a heterodimeric Ig-like molecule from a single cell, wherein said Ig-like molecule comprises two CH3 domains that are capable of forming an interface and wherein contaminating homodimers are less than 5%, preferably less than 2%, more preferably less than 1%, and most preferably essentially absent, said method comprising providing in said cell:

- a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain, and
- a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,

wherein said first CH3 domain-comprising polypeptide chain comprises the amino acid substitutions T366K and L351K, and wherein said second CH3 domain comprising polypeptide chain comprises the amino acid substitutions L351D and L368E, said method further comprising the step of culturing said host cell and allowing for expression of said at two nucleic acid molecules and harvesting said heterodimeric Ig-like molecule from the culture.

In yet another preferred embodiment, threonine (T) is substituted by lysine (K) at position 366 of a first CH3 domain and leucine (L) is substituted by lysine (K) at position 351 of said first CH3 domain and leucine (L) is substituted by aspartic acid (D) at position 351 of a second CH3 domain and tyrosine (Y) is substituted by aspartic acid (D) at position 349 of said second CH3 domain and arginine (R) is substituted by aspartic acid (D) at position 355 of said second CH3 domain. This is denoted as a T366K,L351K/L351'D,Y349'D,R355'D mutation. The T366K-L351K/L351'D-Y349'D pair may be further improved by the R355'D mutation in the B-chain, which results in a higher BB-in silico score, but also the AB in silico score is slightly higher. Further provided is therefore a method for producing a heterodimeric Ig-like molecule from a single cell, wherein said Ig-like molecule comprises two CH3 domains that are capable of forming an interface and wherein contaminating homodimers are less than 5%, more preferably less than 2%, even more preferably less than 1%, and most preferably essentially absent, said method comprising providing in said cell:

- a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain, and
- a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,

wherein said first CH3 domain-comprising polypeptide chain comprises the amino acid substitutions T366K and L351K, and wherein said second CH3 domain comprising polypeptide chain comprises the amino acid substitutions L351D and Y349D and R355D, said method further comprising the step of culturing said host cell and allowing for expression of said at two nucleic acid molecules and harvesting said heterodimeric Ig-like molecule from the culture.

Table B provides an overview of mutations that can be introduced in CH3 domains as preferred means for preferential pairing to create either heterodimers or homodimers.

Table B:

| **AA substitutions in CH3** | **Construct #** | **Preferentially pairs with** |
|---|---|---|
| - (wildtype) | - | Wildtype |
| E356K, D399K | 1 | Construct 2 or 3 |
| K392D, K409D | 2 | Construct 1 |
| K392D, K409D, K439D | 3 | Construct 1 |
| K392D, D399K, K409D | 4 | Construct 4 |
| E356K, E357K, K439D, K370D | 5 | Construct 5 |
| T366W | 6 | Construct 7 |
| T366S, L368A, Y407V | 7 | Construct 6 |
| T366K | 43 | Construct 63, 69, 70, 71, 73 |
| L351D | 63 | Construct 43, 68 |
| T366K, L351K | 68 | Construct 63, 69, 70, 71, 72, 75 |
| L351D, L368E | 69 | Construct 43, 68 |
| L351E, Y349E | 70 | Construct 43, 68 |
| L351D, Y349E | 71 | Construct 43, 68 |
| L351D, R355D | 72 | Construct 43, 68 |
| L351D, Y349E, L368E | 73 | Construct 43 |
| L351D, Y349D, R355D | 75 | Construct 68 |

A method according to the present invention for producing at least two different Ig-like molecules, or a method according to the invention for producing a heterodimeric Ig-like molecule, wherein said means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides and/or said means for preferential pairing of said 3rd and 4th CH3 domain-comprising polypeptides comprise at least one combination of mutations as depicted in Table B is therefore also provided herewith. Preferably, said means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides

and said means for preferential pairing of said 3rd and 4th CH3 domain-comprising polypeptides comprise at least two combinations of mutations as depicted in Table B.

The present invention also provides novel combinations of CH3 mutations with which it has become possible to produce a mixture of at least two monospecific Ig-like molecules in a single cell, wherein contaminating bispecific Ig-like molecules are less than 5%, preferably more than 2%, even more preferably less than 1%, and most preferably even essentially absent. These mutations according to the invention are, therefore, particularly suitable for the production of a mixture of monospecific antibodies, which is for instance advantageous when a high level of crosslinking of two identical target molecules is desired, when the density of antibodies on a target cells needs to be high enough to recruit certain effector functions such as complement-mediated lysis of a tumor cell, or when two targets are located too far away from each order so that they cannot be bound by as single bispecific antibody, or in order to simplify regulatory approval procedures. In such cases, it is often desired to optimize the production platform for such monospecific antibodies. As shown in Example 10, the present invention provides the insight that when lysine (K) at position 392 of a first CH3 domain-comprising polypeptide (for instance having specificity A) is substituted by aspartic acid (D) and when aspartic acid (D) at position 399 of said first CH3 domain-comprising polypeptide is substituted by lysine (K) and when lysine (K) at position 409 of said first CH3 domain-comprising polypeptide is substituted by aspartic acid (D), it has become possible to produce a mixture of at least two different monospecific Ig-like molecules in a single cell, including monospecific Ig-like molecules with specificity AA, wherein the formation of bispecific by-products (bispecific Ig-like molecules) is reduced to below 5%, or even to below 3%, or even essentially not detectable at all. Hence, the above mentioned combination of mutations (denoted herein as K392D, D399K, K409D) is particularly preferred for the production of a mixture of monospecific Ig-like molecules. The skilled person will appreciate that functional variants thereof, i.e., K392E, D399R, K409E, may result in similar effects. Additionally, double mutants comprising D399K and K409D substitutions, or other functional variants such as e.g. K392D and K409D, D399R and K409E and so forth, may also result in similar effects.

The same holds true for a combination of mutations wherein glutamic acid (E) at position 356 of a first CH3 domain-comprising polypeptide is substituted by lysine (K) and wherein glutamic acid (E) at position 357 of said first CH3 domain-comprising polypeptide is substituted by lysine (K) and wherein lysine (K) at position 439 of said first CH3 domain-comprising polypeptide is substituted by aspartic acid (D) and wherein lysine (K) at position 370 of said first CH3 domain-comprising polypeptide is substituted by aspartic acid (D). This combination of mutations (denoted herein as E356K, E357K, K439D, K370D) is also particularly preferred for the production of a mixture of monospecific Ig-like molecules. The skilled person will appreciate that functional variants thereof, i.e., E356R, E357R, K439E, K370E, may result in similar effects. Additionally, triple or double mutants comprising E356K and K439D, and E357K and K370D substitutions, or other functional variants may also result in similar effects. A further embodiment therefore provides a method for producing at least two different monospecific Ig-like molecules from a single host cell, wherein each of said two Ig-like molecules comprises two CH3 domains that are capable of forming an interface, said method comprising providing in said cell

a) a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain having a specificity A,

b) a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain having a specificity B,

wherein said first CH3 domain-comprising polypeptide chain comprises a K392D, D399K, K409D mutation and said second CH3 domain-comprising polypeptide chain comprises either a wildtype CH3 domain or comprises a E356K, E357K, K439D, K370D mutation, said method further comprising the step of culturing said host cell and allowing for expression of said nucleic acid molecules and harvesting said at least two different Ig-like molecules from the culture.

An alternative embodiment provides a method for producing at least two different monospecific Ig-like molecules from a single host cell, wherein each of said two Ig-like molecules comprises two CH3 domains that are capable of forming an interface, said method comprising providing in said cell

a) a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain having a specificity A,

b) a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain having a specificity B,
wherein said first CH3 domain-comprising polypeptide chain comprises either a wildtype CH3 domain or comprises a K392D, D399K, K409D mutation and said second CH3 domain-comprising polypeptide chain comprises a E356K, E357K, K439D, K370D mutation, said method further comprising the step of culturing said host cell and allowing for expression of said nucleic acid molecules and harvesting said at least two different Ig-like molecules from the culture.

As shown in **Example 10**, two monospecific Ig-like molecules can be produced in a single cell, wherein the formation of bispecific Ig-like molecules is essentially undetectable. The skilled person may select a 3rd nucleic acid molecule encoding a wildtype or engineered CH3 domain-comprising polypeptide chain to provide to said host cell such that a mixture of 3 monospecific antibodies is produced, and so forth.

In one aspect of the invention, a method according to the invention for producing at least two different Ig-like molecules or for producing a heterodimeric Ig-like molecule is provided wherein each of the CH3-domain comprising polypeptide chains further comprises a variable region recognizing a different target epitope, wherein the target epitopes are located on the same molecule. This often allows for more efficient counteraction of the (biological) function of said target molecule as compared to a situation wherein only one epitope is targeted. For example, a heterodimeric Ig-like molecule may simultaneously bind to 2 epitopes present on, e.g., growth factor receptors or soluble molecules critical for tumors cells to proliferate, thereby effectively blocking several independent signalling pathways leading to uncontrolled proliferation, and any combination of at least two Ig-like molecules may simultaneously bind to 2, or even 3 or 4 epitopes present on such growth factor receptors or soluble molecules.
In a preferred embodiment, the target molecule is a soluble molecule. In another preferred embodiment, the target molecule is a membrane-bound molecule.

In another aspect of the invention, a method according to the invention for producing at least two different Ig-like molecules or for producing a heterodimeric Ig-like

molecule is provided wherein each of the CH3-domain comprising polypeptide chains further comprises a variable region recognizing a target epitope, wherein the target epitopes are located on different molecules. In this case, each of the different target molecules may either be a soluble molecule or a membrane-bound molecule. In one embodiment, the different target molecules are soluble molecules. Alternatively, one target molecule is a soluble molecule whereas the second target molecule is a membrane bound molecule. In yet another alternative, both target molecules are membrane bound molecules. In one embodiment the different target molecules are expressed on the same cells, whereas in other embodiments the different target molecules are expressed on different cells. As a non-limiting example, any heterodimeric Ig-like molecule or any combination of at least two Ig-like molecules may be suitable for simultaneously blocking multiple membrane-bound receptors, neutralizing multiple soluble molecules such as cytokines or growth factors for tumor cells or for neutralizing different viral serotypes or viral strains.

One preferred embodiment provides a method according to the invention for producing at least two different Ig-like molecules or for producing a heterodimeric Ig-like molecule, wherein at least one of said target epitopes is located on a tumor cell. Alternatively, or additionally, at least one of said target epitopes is located on the surface of an effector cell. This is for instance suitable for recruitment of T cells or NK cells for tumor cell killing. For instance, at least one Ig-like molecule is produced with a method according to the invention that is capable of recruiting immune effector cells, preferably human immune effector cells, by specifically binding to a target molecule located on immune effector cells. In a further embodiment, said immune effector cell is activated upon binding of the Ig-like molecule to the target molecule. Recruitment of effector mechanisms may for instance encompass the redirection of immune modulated cytotoxicity by administering an Ig-like molecule produced by a method according to the invention that is capable of binding to a cytotoxic trigger molecule such as the T cell receptor or an Fc gamma receptor, thereby activating downstream immune effector pathways. The term 'immune effector cell' or 'effector cell' as used herein refers to a cell within the natural repertoire of cells in the mammalian immune system which can be activated to affect the viability of a target cell. Immune effector cells include cells of the lymphoid lineage such as natural killer

(NK) cells, T cells including cytotoxic T cells, or B cells, but also cells of the myeloid lineage can be regarded as immune effector cells, such as monocytes or macrophages, dendritic cells and neutrophilic granulocytes. Hence, said effector cell is preferably an NK cell, a T cell, a B cell, a monocyte, a macrophage, a dendritic cell or a neutrophilic granulocyte.

Target antigens present on immune effector cells may include CD3, CD16, CD25, CD28, CD64, CD89, NKG2D and NKp46. Further provided is therefore a method according to the invention for producing at least two different Ig-like molecules or for producing a heterodimeric Ig-like molecule, wherein said target epitope is located on a CD3, CD16, CD25, CD28, CD64, CD89, NKG2D or a NKp46 molecule.

The viability of a target cell may include cell survival, proliferation and/or ability to interact with other cells.

In one aspect the present invention thus provides methods according to the invention for producing a heterodimeric Ig-like molecule, wherein each of the CH3-domain comprising polypeptide chains further comprises a variable region recognizing a target epitope. In one embodiment, each of the 2 variable regions of the CH3-domain comprising polypeptide chains recognizes the same target epitope but with different affinities. In another embodiment, each of the 2 variable regions of the CH3-domain comprising polypeptide chains recognizes a different target epitope. In another embodiment, the different target epitopes are located on the same target molecule, which can be either a membrane-bound molecule or a soluble molecule. In another embodiment, the different target epitopes are located on different target molecules, which can be either expressed on the same cells or on different cells. Alternatively, the different target molecules can be soluble molecules, or one target molecule can be a soluble molecule whereas the second target molecule is a membrane bound molecule. In a preferred embodiment, at least one of the target molecules of the heterodimeric Ig-like molecule is located on a tumor cell. In yet another preferred embodiment, at least one of the target molecules of the heterodimeric Ig-like molecule is located on an effector cell (i.e. an NK cell, a T cell, a B cell, a monocyte, a macrophage, a dendritic cell or a neutrophilic granulocyte, and said target epitope may be located on a CD3, CD16, CD25, CD28, CD64, CD89, NKG2D or a NKp46 molecule).

In a preferred embodiment, a method according to the invention for producing at least two different Ig-like molecules or for producing a heterodimeric Ig-like molecule is provided, wherein said at least two different Ig-like molecules are antibodies, most preferably antibodies of the IgG isotype, even more preferably the IgG1 isotype, as described herein above.

Further provided is an Ig-like molecule, a heterodimeric Ig-like molecule, or a mixture of at least two Ig-like molecules, obtainable by a method according to the present invention. Said (heterodimeric) Ig-like molecule or mixture of Ig-like molecules preferably comprises at least one CH3 mutation as depicted in Table B. An (heterodimeric) Ig-like molecule or a mixture of at least two Ig-like molecules, comprising at least one mutation as depicted in Table B is therefore also herewith provided, as well as a pharmaceutical composition comprising at least one Ig-like molecule, or a mixture of at least two Ig-like molecules, according to the present invention. In one embodiment said Ig-like molecule is a bispecific Ig-like molecule, such as a bispecific antibody. In another embodiment said Ig-like molecule is a monospecific Ig-like molecule, such as a monospecific antibody. One preferred embodiment provides a mixture of at least two different Ig-like molecules obtainable by a method according to the invention, wherein said at least two different Ig-like molecules bind to different epitopes on the same antigen and/or to different epitopes on different antigens. Further provided is a heterodimeric Ig-like molecule obtainable by a method according to the invention, wherein said heterodimeric Ig-like molecule binds to different epitopes on the same antigen and/or to different epitopes on different antigens. Advantages and preferred uses of such mixtures and antibodies are described herein before. The invention also provides a mixture of at least two different Ig-like molecules obtainable by a method according to the invention, wherein said at least two different Ig-like molecules comprise at least one heterodimeric Ig-like molecule. In one embodiment, two of said at least two different Ig-like molecules are heterodimeric Ig-like molecules. Yet another preferred embodiment provides a heterodimeric antibody comprising two CH3 domains, wherein one of said two CH3 domains comprises the amino acid substitutions L351D and L368E and wherein the other of said two CH3 domains comprises the amino acid substitutions T366K and

L351K. These amino acid substitutions are preferred means for preferential pairing of said two CH3 domains, as explained before. The amino acid substitutions L351D and L368E in one of said two CH3 domains and the amino acid substitutions T366K and L351K in the other of said two CH3 domains are together dubbed the 'DEKK combination of mutations', 'DEKK variant', 'DEKK pair', 'DEKK engineered CH3 domains', 'DEKK' or alternative names referring to DEKK are used. The CH3 domain that carries the amino acid substitutions L351D and L368E is also dubbed 'the DE-side' and the CH3 domain that carries the amino acid substitutions T366K and L351K is also dubbed 'the KK-side'.

Also provided is a pharmaceutical composition comprising a (heterodimeric) Ig-like molecule, or a mixture of at least two Ig-like molecules obtainable by any method according to the invention. Said (heterodimeric) Ig-like molecule, or said at least two Ig-like molecules according to the invention is/are preferably (an) antibody/antibodies. Said pharmaceutical composition may comprise said (heterodimeric) Ig-like molecule, a mixture comprising monospecific or bispecific Ig-like molecules, or a combination of monospecific and bispecific Ig-like molecules. In addition, a pharmaceutical composition according to the invention comprises a pharmaceutically acceptable carrier. As used herein, such 'pharmaceutically acceptable carrier' includes any and all solvents, salts, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Depending on the route of administration (e.g., intravenously, subcutaneously, intra-articularly and the like) the Ig-like molecules may be coated in a material to protect the Ig-like molecules from the action of acids and other natural conditions that may inactivate the Ig-like molecules. In one aspect, a pharmaceutical composition comprising a mixture of at least two Ig-like molecules obtainable by any method according to the invention is provided, wherein said at least two different Ig-like molecules have been produced by recombinant host cells according to the present invention. Furthermore, a pharmaceutical composition is provided comprising a heterodimeric Ig-like molecule obtainable by any method according to the invention, wherein said heterodimeric Ig-like molecule has been produced by recombinant host cells according to the present invention.

A nucleic acid molecule encoding a CH3 domain-comprising polypeptide chain that comprises at least one mutation as depicted in Table B is also provided herewith, as well as a recombinant host cell comprising at least one nucleic acid molecule encoding a CH3 domain-comprising polypeptide chain that comprises at least one mutation as depicted in Table B.

The invention is further illustrated by the following examples. These examples are not limiting the invention in any way, but merely serve to clarify the invention.

Brief description of the drawings

Figure 1: A) schematic representation of construct vector MV1057. The stuffer region is the region into which an antibody VH region is cloned. B) schematic representation of phage display vector MV1043.

Figure 2: amino acid sequence of wildtype IgG1 Fc, as present in construct vector MV1057 (EU numbering scheme applied).

Figure 3: nucleotide and amino acid sequences of VH regions used for cloning into the various constructs.

Figure 4: mass spec data of transfections A, G and H.

Figure 5: mass spec data of transfections M and U.

Figure 6: mass spec data of transfection O.

Figure 7: prevention of homodimerisation by substitution of neutral amino acids for charged amino acids.

Figure 8: Native MS spectrum of transfection sample ZO (T366K/L351'D) (A) and Convoluted MS spectrum of transfection sample ZO (T366K/L351'D). The second/main peak represents the bispecific molecule (B).

Figure 9: HADDOCK scores on experimentally verified mutation pairs

Figure 10: Cartoons of interactions in the CH3-CH3 interface; A) K409D:K392D/D399'K:E356'K, B) D399K:E356K/D399'K:E356'K, C) K409D:K392D/ K409'D:K392'D

Figure 11: HADDOCK scores for various 366/351' charge mutants

Figure 12: Cartoons of interactions in the CH3-CH3 interface ; A) L351D/L351'D, B) L351D:S354A:R355D/ L351'D:S354'A:R355'D

Figure 13: HADDOCK scores for additional charge mutations around position L351

Figure 14: HADDOCK scores for additional charge mutations around position T366 in chain A and position L351 in chain B.

Figure 15: Cartoons of interactions in the CH3-CH3 interface

Figure 16: HADDOCK scores for variants around T366/L351

Figure 17: HADDOCK scores for additional variants around T366/L351

Figure 18: Examples of nMS spectra for bispecific IgG obtained after the co-expression of construct T366K,L351K with either construct L351D (left hand panel) or

L351D,Y349E (right hand panel), zoomed in on a single charge state of the full IgG (half bodies not shown)

Figure 19: A) Results of native MS showing relative abundances of AA, AB, BB, A and B (total of all species is 100%); B) idem but now without AB to have a better overview on the undesired species AA, BB, A and B

Figure 20: Results of thermostability assay. Squares: wildtype; triangles: charge reversal pair E356K:D399K/K392D:K409D; circles: mutant CH3 combinations as indicated above each graph.

Figure 21: Results of 10x freeze-thaw experiment. 1122= 1st parental antibody BB; 1337= 2nd parental antibody AA; wildtype=AA, AB, BB; CR= bispecific of charge reversal pair E356K:D399K/K392D:K409D; 3-6 and 9-12=bispecific molecules from combinations 3-6 and 9-12 from Table 15.

Figure 22: Results in serum stability, measured by ELISA using fibrinogen as coated antigen. A) ELISA data with IgG samples diluted to 0.5 µg/ml; B) ELISA data with IgG samples diluted to 0.05 µg/ml;. Results are normalized to the T=0 days time point (100%). 1337= 2nd parental antibody AA; wildtype=AA, AB, BB; CR= bispecific of charge reversal pair E356K:D399K/K392D:K409D; 3-6 and 9-12=bispecific molecules from combinations 3-6 and 9-12 from Table 15.

Figure 23: nMS results of ratio experiments with transfection ratios from 1:5 to 5:1. A) DEKK combination of mutations, with specificity 'A' on the DE-side and 'B' on the KK-side; B) DEKK combination of mutations, with specificity 'C' on the DE-side and 'B' on the KK-side; C) charge reversal combination of mutations, with specificity 'A' on the E356K:D399K-side and 'B' on the K392D:K409D-side

Figure 24: nMS results of transfections # 1-11 from Table 20.

Figure 25: HADDOCK scores for dimers with different CH3 engineered vectors. Grey bars: Desired species AB and CD; black bars: undesired species AA, BB, CC, DD, AC, BC, AD, BD.

Figure 26: SDS-PAGE of transfections # 1-11 from Table 20. Control samples DE/KK, DE/DE and KK/KK are also included.

Figure 27: nMS of transfections # 9 (A) and # 11 (B).

Figure 28: nMS of gel filtrated samples 1516:1516 (A), 1337:1337 (B) and 1516:1337 (C).

Figure 29: serum levels of samples of DEKK engineered antibody and its two parental antibodies (pK study).

5

Examples

**Example 1: amino acid substitutions to create various different CH3-domains**

In order to have a wide variety of Ig-like molecules that differ in their CH3 domains such that pairing of CH3-domain comprising Ig-like molecules is preferentially promoted or inhibited, a number of amino acid substitutions that were known to promote heterodimer formation, as well as a number of alternative amino acid substitutions that were not previously reported nor tested but that were chosen to promote homodimer formation, were introduced into a construct vector (construct vector MV1057; Figure 1A). The construct vector MV1057 comprises nucleic acid sequences encoding the normal wildtype IgG1 Fc part, as depicted in figure 2. Table 1 lists the amino acid substitutions that were introduced in this wildtype Fc, resulting in a series of seven constructs. All constructs were made at Geneart. Constructs 1, 2 and 3, or alternatives thereof, have previously been described to drive heterodimerization (EP01870459, WO2009/089004) as have constructs 6 and 7 (WO98/50431). Constructs 4 and 5 are new and are designed to promote homodimerization.

Table 1

| **AA substitutions in CH3** | **construct #** | **Will pair with** | **% bispecific product reported** |
|---|---|---|---|
| - (wildtype) | - | - (wildtype) | ~50% |
| E356K, D399K | 1 | Construct 2 or 3 | ~100% |
| K392D, K409D | 2 | Construct 1 | ~100% |
| K392D, K409D, K439D | 3 | Construct 1 | ~100% |
| K392D, D399K, K409D | 4 | Construct 4 | |
| E356K, E357K, K439D, K370D | 5 | Construct 5 | |
| T366W | 6 | Construct 7 | ~86,7% |
| T366S, L368A, Y407V | 7 | Construct 6 | ~86,7% |

**Example 2: cloning of VH into constructs with CH3 mutations**

Several antibody VH regions with known specificities and known ability to pair with the human IGKV1-39 light chain were used for cloning into these constructs.

As indicated earlier, all CH3 variants can be used in association with other antibody domains to generate full length antibodies that are either bispecific or monospecific. The specificity of the antibody as defined by the VH/VL combinations will not affect the heavy chain dimerization behaviour that is driven by the CH3 domains. Model VH/VL combinations were used throughout the studies, wherein all VLs are based on the germline human IGKV1-39 and VHs vary. Figure 3 provides full sequences and specificities of the antibody VH regions used throughout the studies. The MF coding refers to internal Merus designation for various VHs, e.g. VH MF1337 has specificity for tetanus toxoid, MF1025 for porcine thyroglobulin, MF1122 for bovine fibrinogen.

VH regions present in phage display vector MV1043 (Figure 1B) are digested with restriction enzymes SfiI and BstEII (New England Biolabs/ cat# R0123L and R0162L/ according to manufacturer's instructions) that release the VH fragment from this vector. Vector MV1057 is digested with SfiI and BstEII according to standard procedures (according to manufacturer's instructions). Fragments and vector are purified over gel (Promega/ cat# V3125/ according to manufacturer's instructions) to isolate the cut vector and VH gene inserts. Both are combined by ligation after which the ligation is transformed into E. coli DH5α (Invitrogen/ cat# 12297-016/ according to manufacturer's instructions). After overnight selection single colonies are picked and vectors with a correct insert identified by sequencing.

**Example 3: transfection and expression of full IgG in HEK293T cells**

Transfection of the various plasmids encoding the recloned VH variants, and further encoding the common light chain huIGKV1-39, in HEK293T cells was performed according to standard procedures such that IgG could express (de Kruif etal Biotech Bioeng. 2010). After transfection, IgG expression levels in supernatants were measured using the ForteBIO Octet-QK system, which is based on Bio-Layer Interferometry (BLI) and which enables real-time quantitation and kinetic characterization of biomolecular interactions; for details see www.fortebio.com. When expression levels exceeding 5 µg/ml were measured, the IgG was purified using Protein A affinity purification.

**Example 4: purification of IgG**

Culture supernatants were purified using protein A columns (GE Healthcare/ cat# 11-0034-95/ according to manufacturer's instructions) and eluted in 0,1 M citrate buffer pH 3.0 and immediately neutralized in an equal volume of 1,0 M Tris-HCL pH 8.0 or directly rebuffered to PBS using a desalting column. Alternatively one could purify IgG using protein A beads (sepharose beads CL-4B, GE healthcare cat #170780-01)

**Example 5: Ag-specific ELISA's**

Antigen specific ELISAs were performed to establish binding activity against the antigens and capture ELISAs were carried out to demonstrate binding activity of the bispecific antibodies. Biotinylated second antigen was used for detection of the complex. (de Kruif etal Biotech Bioeng. 2010)

**Example 6: SDS-PAGE**

The purified IgG mixtures were analysed by SDS-PAGE (NuPAGE® 4-12% bis-tris gel/ Invitrogen/ cat# NP0323BOX) under reduced and non-reducing conditions according to standard procedures, and staining of proteins in gel was carried out with colloidal blue (PageBlue™ protein staining solution/ Fermentas/ cat# RO571).

**Example 7: Enzymatic deglycosylation of IgG1**

As there is heterogeneity in the glycosylation of the IgGs, the proteins were deglycosylated in order to create a single product with a distinct mass, suitable for mass spectrometric analysis. One unit of N-glycosidase F (PNGase F; Roche Diagnostics, Mannheim, Germany) was incubated per 10 µg of IgG1, overnight at 37°C. Buffer exchange using 10 kDa MWCO centrifugal filter columns (Millipore) was performed to remove the original purification buffer (0,1 M citrate buffer pH 3.0 / 1,0 M Tris-HCL pH 8.0) and to rebuffer to PBS. Similar buffer exchange procedures were performed to remove the detached glycan chains, and to change the buffer to 150 mM ammonium acetate pH 7.5. Filters were washed with 200 µl 150 mM ammonium acetate pH 7.5, for 12 min 11,000 rpm and 4°C. After washing 50 µl deglycosylated IgG was loaded on the filter and 450 µl of 150 mM ammonium acetate pH 7.5 was added, subsequently followed by another centrifugation round of 12 min at 11,000

rpm at 4°C. In total the centrifugation was repeated 5 times, each time fresh 150 mM ammonium acetate pH 7.5 buffer was added to a total volume of 500 µl. After the last centrifugation step the remaining buffer exchanged deglycosylated IgG1, approximately 25 µl, was collected and transferred to an eppendorf tube, ready for mass spectrometric analysis.

**Example 8: Native mass spectrometric analysis**

Mass Spectrometry was used to identify the different IgG species in the purified IgG mixtures and to establish in what ratios these IgG species are present. Briefly, 2-3 µl at a 1 µM concentration in 150 mM ammonium acetate pH 7.5 of IgG's were loaded into gold-plated borosilicate capillaries made in-house (using a Sutter P-97 puller [Sutter Instruments Co., Novato, CA, USA] and an Edwards Scancoat six sputter-coater [Edwards Laboratories, Milpitas, CA, USA]) for analysis on a LCT 1 mass spectrometer (Waters Corp., Milford, MA, USA), adjusted for optimal performance in high mass detection (Tahallah et al., RCM 2001). A capillary voltage of 1300 V was used and a sampling cone voltage of 200 V; however, these settings were adjusted when a higher resolution of the 'signal-to-noise' ratio was required. The source backing pressure was elevated in order to promote collisional cooling to approximately 7.5 mbar. To measure the IgG1's under denaturing conditions the proteins were sprayed at a 1 µM concentration in 5% formic acid.

**Example 9: Data processing and quantification**

Processing of the acquired spectra was performed using MassLynx 4.1 software (Waters Corp., Milford, MA, USA). Minimal smoothing was used, after which the spectra were centered. The mass of the species was calculated using each charge state in a series. The corresponding intensities of each charge state were assigned by MassLynx and summed. This approach allowed the relative quantification of all species in a sample. Alternatively, quantification of the peaks can be performed using area-under-the-curve (AUC) methods, known in the art. All analyses were repeated three times to calculate standard deviations of both the masses of the IgG's as well as their relative abundance.

**Example 10: mixtures of 2 or 3 monospecific antibodies from a single cell**

Several antibody VH regions with known specificities and known ability to pair with the human IGKV1-39 light chain (Figure 3) were used for recloning into the wildtype construct vector MV1057, or in construct 4 or construct 5 of Table 1, resulting in vectors I-III (Table 2). The resulting vectors I, II and III, each containing nucleic acid sequences encoding for the common human light chain as well as an Ig heavy chain with different CH3 region and different VH specificity, were subsequently transfected into cells, either alone to demonstrate formation of intact monospecific antibodies only, or in combination with one or two other construct vectors to obtain mixtures of two monospecific or three monospecific antibodies. Table 3 depicts the transfection schedule and results.

Table 2: **VH specificity inserted in different constructs**

| **Vector** | **VH gene** | **Antigen specificity** | **VH mass (Da)** | **Merus designation** | **Cloned in construct #** |
|---|---|---|---|---|---|
| I | IGHV 1.08 | Tetanus (A) | 13703 | MF1337 | wildtype |
| II | IGHV 3.23 | Thyroglobulin (B) | 12472 | MF1025 | 4 |
| III | IGHV 3.30 | Fibrinogen (C) | 12794 | MF1122 | 5 |

Table 3: **transfection schedule and results**

| **# different mono-specifics produced** | **Transfection of** | **Transfection code and ratio** | **Expected species** | **Calculated mass - 2LYS** | **Experimental mass** | **AA found (%)** | **BB found (%)** | **CC found (%)** | **Other molecules (%)** |
|---|---|---|---|---|---|---|---|---|---|

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Only vector I | A | AA | 146521 | 146503 | 100 | | | |
| 1 | Only vector II | G | BB | 144032 | 144087 | | 100 | | |
| 1 | Only vector III | H | CC | 144647 | 144656 | | | 100 | |
| 2 | Vector I and II | M (I:II=1:1) | AA<br>BB | 146521<br>144032 | 146518<br>144030 | 51 | 45 | | 4 |
| 2 | Vector I and III | N (I:III=1:1) | AA<br>CC | 146521<br>144647 | 146509<br>144633 | 88 | | 9 | 3 |
| | | U (I:III= 1:5) | AA<br>CC | 146521<br>144647 | 146522<br>144643 | 47 | | 48 | 5 |
| 2 | Vector II and III | nd | BB<br>CC | | | | | | |
| 3 | Vector I, II and III | O (I:II:III=1: 1:1) | AA<br>BB<br>CC | 146521<br>144032<br>144647 | 146525<br>144032<br>144650 | 66 | 4 | 30 | |
| | | V (I:II:III=1: 1:10) | AA<br>BB<br>CC | 146521<br>144032<br>144647 | 146531<br>144043<br>144654 | 8 | 81 | 9 | 2 |

nd= not done.

It was observed that transfections A, G and H resulted in formation of homodimers only, and 100% of bivalent monospecific AA, BB or CC was retrieved from cells transfected with any one of vectors I, II or III (Fig. 4). Although this was to be expected and previously demonstrated for transfection A, it is actually now shown for the first time that homodimerisation of CH3-engineered Ig heavy chains containing either the triple amino acid substitution of construct 4 (i.e., K392D, D399K, K409D) or the quadruple amino acid substitution of construct 5 (i.e., E356K, E357K, K439D, K370D) is reported (transfections G and H).

Next, co-expression experiments of two vectors in a single cell were performed. Interestingly, transfections M and N show that wildtype and CH3 engineered Ig heavy chains can be co-expressed in a single cell together with a common light chain resulting in mixtures of two species of monospecific antibodies without the presence of undesired bispecific antibodies and with as little as 4-5% contaminating 'other molecules' present in the mixture. 'Other molecules' is defined as all molecules that do not have the mass of an intact IgG, and includes half molecules consisting of a single heavy and light chain pair. Importantly, the fraction 'other' does not include bispecific product. In transfection M, the ratio of AA:BB was close to 1:1 upon transfection of equal ratios of vector DNA. However, transfection N resulted in an almost 10:1 ratio of AA:CC. Therefore, this transfection was repeated with adjusted ratios of DNA (transfection U). Indeed, a 1:5 ratio of vector DNA I:III equalized the ratio of AA:CC antibody product in the mixture towards an almost 1:1 ratio. Thus, transfections M and U show that it is possible to express two different, essentially pure, monospecific antibodies in a single cell, without undesired by products (i.e., no abundant presence of AC or half molecules A or C) (fig. 5). The novel CH3 modifications of constructs 4 and 5 differ substantially from wildtype CH3 such that heterodimerization between wildtype and 4, or wildtype and 5, does not occur, which is advantageous for application in large scale production of mixtures of monospecific antibodies from single cells.

Analogous to these results, also transfection of two different CH3 engineered Ig heavy chains (constructs 4 and 5) are expected to result in mixtures of two different monospecific antibodies only, without further undesired species present. It is reasoned that the CH3 modifications of construct 4 differ substantially from the CH3 modifications of constructs 5 such that heterodimerization does not occur. In that case, co-expression of CH3-engineered heavy chains of constructs 4 and 5, together with wildtype CH3 heavy chains in a single cell would results in 3 monospecific antibodies only.

Indeed, this was observed to be the case as it was found that also a mixture of three pure monospecific antibodies could be obtained by expression of three different Ig heavy chains, designed to form homodimers over heterodimers, together with a common light chain in a single cell, with no contaminations present in the mixture (transfection O) (Fig. 6). As is clear from Table 3, with equal ratios of vector DNA

used during transfection O, no 1:1:1 ratio of AA:BB:CC antibodies was obtained. Transfections with altered vector DNA ratios (1:1:10, transfection V) demonstrated that ratios of AA:BB:CC in the mixtures can be steered towards desired ratios. Taken together, these experiments show that two or three essentially pure monospecific antibodies can be expressed in a single cell without undesired by products, offering advantages for large scale production of mixtures of therapeutic monospecific antibodies.

**Example 11: mixtures of 2 bispecific antibodies from a single cell**

Whereas use of CH3-engineered heavy chains for production of single bispecific antibodies has been reported elsewhere, this experiment was designed to investigate whether it is feasible to produce mixtures of 2 different bispecific antibodies from a single cell.

Antibody VH regions with known specificities and known ability to pair with the human IGKV1-39 light chain (fig. 3) were used for recloning into vectors containing constructs 1-3 or 6-7 of Table 1 resulting in vectors IV-X (Table 4). Vectors IV-X, each containing nucleic acid sequences encoding the common human light chain as well as an Ig heavy chain with different CH3 region and different VH specificity, were subsequently transfected into cells, either alone to demonstrate that formation of intact monospecific antibodies was hampered, or in combination with another construct vector to obtain bispecific antibodies or mixtures of two bispecific antibodies. Table 5 depicts the transfection schedule and results.

Table 4: **VH specificity inserted in different constructs**

| **Vector** | **VH gene** | **Antigen specificity** | **VH mass (Da)** | **Cloned in construct #** |
|---|---|---|---|---|
| IV | IGHV 3.23 | Thyroglobulin (B) | 12472 | 1 |
| V | IGHV 3.30 | Fibrinogen (C) | 12794 | 2 |
| VI | IGHV 1.08 | Tetanus (A) | 13703 | 2 |

| | | | | |
|---|---|---|---|---|
| VII | IGHV 3.30 | Fibrinogen (C) | 12794 | 3 |
| VIII | IGHV 1.08 | Tetanus (A) | 13703 | 3 |
| IX | IGHV 1.08 | Tetanus (A) | 13703 | 6 |
| X | IGHV 3.23 | Thyroglobulin (B) | 12472 | 7 |

Table 5:

| **# different-bispe-cifics produ-ced** | **Trans-fection of** | **Trans fectio n code and ratio** | **Expec ted spe-cies** | **Calculate d mass - 2LYS** | **Experim ental mass** | **Half molecu les found (%)** | **Full IgG foun d (%)** | **Bispe cific found (%)** | **Other molec ules (%)** |
|---|---|---|---|---|---|---|---|---|---|
| 0 | vector IV | B | Half B | 144082 | 144066 | 40 | 60 | | |
| 0 | vector V | C | Half C | 144651 | 144622 | 77 | 23 | | |
| 0 | vector VI | D | Half A | 146469 | 146459 | 23 | 77 | | |
| 0 | vector VII | E | Half C | 144625 | 144643 | 76 | 24 | | |
| 0 | vector VIII | F | Half A | 146443 | 146468 | 64 | 36 | | |
| 0 | vector IX | P | Half A | 146691 | 146677 | 82 | 18 | | |
| 0 | vector X | Q | Half B | 143818 | 143844 | 58 | 42 | | |
| 1 | Vector IV and V | I (1:1) | BC | 144367 | 144352 | | | 96 | 4 |
| 1 | Vector IV and | J (1:1) | BC | 144354 | 144382 | | | 96 | 4 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | VII | | | | | | | | |
| 2 | Vector IV, V and VI | K(1:1:1) | BC + AB | 144367 + 145276 | 144351+ 145260 | | | 38 + 47 | 15 (A + C) |
| | | S(2:1:1) | BC + AB | 144367 + 145276 | 144371 + 145277 | | | 42 + 55 | 3 (BB) |
| 2 | Vector IV, VII and VIII | L (1:1:1) | BC + AB | 144354 + 145263 | 144346 + 145255 | | | 16 + 60 | 24 (A + C) |
| | | T (2:1:1) | BC + AB | 144354 + 145263 | 144385 + 145292 | | | 58 + 39 | 3 (BB) |

It was previously demonstrated that CH3-engineered Ig heavy chains encoded by constructs 1 and 2 are still able to form homodimers when expressed alone in single cells (WO2009/089004). However, WO2009/089004 further reports that CH3 domains that are engineered to comprise triple charge pair mutations, such as present in construct 3, are no longer capable of forming homodimers when expressed alone.

In the present study, these findings were only partly confirmed. Indeed, the results of transfections B, C and D demonstrated the presence of full IgGs, in addition to a high proportion of unpaired half molecules, demonstrating some homodimerization of CH3 domains encoded by constructs 1 and 2. Transfections E and F also resulted in production of full IgGs in addition to unpaired half molecules, demonstrating that the triple charge mutations of construct 3 do not fully impair homodimerisation.

It was furthermore demonstrated that also the 'knob' and 'hole' CH3 variants of constructs 6 and 7 form homodimers (18% homodimers for 'knob-knob' and 42% homodimers for 'hole-hole').

CH3 variants that fully prevent homodimerisation when expressed alone are preferred, to prevent or minimize undesired byproducts (homodimers) upon co-expression with a second CH3 variant for heterodimerization.

Interestingly, the present experiments demonstrate for the first time that also mixtures of bispecific antibodies can be expressed in single cells with virtually no homodimers in the mixture. Transfections K and L clearly show that the expected bispecific species BC + AB are indeed obtained (38% + 47% in transfection K, and 16%

+ 60% in transfection L). In both transfections a relatively high percentage of undesired half molecules was observed (15% half molecule A + half molecule C in transfection K, and 24% half molecule A + half molecule C in transfection L). The relatively high percentage of half molecules still present was attributed to low amounts of matching heavy chains of vector IV due to unbalanced expression of heavy chains in a matched pair. Therefore, transfections were repeated with an adjusted ratio of vector DNA, 2:1:1, in transfections S and T. This resulted in equal amounts of IgG heavy chains constituting a matched pair and pure mixtures of bispecific IgG without the presence of half IgG molecules and with as little as 3% homodimeric BB present. Ideally, this low proportion of contaminating monospecific product should be reduced to essentially zero. It is therefore desired to find additional CH3-mutants that would result in mixtures of bispecific antibodies with minimal contaminating monospecific antibodies present.

The present study demonstrates for the first time that essentially pure mixtures of two bispecific antibodies recognizing 3 different target epitopes can be produced in a single cell, with minimal presence of monospecific antibodies in the mixture.

**Example 12: varieties of mixtures**

As it was demonstrated that production of mixtures of 2 bispecific antibodies recognizing 3 epitopes from a single cell, or production of mixtures of 2 or 3 monospecific antibodies from a single cell is technically feasible, we next explored the feasibility of controlled production of a variety of other mixtures. A fourth antibody VH region with known specificity and known ability to pair with the human IGKV1-39 light chain will be used for recloning into vectors containing constructs 1-3 or 7 of Table 1, resulting in vectors I', II', III' or X' (the ' indicating a different specificity as compared to corresponding vector numbers). The resulting vectors I'-III', X' and IV-IX, each containing nucleic acid sequences encoding for the common human light chain as well as an Ig heavy chain with different CH3 region and different VH specificity, will subsequently be transfected into cells, in combination with other construct vectors to obtain a variety of mixtures of bispecific and/or monospecific antibodies. The variety of mixtures that will be obtained include mixtures of 2 bispecific antibodies recognizing 4 epitopes, 2 bispecific antibodies and one

monospecific antibody, or mixtures of 1 bispecific and one monospecific antibody from a single cell. Table 6 depicts the transfection schedule and expected results.

Table 6

| **Variety of mixture** | **Transfect -ion of** | **Transfecti on code and ratio** | **Expecte d species** | **Expected % monospecifi c IgG** | **Expected % Bispecific** |
|---|---|---|---|---|---|
| 2 BsAbs, 4 epitopes | IV+V+IX+ X’ | ZA (1:1:1:1) | BC + AD | 0 | 50 + 50 |
| 2 BsAbs, 4 epitopes | IV+VII+IX +X’ | ZB (1:1:1:1) | BC + AD | 0 | 50 + 50 |
| 2 bsAbs + 1 mAb | IV+V+VI+ wt’ | ZC (2:1:1:2) | BC + AB + DD | 33 | 33 + 33 |
| 2 bsAbs + 1 mAb | IV+V+VI+ II’ | ZD (2:1:1:2) | BC + AB + DD | 33 | 33 + 33 |
| 2 bsAbs + 1 mAb | IV+V+VI+ III’ | ZE (2:1:1:2) | BC + AB + DD | 33 | 33 + 33 |
| 1 bsAb + 1 mAb | IV+V+wt’ | ZF (1:1:2) | BC + DD | 50 | 50 |
| 1 bsAb + 1 mAb | IV+V+II’ | ZG(1:1:2) | BC + DD | 50 | 50 |
| 1 bsAb + 1 mAb | IV+V+III’ | ZH(1:1:2) | BC + DD | 50 | 50 |
| 1 bsAb + 1 mAb | IV+VII+wt ’ | ZI (1:1:2) | BC + DD | 50 | 50 |
| 1 bsAb + 1 mAb | IV+VII+II’ | ZJ (1:1:2) | BC + DD | 50 | 50 |
| 1 bsAb + 1 mAb | IV+VII+III ’ | ZK (1:1:2) | BC + DD | 50 | 50 |
| 1 bsAb + 1 mAb | IX+X+wt’ | ZL (1:1:2) | AB + DD | 50 | 50 |
| 1 bsAb + 1 | IX+X+II’ | ZM (1:1:2) | AB + DD | 50 | 50 |

| | | | | | |
|---|---|---|---|---|---|
| mAb | | | | | |
| 1 bsAb + 1 mAb | IX+X+III' | ZN (1:1:2) | AB + DD | 50 | 50 |

Although, theoretically, production of all mixtures should be feasible, it is known from previous work by others that large scale production of classical knob-into-hole variants is hampered by instability issues. Mixtures resulting from transfections ZA, ZB, ZL, ZM and ZN are thus expected to become problematic when transferred to larger scale production.

Thus, the current set of constructs present in Table 1 would not allow production of all theoretical mixtures from single cells at a larger scale, as knob-into-hole variants are reported to be unstable, and it cannot be excluded that CH3 domains comprising a 'knob' or a 'hole' will dimerize with either charge variants or wildtype CH3 domains. It is thus desired to design new CH3-variants that are engineered to preferentially form homodimers or heterodimers only and which will not homo- or heterodimerize with constructs 1-5 of Table 1 as to allow for co-expression in single cells.

**Example 13: identification of novel charge pair mutants**

The objective of this study was to engineer the IgG CH3 region to result in the production of only heterodimers or only homodimers upon mixed expression of different IgG heavy chains in a single cell, wherein the novel engineered CH3 domains will not homo- or heterodimerize with known engineered CH3 domains, or with wildtype CH3 domains. Therefore, as a first step in identifying novel engineered CH3 domains that would meet the criteria, many interface contact residues in the IgG CH3 domain were scanned one by one or in groups for substitutions that would result in repulsion of identical heavy chains – i.e., reduced homodimer formation - via electrostatic interactions. The objective was to obtain a list of residues that, when substituted by a charged residue, would result in repulsion of identical chains such that these mutations may be used to drive homo- and/or heterodimer formation upon mixed expression of different IgG heavy chains, whereby the obtained full length IgGs are stable and are produced with high proportions. In a follow up, the identified substitutions will be used to generate bispecific antibodies or mixtures of bispecific or monospecific antibodies by engineering matched pairs of CH3 residues in one or more

IgG heavy chains - CH3 regions. Additionally, newly identified charge mutant pairs may be combined with existing pairs, such that multiple nucleic acid molecules encoding different heavy chains, all carrying different and complementing CH3 mutations, can be used for expression in cells such that mixtures of monospecific antibodies only, or bispecific antibodies only, or mixtures of defined monospecific and bispecific antibodies can preferentially be obtained. The residues to be tested in the present study are contact residues as previously identified (Deisenhofer J., 1981; Miller S., 1990; Padlan, 1996, Gunasekaran, 2010). The rationale for this approach is that repulsive charges are engineered into each available pair of contacting residues. Samples are subsequently analyzed on non-reducing SDS-PAGE to identify pairs in which dimer formation is reduced, as visualized by the presence of bands of approximately 72 kD. All available pairs will be screened as single mutations or in combination with a single other mutation as the repulsive electrostatic interaction between one non-matching pair may or may not be sufficient to result in sufficient amounts of half-molecules for detection by this method, the mutations are also combined.

Amino acid substitutions were introduced in construct vector MV1057 by Geneart according to the table 7 and expression of constructs was performed by transfection in HEK293T cells, according to standard procedures. IgG expression levels were measured in Octet. When production failed twice, the mutation was considered to be detrimental to expression and the mutation was not pursued further.

Table 7: list of amino acid substitutions in the various constructs that were made (EU numbering)

| **AA substitutions in CH3** | **construct #** | **Effect on homodimer formation (- = no effect; +++ = max. inhibition; NT= not tested on gel)** |
|---|---|---|
| Q347K | 8 | - |
| Y349D | 9 | +- |
| Y349K | 10 | +- |

| **AA substitutions in CH3** | **construct #** | **Effect on homodimer formation (- = no effect; +++ = max. inhibition; NT= not tested on gel)** |
|---|---|---|
| T350K | 11 | - |
| T350K, S354K | 12 | +- |
| L351K, S354K | 13 | +- |
| L351K, T366K | 14 | ++ |
| L351K, P352K | 15 | +- |
| L351K, P353K | 16 | ++ |
| S354K, Y349K | 17 | ++ |
| D356K | 18 | - |
| E357K | 19 | - |
| S364K | 20 | ++ |
| T366K, L351K | 21 | ++ |
| T366K, Y407K | 22 | +++ |
| L368K | 23 | NT |
| L368K, S364K | 24 | ++ |
| N390K, S400K | 25 | +- |
| T394K, V397K | 26 | + |
| T394K, F405K | 27 | +++ |
| T394K, Y407K | 28 | +++ |
| P395K, V397K | 29 | +- |
| S400K | 30 | - |
| F405K | 31 | +++ |
| Y407K | 32 | ++ |
| Q347K, V397K, T394K | 33 | + |
| Y349D, P395K, V397K | 34 | + |
| T350K, T394K, | 35 | NT |

| **AA substitutions in CH3** | **construct #** | **Effect on homodimer formation (- = no effect; +++ = max. inhibition; NT= not tested on gel)** |
|---|---|---|
| V397K | | |
| L351K, S354K, S400K | 36 | + |
| S354K, Y349K, Y407K | 37 | +- |
| T350K, N390K, S400K | 38 | +- |
| L368K, F405K | 39 | ++ |
| D356K, T366K, L351K | 40 | +++ |
| Q347K, S364K | 41 | +++ |
| L368D, Y407F | 42 | + |
| T366K | 43 | + |
| L351K, S354K, T366K | 44 | + |
| Y349D, Y407D | 45 | + |
| Y349D, S364K, Y407D | 46 | + |
| Y349D, S364K, S400K, T407D | 47 | + |
| D399K | 48 | +- |
| D399R | 49 | +- |
| D399H | 50 | +- |
| K392D | 51 | +- |
| K392E | 52 | +- |
| K409D | 53 | + |

Supernatants containing ≥5 µg/ml IgG were analyzed in SDS-PAGE and IgG was purified using protein A. The proteins were stained using colloidal blue. Homodimers were visible as a band of approximately 150 kD. Smaller bands of approx 75 kD represented the presence of half molecules (see negative control: K392D, K409D). Blots are shown in Figure 7.

The results of SDS-PAGE gels were analyzed and scored as presented in table 7, right hand column. A number of residues were considered promising for further testing in combination, including residues Q347, S354, Y349, L351, K360, T366, T394, and V397. The choice was based on high scores in the inhibition of formation of homodimers combined with the availability of contacting residues that can be modified without running into issues such as other non-complementary charges. For example, it is known that residues F405 and Y407 have multiple interactions at the CH3-CH3 interface, including interactions with residues that are already charged, which may be problematic after introduction of multiple charge mutations among these interacting residues (see Table A). New constructs were made in vector MV1057 (Table 8), and antibody VH regions with known specificities and known ability to pair with the human IGKV1-39 light chain were used for recloning into vectors containing these new constructs (see Table 9) such that combinations could further be tested. Table 10 depicts the transfection schedules and results.

Table 8:

| **AA substitutions in CH3** | **construct #** |
|---|---|
| L351K | 61 |
| T394K | 62 |
| L351D | 63 |
| T366D | 64 |
| S354D, Y349D | 65 |
| V397D | 66 |
| K360D | 67 |

Table 9: VH specificity inserted in different constructs

| Vector | VH gene | Antigen specificity | VH mass (Da) | Cloned in construct # |
|---|---|---|---|---|
| XI | IGHV 1.08 | Tetanus (A) | 13703 | 8 |
| XII | IGHV 1.08 | Tetanus (A) | 13703 | 17 |
| XIII | IGHV 1.08 | Tetanus (A) | 13703 | 43 |
| XIV | IGHV 1.08 | Tetanus (A) | 13703 | 61 |
| XV | IGHV 1.08 | Tetanus (A) | 13703 | 62 |
| XVI | IGHV 3.30 | Fibrinogen (C) | 12794 | 63 |
| XVII | IGHV 3.30 | Fibrinogen (C) | 12794 | 64 |
| XVIII | IGHV 3.30 | Fibrinogen (C) | 12794 | 65 |
| XIX | IGHV 3.30 | Fibrinogen (C) | 12794 | 66 |
| XX | IGHV 3.30 | Fibrinogen (C) | 12794 | 67 |

Table 10:

| Transfection of | Transfection code (ratio) | Expected species | AA found (%) | AC found (%) | CC found (%) | Half A found (%) | Half C found (%) | other (%) |
|---|---|---|---|---|---|---|---|---|
| XIII + XVI | ZO (1:1) | AC | 0 | 69 | 7 | 24 | 0 | 0 |
| | ZT (3:1) | AC | 10 | 45 | 16 | 27 | 0 | 0 |
| | ZU (1:1) | AC | 5 | 61 | 10 | 13 | 0 | 0 |
| | ZV (1:3) | AC | 3 | 61 | 23 | 13 | 0 | 0 |
| | ZW (1:1) | AC | 0 | 88.3 | 2.4 | 7 | 0 | 2.3 |
| XIV + XVII | ZP | AC | 30 | 52 | 13 | 0 | 0 | 5 |
| XII + XVIII | ZQ | AC | 4 | 51 | 33 | 2 | 1 | 8 |
| XV + XIX | ZR | AC | 20 | 42 | 11 | 0 | 1 | 26 |
| XI + XX | ZS | AC | 34 | 41 | 15 | 0 | 0 | 10 |

Combinations of CH3 variants were expressed, and analyzed in SDS-PAGE (data not shown) and in native mass spectrometry (MS). Results are summarized in Table 10.

The ZO transfection resulted in the highest proportion of heterodimers in the mixtures (69% AC). Interestingly, in the ZO transfection, the AA homodimer was not present whereas the CC homodimer comprised a small proportion (7%). Mass spectrometric analysis unveiled that the remaining protein in the mixture consisted of half A molecules, probably resulting from unequal expression of the A and C heavy chains. The raw MS data from transfection sample ZO are shown in Figure 8. Surprisingly, whereas transfection ZO resulted in fair amounts of bispecific product, the reverse charge pair of transfection ZP (L351K/T366'D versus T366K/L351'D of ZO) did not result in similar results, and only 52% of bispecific product was observed, with considerable amounts of the two homodimers being present (30% AA and 13% CC). An explanation for this may be that the negatively charged D structurally closely resembles T, hence the T366D may not be potent enough to repulse itself and T366D will thus still form homodimers, as was indeed observed.

It can be envisaged that subtle variants of the newly found T366K/L351'D pair (e.g. by testing all permutations including new constructs T366R and L351E) may result in similar percentages of BsAbs.

**Example 14: HADDOCK for design of new CH3 mutants to drive efficient heterodimerization.**

As described in example 13, the newly found charge pair T366K/L351'D increases the proportion of heterodimers in the mixture (69 %) with a small fraction of undesired CC homodimers (7%) (L351D/L351'D) and a substantial fraction of half A molecules (24%) 'contaminating' the mixture. In this example, an in silico approach was used to generate further insight in amino acid residues involved CH3 interface interactions, to test complementary substitutions in opposing CH3 regions and to find novel CH3 pairs containing complementary substitutions that further increase efficient heterodimerization while preventing efficient formation of homodimers of the two heavy chains.

HADDOCK (High Ambiguity Driven protein-protein DOCKing) is an information-driven flexible docking approach for the modeling of biomolecular complexes. HADDOCK distinguishes itself from ab-initio docking methods in the fact that it encodes information from identified or predicted protein interfaces in ambiguous interaction restraints (AIRs) to drive the docking process. (de Vries et al., 2010).

The input for the HADDOCK web server consists of a protein structure file, which can be a crystal structure, NMR structure cluster or a modeled structure. After the docking or refinement, HADDOCK returns a so-called HADDOCK score, which is a weighted average of VanderWaals energy, electrostatic energy, buried surface area and desolvation energy. The HADDOCK score can be interpreted as an indication of binding energy or affinity, even though a direct translation to experimental data is often hard to achieve. In addition to this, HADDOCK provides structure files for the 'top four' structures that resulted from the docking run. These structure files can be downloaded and visualized, enabling the detailed analysis of the interactions of the individual residues.

In this example, the interactions between the CH3-domains of the IgG1 heavy chains were studied. A high-resolution crystal structure of the Fc part of the IgG (structure 1L6X) was used as starting structure (http://www.rcsb.org/pdb/explore/explore.do?structureId=1l6x ; Idusogie, E.E. et al., J.I. 2000(164)4178-4184).

In example 13, it was found that co-transfection of vectors XIII and XVI resulted in the formation of the CC homodimeric contaminant (Table 10). HADDOCK was used to search for additional mutations to the T366K/L351'D pair that prevent homodimerization.

The HADDOCK output consists of a set of calculated energies, a HADDOCK score (which is a weighted average of the energies) and four structure files corresponding to the four lowest-energy structures found by the program. The HADDOCK-scores are used to compare different structures; the other energies are merely used to get an indication about what is happening in the structures (e.g. good electrostatic interactions, smaller buried surface, high Van der Waals energy). The lower the HADDOCK score, the better. For each mutation pair, the scores were calculated for the AA, AB and BB dimers.

Sets of mutation pairs from example 12 were run in HADDOCK to see whether the calculated energies would correlate to the experimental data. Table 11 presents all theoretical energies, which are visualized in Figure 9.

Table 11:

| **Construct combinations** | **HADDOCK** | **VdW** | **Electrostatic** | **Desolvation** | **Buried** |
|---|---|---|---|---|---|

| | **Score** | **energy** | **energy** | **energy** | **surface area** |
|---|---|---|---|---|---|
| Wildtype-wildtype | -208.2 | -62.8 | -773 | 9.2 | 2505.8 |
| 1-2 (E356KD399K – K392DK409D) | -225.8 | -56.4 | -862 | 3 | 2458.3 |
| 2-2 (K392DK409D – K392DK409D) | -180.3 | -67.9 | -562.1 | 0.1 | 2312.5 |
| 1-1 (E356KD399K – E356KD399K) | -176.7 | -75.5 | -469.3 | -7.3 | 2349.6 |
| 1-3 (E356KD399K – K392DK409DK439D) | -220.6 | -67.9 | -793.8 | 6.1 | 2499.8 |
| 3-3 (K392DK409DK439D - K392DK409DK439D) | -150.1 | -76.6 | -387.6 | 4.1 | 2261.2 |
| 6-7 (T366W – T366SL368AY407V) | -221.3 | -65.8 | -735.5 | -8.3 | 2509.0 |
| 6-6 (T366W – T366W) | 1916.9* | 2072.3 | -681.3 | -19.2 | 2499.9 |
| 7-7 (T366SL368AY407V – T366SL368AY407V) | -191.9 | -55.0 | -683.2 | -0.2 | 2427.2 |
| 43-63 (T366K – L351D) | -210.6 | -64 | -758.4 | 5.1 | 2456.5 |
| 43-43 (T366K – T366K) | -191.7 | -71.2 | -634.1 | 6.3 | 2533.5 |
| 63-63 (L351D – L351D) | -212.5 | -60.4 | -774 | 2.6 | 2445.6 |

*this value is unusually high due to high VanderWaals energy score, probably due to steric clash of T366W/T366'W

With 2 wildtype CH3 domains, the HADDOCK scores are the same for AA, AB and BB because the A and B CH3 regions are identical. In most other cases, the AB pair has the lowest score, which is as expected. For the T366K/L351D pair the BB score is slightly better than the AB score (-210.6 vs. -212.5), but this difference is within the error of the calculations. Using HADDOCK, the structures of the heterodimers of these pairs were visualized. For example, the construct combinations 1-2, 1-1 and 2-2 are presented in Figure 10. From these visualizations it is apparent that salt bridges are formed in the heterodimer (Figure 10A *left hand panel*) whereas electrostatic repulsion occurs between residues of identical chains (Figure 10B and C, *middle and right hand panel*). The higher HADDOCK scores for the homodimers can thus be explained by the electrostatic repulsion of the mutated interface residues. These residues have to bend away from each other and don't have interaction with residues on the other chain, causing a drop in the affinity.

Table 11 and Figure 9 confirm what was observed in example 13. The T366K/L351'D AC heterodimer and the L351D/L351'D CC homodimer form with a similar energy, explaining the presence of both the heterodimer and homodimer in the mixture. The T366K/T366'K AA homodimer, on the other hand, is barely detectable in the mixture although T366K half A molecules are present. Table 11 and Figure 9 indeed show that the HADDOCK score for the T366K/T366'K AA homodimer is higher than the score for the AC heterodimer; hence formation of this homodimer is energetically less favorable.

**Example 15: 366/351 variations**

In example 13, it is hypothesized that alternatives for the T366K/L351'D mutant charge pair can be designed that may have similar results in terms of percentage of bispecific antibodies in the mixture. Alternatives may include substitutions T366R, T366D, T366E, L351E, L351K and L351R. The proportion of CC homodimers of L351D/L351'D may be diminished by creating variants of the 366/351 pair. All possible mutation pairs were run in HADDOCK and the resulting scores are presented in Table 12 and visualized in Figure 11.

Table 12

| **Construct combinations** | **HADDOCK Score** | **VdW energy** | **Electrostatic energy** | **Desolvation energy** | **Buried surface area** |
|---|---|---|---|---|---|
| T366K – L351D | -210.6 | -64 | -758.4 | 5.1 | 2456.5 |
| T366K – T366K | -191.7 | -71.2 | -634.1 | 6.3 | 2533.5 |
| L351D – L351D | -212.5 | -60.4 | -774 | 2.6 | 2445.6 |
| T366K – L351E | -216.9 | -55.7 | -854.7 | 9.8 | 2532.7 |
| L351E – L351E | -217.9 | -65.5 | -802.2 | 8 | 2532 |
| T366R – L351D | -210.5 | -68.8 | -760.8 | 10.4 | 2514.5 |
| T366R – T366R | -201.8 | -77.4 | -626.4 | 0.9 | 2608 |
| T366R – L351E | -225.8 | -56.2 | -874.8 | 5.4 | 2579.2 |
| T366D – L351R | -211.2 | -71.3 | -723.6 | 4.8 | 2455.6 |
| T366D – T366D | -198.1 | -58.1 | -713.4 | 2.1 | 2477 |

| | | | | | |
|---|---|---|---|---|---|
| L351R – L351R | -220.7 | -75.5 | -806.5 | 16.1 | 2552.2 |
| T366D – L351K | -223.9 | -62.1 | -810.1 | 0.3 | 2487.8 |
| L351K – L351K | -224.4 | -75.6 | -812.1 | 13.6 | 204.5 |
| T366E – L351R | -222.3 | -69 | -783 | 3.4 | 2557.2 |
| T366E – T366E | -201.9 | -57.6 | -741 | 4 | 2487.5 |
| T366E – L351K | -215.9 | -58.4 | -808.9 | 4.3 | 2486 |

When looking at the HADDOCK scores, it was observed that some of the mutations have a similar 'pattern' when compared to T366K/L351'D. For most permutations the AA homodimer was found to have a higher HADDOCK-score than the AB heterodimer, but the BB homodimer appeared as favorable as the AB heterodimer. Even though the 351 residue is known to be a 'neighbor' to itself on the other chain, i.e. residue 351 of chain A pairs with residue 351 of chain B at the CH3-CH3 interface, there is barely a negative influence of the identical charges when the BB dimer is formed. Looking at the L351D/L351'D structure this is explained by the aspartic acids bending away from each other and the stabilizing influence of at least the naturally occurring Arginine at position 355 and also some stabilization of negative charge by the naturally occurring Serine at position 354 (see Figure 12A). Mutation of these residues (S354A and R355D) provides only little improvement. From figure 12B it is clear that the backbone-hydrogen of A354 causes stabilization of the homodimer. From this series, the T366R/L351'E pair seems to be the most favorable, with the lowest HADDOCK score for the bispecific molecule.

**Example 16: mutations around T366K/L351'D**

In the series of HADDOCK analyses in this example, the T366K/L351'D or T366K/L351'E pair were taken as a starting structure. In order to identify additional mutations that would further increase the predicted percentage of bispecifics of these A and B chains, additional mutations on the B-chain were used to calculate the HADDOCK-scores and energies. When the structure of the CH3 domain is studied using a viewer for visualization of protein structures at a molecular level (YASARA, www.yasara.org), one can calculate the distances between individual residues. While doing so, it was observed that the two residues Y349 and L368 are neighboring residues that may contribute positively or negatively to dimer interactions and these

have been mutated in this example –in addition to the L351D mutation– to study the result on dimer formation of the homo- and heterodimers (see figure 13). Both residues seem to add to the stability of the heterodimer (lower HADDOCK scores) as well as to the destabilization of the BB dimer (higher HADDOCK scores). Glutamic acids (E) on positions 349 and 368 seem to be more favorable than aspartic acids (D). Thus, introduction of a second amino acid substitution in the B-chain, comprising already the amino acid substitution at position 351, seems to favor heterodimerization further.

In a next set of HADDOCK analyses, the T366K/L351'D pair was again taken as starting structure. In addition to the substitutions in the B chain that further increased heterodimerization (i.e. Y349D/E and L368E), additional mutations were added to the A-chain which already comprises the T366K substitution. As shown in Figure 14, there are several mutation pairs that seem favorable towards the formation of bispecific heterodimers. In the T366K-L351K/L351'D-Y349'D pair, all four mutated residues are involved in the heterodimeric pairing, which is not de case for T366K-L351K/L351'E-L368'E in which K351 is not directly involved in the binding. However, the HADDOCK-score for this latter heterodimer is -228.9; significantly lower than the -214.2 for the T366K/ L351'E-L368'E, which can be explained by hydrogen bonding interactions of the K at position 351 (see Figure 15). The T366K-L351K/L351'D-Y349'D pair may be further improved by the R355'D mutation in the B-chain, which results in a higher BB-HADDOCK score, but also the AB HADDOCK score is slightly higher. Overall the additional L351K results in lower AB scores and similar AA and BB scores when compared to the sole T366K mutation in the A chain. Theoretically this would result in higher amounts of bispecific heterodimers in the samples.

As is apparent from figure 11, having an R rather than a K at position 366 may be more potent in driving heterodimerization. Therefore, some of the HADDOCK analyses shown in figure 13 were repeated but now with T366R rather than T366K in the A-chain. It was demonstrated that it is not favourable to combine an R366 in chain A with double mutations in chain B (figure 16). This may be due to the large size of this residue, interfering with other interface interactions, even though all the expected salt-bridges with R366 are present in the structures. Also, the HADDOCK score for the AA homodimer is lower for R366 than for K366, which also doesn't

contribute favorably to heterodimer formation. Therefore no further HADDOCK analyses were performed using R366 in the interface.

A total of 14 best performing pairs, according to HADDOCK predictions, have been selected (see Table 13 and Figure 17). In some pairs, an R355D substitution is included to remove the stabilizing influence of the naturally occurring R355 on the L351/L351'D interaction.

Table 13:

| **Construct combinations** | **HADDOCK Score AB** | **HADDOCK Score AA** | **HADDOCK Score BB** |
|---|---|---|---|
| Wildtype-wildtype | -208.2 | -208.2 | -208.2 |
| T366K – L351D | -210.6 | -191.7 | -212.5 |
| T366K – L351E | -216.9 | -191.7 | -217.9 |
| T366R – L351E | -225.8 | -201.8 | -217.9 |
| T366E – L351R | -222.3 | -201.9 | -220.3 |
| T366K – L351DY349E | -215.9 | -191.7 | -190 |
| T366K – L351DL368E | -223.3 | -191.7 | -198.9 |
| T366K – L351EY349E | -214.5 | -191.7 | -187.5 |
| T366KL351K – L351D | -233.2 | -205 | -212.5 |
| T366K – L351DY349EL368E | -207.5 | -191.7 | -179.5 |
| T366KL351K – L351DY349D | -255.2 | -205 | -204.3 |
| T366KL351K – L351DY349E | -227.2 | -205 | -190 |
| T366KL351K – L351DL368E | -243.9 | -205 | -198.9 |
| T366KL351K – L351DR355D | -233.6 | -205 | -211.9 |
| T366KL351K – L351DY349DR355D | -242.8 | -205 | -183.5 |
| T366D – L351KY349K | -237.9 | -198.1 | -228.4 |

**Example 17: in vitro expression of bispecifics using CH3 mutants based on HADDOCK predictions**

The analysis in example 16 suggested that some CH3 variants with additional mutations around the T366K/L351'D pair would yield mixtures with higher proportions of the bispecific component and potentially lower proportions of the homodimeric component. These best performing pairs were selected for production and further analysis. In addition, the constructs T366R and L351E were also generated. Table 14 lists the constructs that were made and which were used for recloning antibody VH regions with known specificities and known ability to pair with the human IGKV1-39 light chain. Expression of the IgGs that contain the individual constructs was previously reported in example 13, and was repeated for the constructs as listed in Table 14. Aim was to assess which of the constructs homodimerize in the absence of a matching heterodimerization partner. Ideally, high percentages of half bodies would be formed and low percentages of homodimers. As a control, constructs containing previously reported charge mutations and constructs containing the previously reported knob-in-hole mutations were also used for expression as whole IgG by recombinant cells. Protein A purified supernatants were analyzed in SDS-PAGE; results were analyzed and scored as presented in Table 14

Table 14:

| **AA substitutions in CH3** | **Construct #** | **% IgG** | **% half molecule** |
|---|---|---|---|
| E356K, D399K | 1 | 64.2 | 35.8 |
| K392D, K409D | 2 | 30.9 | 69.1 |
| K392D, K409D, K439D | 3 | 24.5 | 75.5 |
| T366W | 6 | 27.6 | 72.4 |
| T366S, L368A, Y407V | 7 | 58.6 | 41.4 |
| T366K | 43 | 32.9 | 67.1 |
| L351D | 63 | 89.8 | 10.2 |
| T366D | 64 | 89.6 | 10.4 |

| | | | |
|---|---|---|---|
| T366K, L351K | 68 | 34.7 | 65.3 |
| L351D, L368E | 69 | 83.7 | 16.3 |
| L351E, Y349E | 70 | 67.8 | 32.2 |
| L351D, Y349E | 71 | 79.7 | 20.3 |
| L351D, R355D | 72 | 100 | - |
| L351D, Y349E, L368E | 73 | 79.3 | 20.7 |
| L351D, Y349D | 74 | 88.6 | 11.4 |
| L351D, Y349D, R355D | 75 | 89.9 | 10.1 |
| L351K, L368K | 76 | 56.6 | 43.4 |
| L351R | 77 | 100 | - |
| T366E | 78 | 44.4 | 55.6 |
| T366R | 79 | 29.6 | 70.4 |
| L351E | 80 | 100 | - |

The results of co-expression of a common light chain and two different heavy chains carrying the amino acid substitutions of constructs shown in Table 14 or heavy chains carrying the amino acid substitutions of previous constructs are presented in Table 15. Expression of two different heavy chains comprising the amino acid substitutions T366K and L351'D:L368'E respectively resulted in approximately 87% of the bispecific AB heterodimer in the mixture with no AA or BB homodimers present (combination nr. 3 of Table 15). About 12% half molecules (half A) comprising the T366K substitution was observed. Furthermore, it was found that the percentage of bispecific AB heterodimer increased when the additional amino acid substitution L351K was introduced in the first heavy chain. For example, co-expression of two different heavy chains comprising the amino acid substitutions T366K:L351K and L351'D:L368'E respectively resulted in approximately 92% of bispecific AB heterodimer whereas AA and BB homodimers are essentially absent in the mixture (combination nr. 12 of Table 15). Combinations 10 and 11 also resulted in favorable distributions of high percentages heterodimers and virtually absence of homodimers. The absence of homodimers is advantageous, because the fraction containing the intact IgG molecules is composed of AB heterodimer only. For purification and subsequent therapeutic application, the half molecules can be removed by standard approaches such as size exclusion chromatography. Hence, applying these newly

identified charge mutants in the production process for generating bispecific antibodies provides advantages over known charge mutants and knobs-into-holes mutants where the presence of 'contaminating' homodimeric antibodies is not excluded. In addition, the T366K/L351'D:L368'E and T366K:L351K/ L351'D:L368'E charge pairs have an additional advantage over the previously described E356K:D399K/K392'D:K409'D and E356K:D399K/K392'D:K409'D:K439'D charge reversal pairs, in that the previously described charge variants are based on the reversal of existing charges within the CH3-CH3 interface whereas the newly identified charge variants are adding additional charge pairs (charge-charge interactions) to the CH3-CH3 interface. The introduction of additional charge pairs in the CH3-CH3 interface may further increase the stability of the interface and thereby of the intact antibody. The same holds true for the mutations used in combinations nrs. 4, 5, 6, 9, 10, and 11, which also resulted in favorable proportions of bispecific heterodimer with exceedingly low proportions of AA and BB homodimers present in the mixtures.

Table 15:

| Combination of 2 different heavy chains | chain A* / mutations (construct #) | chain B**/ mutations (construct #) | % AA found | % AB found | % BB found | % half A found | % half B found |
|---|---|---|---|---|---|---|---|
| 1 | T366E (78) | L351R (77) | 3 | 81 | 2 | 13 | 0 |
| 2 | T366K (43) | L351D (63) | 0 | 88 | 3 | 9 | 0 |
| 3 | T366K (43) | L351D,L368E (69) | 0 | 87 | 0 | 12 | 0 |
| 4 | T366K (43) | L351E,Y349E (70) | 2 | 85 | 0 | 11 | 0 |
| 5 | T366K (43) | L351D,Y349E (71) | 2 | 92 | 1 | 5 | 0 |
| 6 | T366K (43) | L351D,Y349E,L368E (73) | 0 | 96 | 1 | 4 | 0 |
| 7 | T366K,L351K (68) | L351D (63) | 0 | 77 | 12 | 10 | 1 |
| 8 | T366K,L351K (68) | L351D,R355D (72) | 0 | 79 | 8 | 10 | 1 |
| 9 | T366K,L351K (68) | L351D,Y349D,R355D (75) | 1 | 93 | 2 | 4 | 1 |
| 10 | T366K,L351K (68) | L351D,Y349D (74) | 1 | 95 | 1 | 3 | 0 |
| 11 | T366K,L351K (68) | L351D,Y349E (71) | 1 | 95 | 0 | 3 | 1 |
| 12 | T366K,L351K (68) | L351D,L368E (69) | 0 | 92 | 0 | 8 | 0 |
| 13 | T366K (43) | L351E (80) | 0 | 70 | 10 | 18 | 2 |
| 14 | T366R (79) | L351E (80) | 4 | 38 | 36 | 21 | 1 |
| 15 | T366D (64) | L351K, L368K (76) | 3 | 92 | 2,5 | 2,5 | 0 |
| 16 | T366D (64) | L351R (77) | 30 | 69 | 1 | 0 | 0 |

* chain A carries specificity of MF1337 (=tetanus toxoid); ** chain B carries specificity of MF1122 (=fibrinogen)

**Native MS**

Native MS was performed on all bispecific samples. The obtained graphs were analyzed to determine the relative ratio's of the present species in two ways: by peak height and by peak area. Peak area is the more scientifically correct way of analysis, but since all previous analyses for other studies were done based on peak height, both methods were included in the analysis, for comparison purposes. The differences

between the methods were within the error of measurement, and therefore only the peak area values were used for future measurements. Two typical spectra are shown in Figure 18. An overview of the results is shown graphically in Figure 19, the numerical values can be found in Table 15. In about half of the samples the total contamination of monospecific lgG is less than 5%, and only in three cases it is > 10% while for wt lgG it is expected to find about 50% of monospecific lgG in the mixture.

A panel of ten combinations of 2 different heavy chains was selected from Table 15 for further analyses. These ten combinations included combinations 1, 2, 3, 4, 5, 6, 9, 10, 11 and 12 (Table 15). Selection of these ten was based on low percentages of homodimers present in the mixtures as determined by nMS, but also based on their overall physico-chemical properties, including production yields, SDS-PAGE, as well as the number of mutations present in the CH3 domain.

**Example 18: IgG stability analyses**

In this study, a series of CH3 mutation pairs that resulted in high proportions of bispecific heterodimers in the intact IgG fraction and very low amounts (<5%) of parental IgGs will be further analyzed for stability of the Fc part of the IgG molecule. The mutated CH3 domains that are used to promote the heterodimerization of the heavy chains may have unexpected destabilizing effects on the Fc region of the IgG, that may result in undesirable properties such as a reduction of *in vivo* half life, reduction in effector function and/or an increase in immunogenicity. The newly identified charge pairs will be compared to wildtype bispecifics and a bispecific containing previously identified charge mutations (chain A comprising construct 1 and chain B comprising construct 2). All bispecifics in this study will contain the same heavy and light chain variable regions, ensuring that the observed effects are caused by mutations in the Fc-part of the molecule and not by variation in the variable regions.

A series of stability studies will be performed on these bispecifics. These studies include spectroscopic (UV-Vis absorbance, fluorescence and light-scatter) and microscopic (light and fluorescence microscopy with Nile Red staining) analyses that provide information on the aggregation state of the CH3 variants.

The UV-Vis absorbance spectra will be recorded with a double beam, two monochromators Cary 300 Bio spectrophotometer at 25°C. The spectra will be monitored between 250 and 400 nm using a path length of 1 cm. The absorbance at wavelengths of 320 nm and longer provides information on the aggregation state of the IgG.

Intrinsic fluorescence spectra will be monitored at 25°C using a FluoroMax spectrofluorimeter. The fluorescence method will be optimized. The fluorescence emission will provide information on conformation and aggregation properties.

90° light-scattering spectra will be monitored at 25°C using a FluoroMax spectrofluorimeter by running a synchronous scan ($\lambda_{em} = \lambda_{ex}$) between 400 nm and 750 nm with an integration time of 0.01s. Excitation and emission slits will be optimized. For example, right angle light-scattering can distinguish between IgG samples that have no and 5% dimers.

For fluorescence microscopy with Nile Red staining, just prior to measurements, Nile Red in ethanol will be added to the sample. The samples will be filled in a microscopy slide and analyzed by fluorescence microscopy. Particles will be counted. The lower size limit of the particles that can be observed by fluorescence microscopy is approximately 0.5 µm.

Application of stress such as temperature, pH, mechanical stress or denaturants on proteins might result in a conformation change (e.g. unfolding) and/or aggregation. As it was previously reported that charge-engineered bispecific antibodies have reduced melting temperature of the modified CH3 (Gunasekaran 2010), these studies aim to discriminate between the novel charge mutants of the present invention and existing known charge mutants.

Thermo-stability studies using the Octet are explored, both with Protein A biosensors and by using FcRn binding to IgG. To examine the thermal stability of CH3-engineered IgGs, the samples will be incubated at a concentration of 100 ug/ml (in PBS) at 4, 50, 55, 60, 65, 70 and 75°C for 1 hour using a PCR machine. Following this the samples will be cooled down slowly during a period of 15 minutes to 25°C and kept at this temperature for 2 hours, after which they will be stored overnight at 4°C. Precipitated antibodies will be removed by centrifugation, after which the total IgG concentration of soluble antibodies will be determined by Octet using the protein A Biosensor (1/10 dilution in PBS). Assays that measure binding of the CH3 engineered

IgG to FcRn using the Octet are being explored. Either protein L biosensors are used to bind the light chain of IgG to the sensor, followed by incubation with FcRn in solution, or anti-penta-HIS biosensors are used to bind His-tagged FcRn protein, followed by incubation with the IgG of interest. These methods may be more sensitive than using the protein A Biosensor and can also be used for thermal stability studies. All samples will also be analyzed for serum stability. Briefly, (engineered) IgG samples will be incubated at 37°C in human serum, control samples will be kept at 4°C. After 1, 2, 3 and 4 weeks, samples are centrifuged to remove precipitated IgG. Subsequently the sample is titrated in antigen-specific ELISA to determine the relative amounts of functional IgG. Purified control antibody freshly spiked in human serum will be used as a reference.

**Example 19: stability analyses**

In previous experiments, high percentages of bispecific antibodies were obtained by co-expression of two different heavy chains comprising CH3 mutations, and a common light chain (example 17).

A panel of eight combinations of 2 different heavy chains was selected from Table 15 for further analyses. These eight combinations included combinations 3, 4, 5, 6, 9, 10, 11 and 12 (Table 15). In this study, these eight combinations were analyzed, with a strong focus on stability of the Fc part of the IgG. As controls, wildtype bispecifics (i.e. without CH3 mutations) and/or bispecifics based on previously reported CH3 charge mutations were included. Note that for wildtype bispecifics, 2 heavy chains and the common light chain are co-expressed without means for preferential steering towards heterodimers. These 'wildtype bispecifics' thus represent a mixture of AA, AB and BB. All bispecifics in this study were designed to carry the same VH/VL-combinations, ensuring that the observed effects are caused by mutations in the Fc-part of the molecule and not by variation(s) in the Fab parts.

It was hypothesized that the mutational pairs that were used to promote the heterodimeric pairing of the two different heavy chains could be associated with unexpected structural or otherwise destabilizing effects on the Fc region of the IgG. This could subsequently result in undesired issues that would hamper further clinical development, such as a reduction of *in vivo* half life, a reduced effector function and/or increased immunogenicity due to the presence of these mutations.

**Thermo stability**

Application of stress such as increases or decreases in temperature might result in a conformation change (e.g. unfolding) and/or aggregation of proteins. To examine the thermal stability of CH3-engineered IgGs, the bispecific molecules from combinations 3-6 and 9-12 (Table 15), as well as wildtype bispecifics and bispecific molecules obtained when using constructs 1 and 2 (E356K:D399K/ K392D':K409D' combination, also dubbed 'charge reversal' pair) were incubated at a concentration of 100 µg/ml (in PBS) at 4, 60, 62.5, 65, 67.5, 70 and 72.5°C for 1 hour using a PCR machine. Following this the samples were cooled down slowly during a period of 15 minutes to 25°C and kept at this temperature for 2 hours, after which they were stored overnight at 4°C. The next day, precipitated antibodies were removed by centrifugation (18,000 rpm; 4°C, 20 min), after which the total IgG concentration of soluble antibodies was determined by Octet using the protein A Biosensor (1/10 dilution in PBS). Results are shown in figure 20. It was observed that the control CH3 engineered bispecific antibody (the charge reversal E356K:D399K/ K392D':K409D' combination (triangles)) has a reduced thermal stability as compared to the wildtype bispecific (squares). The bispecific molecules from combinations 3-6 and 9-12 (diamonds) also demonstrated a reduced thermal stability as compared to wildtype. Remarkably, three combinations, however, demonstrated an improved stability as compared to the control CH3 engineered bispecific antibody. Bispecifics of combinations 9, 10 and 11 are significantly more stable than the other CH3 engineered (charge reversal) bispecifics and are as stable as wildtype bispecifics at the highest temperature measured.

**Freeze-thaw stability**

To examine the stability of CH3-engineered IgGs upon repetitive freezing and thawing, the bispecific molecules from combinations 3-6 and 9-12 (Table 15), as well as wildtype bispecifics and bispecific molecules obtained when using constructs 1 and 2 (E356K:D399K/ K392D':K409D' combination (charge reversal pair)) were exposed to ten subsequent freeze-thaw cycles by putting the samples at -80°C for at least 15 minutes until they were completely frozen. Thereafter, samples were thawed at room temperature. When they were completely thawed, the freeze-thaw cycle was repeated. After 10 freeze-thaw cycles, precipitated antibodies were removed by centrifugation (18,000 rpm; 4°C, 20 min), after which the total IgG concentration of soluble

antibodies was determined by Octet using the protein A Biosensor (1/10 dilution in PBS). The freeze-thaw stability test was repeated three times. Results are shown in figure 21. It was observed that the control charge reversal CH3 engineered bispecific antibody seemed to have a slightly reduced stability as compared to the wildtype bispecific. In contrast, the bispecific molecules from combinations 3, 4 and 9 seemed to have a slightly improved stability as compared to the wildtype bispecific. Overall, it can be concluded that the stringent conditions of freeze-thaw cycles do not cause major stability issues for the CH3 engineered variants.

**In vitro serum stability**

To examine the stability of CH3-engineered IgGs in serum kept at 37°C, the bispecific molecules from combinations 3-6 and 9-12 (Table 15), as well as wildtype bispecifics and the charge reversal bispecific molecules were incubated at 37°C in 10% human serum. Control samples were kept in human serum at 4°C. After 1, 2 or 5 days, precipitated antibodies were removed by centrifugation. Thereafter, the samples were titrated in a fibrinogen-specific ELISA, to determine the relative amounts of functional IgG. Purified control antibody freshly spiked in human serum was used as reference.

Data of the fibrinogen ELISA show that all samples were quite stable in 10% human serum at 37°C for 5 days. At the lower IgG concentration bispecific molecules from combinations 4 and 5 seem to be slightly less stable, especially at T=1 and T=2, but the difference is only minimal at the end-point of this experiment (see Figure 22).

**Example 20: Further stability tests**

A further series of analytical methods was used to assess the stability of the variant IgGs. Bispecific molecules from combinations 3-6 and 9-12 (Table 15), as well as wildtype bispecifics (AA, AB, BB), the individual parental antibodies (AA and BB) and bispecific molecules obtained when using constructs 1 and 2 (E356K:D399K / K392D':K409D' combination (charge reversal pair)) were used as samples in these stability assays. All IgGs were diluted to 0.2 mg/ml and several stress conditions (2 days at 50°C, 2 weeks at 40°C, 5x freeze-thawing) were applied, aiming to be able to discriminate between the different samples. Of note, these high stress levels resulted in conditions in which one of the parental antibodies (the BB parental, carrying two

1122 Fabs) as used in all bispecifics became unstable. At 2 days at 50°C, aggregation of this protein was detected by UV absorbance. This suggested that this stress condition may not differentiate between instability of the Fab and the CH3 in the bispecific and data resulting from the 50°C incubation should be used cautiously.

The results are summarized in Table 16. Analytical methods that were used included:

- Fluorescence microscopy with Nile Red ('Nile Red particles' in Table 16); to observe the amount of particles > 0.5 µm after addition of Nile Red dye.
- UV spectrometry at 350 nm ('UV 350 nm'); a change in absorption at wavelengths > 320 nm gives information about the aggregation state of the protein.
- 90° Light scatter at 400 nm ('LS 400 nm'); a sensitive technique to observe changes in protein aggregation, e.g. the difference between monomers and dimers of IgG.
- Intrinsic fluorescence; the fluorescence wavelength maximum and intensity of the aromatic residues in a protein change upon changes in the environment (e.g. unfolding)
- 1,8-ANS fluorescence spectroscopy; 1,8-ANS binds through electrostatic interactions to cationic groups through ion pair formation and changes in protein structure and/or conformation can be detected

**UV-VIS spectroscopy**

UV-Vis absorbance spectra were measured at 25°C with a double beam, two monochromators Cary 300 Bio spectrophotometer from Varian in different quartz cuvettes (such as black low volume Hellma cuvettes with a pathlength of 1.0 cm and clear Hellma cuvettes of 0.2 cm x 1.0 cm). The spectra were monitored between 220 and 450 nm using a pathlength of 1.0 cm. The absorbance around 280 nm provides information on the protein concentration. The region between 320 nm and 450 nm can provide information on the aggregation state of the samples.

**90° light-scattering**

The 90° light-scattering spectral method was developed to study protein aggregation and was performed as described in Capelle, 2005; Demeule, 2007a. 90° light-

scattering spectra were monitored at 25°C using a FluoroMax spectrofluorimeter (Spex, Instruments S.A., Inc. U.K.) by running a synchronous scan ($\lambda_{em} = \lambda_{ex}$) between 400 nm and 750 nm with an integration time of 0.01 s. Different slits settings were tested in order to find the optimal conditions. After optimization, the same slit settings were used for all measurements.

**Steady-state fluorescence emission**

The fluorescence emission of tryptophan, tyrosine and phenylalanine residues gives information on the local environment of these fluorophores. Changes or differences in hydrophobicity and/or rigidity are measured. Typically, a more hydrophobic and rigid environment leads to an increase in the fluorescence intensity and a blue shift of the emission maximum. Intrinsic fluorescence spectroscopy can provide information on the current state of the protein and monitor changes in the physical and chemical properties. More information on the fluorescence of tyrosine and tryptophan can be found in the book of Lakowicz [Lakowicz, 2006].

The fluorescence emission and excitation spectra were recorded at 25°C in different quartz cuvettes. The samples were excited at different wavelengths. Integration times and slit settings were optimized. After optimization, the same integration times and slit settings were applied for all samples.

**Fluorescence microscopy with Nile Red staining**

The Nile Red staining method was developed to visualize protein aggregates and was performed as described in Demeule et al., 2007b.

The microscopy observations were performed on a Leica DM RXE microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a mercury lamp. The images were acquired with a Sony NEX-5 camera and its firmware. The objectives were 10x, 20x and 40x. For microscopy investigations slides with a fixed distance of 0.1 mm between the slide and the cover glass were used. The size of the 4x4 grids is 1 mm x 1 mm and corresponds to 0.1 µl.

**1,8-ANS fluorescence spectroscopy**

1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) is an uncharged small hydrophobic fluorescent probe (Mw 299.34 Da) used to study both membrane surfaces and

proteins.

1,8-ANS is essentially non-fluorescent in water and only becomes appreciably fluorescent when bound to membranes (quantum yields ~0.25) or proteins (quantum yields ~0.7). This property of 1,8-ANS makes it a sensitive indicator of protein folding, conformational changes and other processes that modify the exposure of the probe to water. References on 1,8-ANS can be found on the Internet home page of Molecular Probes, www.probes.com.

The fluorescence emission spectra of 1,8-ANS were recorded using a FluoroMax spectrometer. A direct comparison of the 1,8-ANS fluorescence between IgGs will not be performed. Each IgG can have different number of 1,8-ANS binding sites and can therefore not be compared. In principle, the lower the 1,8-ANS fluorescence, the less 1,8-ANS molecules are bound to the antibody. The changes in the 1,8-ANS fluorescence intensity and emission wavelength due to stress will be evaluated.

Table 16: Overview of the different forced degradation results on various IgG samples after dilution to 0.2 mg/ml. The colour of the cells indicate the variations between T= 0 and after stress: dark grey = large change, light grey = small change and no colour = no change (=stable).

* 'combi. #' refers to the combination of mutations as listed in Table 15; ** very small particles by fluorescence microscopy, relevance of these particles unknown; 2d4°C= 2 days at 4°C; 2d50°C= 2 days at 50°C; 2w4°C= 2 weeks at 4°C; 2w40°C= 2 weeks at 40°C; T0= start of experiment; 5FT=5 freeze thaw cycles

| **Protein sample** | **Stress** | **Nile red particles** | **UV 350 nm** | **LS 400 nm ($10^7$ cps)** | **Intrinsic fluorescence** | | **1,8-ANS fluorescence** | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | **fluo.int. ($10^6$cps)** | **λ Max. (nm)** | **1,8-ANS int. ($10^6$cps)** | **λ Max. (nm)** | **Shift (nm)** |
| BB | 2d4°C | 0-10 | 0.001 | 0.7 | 4.2 | 335 | | | |
| | 2d50°C | 0-10 | 0.013 | 0.8 | 4.2 | 335 | | | |
| AA | 2d4°C | 10-20 | 0 | 1.2 | 5.7 | 338 | | | |
| | 2d50°C | 10-20 | 0.002 | 1.0 | 5.5 | 338 | | | |
| Wildtype | 2d4°C | 30-50 | 0.003 | 0.9 | 5.1 | 336 | 7.1 | 507 | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| bispecific (AA AB BB) | 2d50°C | >10000** | 0.007 | 0.9 | 5.0 | 336 | 7.1 | 507 | |
| | 2w4°C | | 0 | 0.9 | 5.0 | 336 | | | |
| | 2w40°C | >2000** | 0 | 0.8 | 5.0 | 336 | | | |
| | T0 | | 0.001 | 0.8 | 5.0 | 336 | | | |
| | 5FT | >2000** | 0.009 | 1.2 | 4.8 | 336 | | | |
| Charge reversal bispecific (E356K,D399K/ K392D,K409D) | 2d4°C | 10-20 | 0.001 | 1.3 | 5.9 | 336 | 7.0 | 507 | |
| | 2d50°C | 10-20 | 0.002 | 1.2 | 5.7 | 336 | 7.0 | 507 | |
| | 2w4°C | >2000** | 0 | 1.1 | 5.5 | 336 | | | |
| | 2w40°C | >2000** | 0.002 | 1.1 | 5.5 | 336 | | | |
| | T0 | | 0.001 | 1.3 | 5.7 | 336 | | | |
| | 5FT | 30-50 | 0.007 | 1.8 | 5.5 | 336 | | | |
| Combi. # 3* | 2d4°C | 30-50 | 0 | 0.9 | 5.0 | 337 | | | |
| | 2d50°C | 30-50 | 0.001 | 0.8 | 4.9 | 337 | | | |
| Combi. # 4 | 2d4°C | 20-30 | 0 | 1.0 | 6.2 | 337 | 7.5 | 505 | |
| | 2d50°C | >3000** | 0.001 | 1.0 | 6.2 | 337 | 7.5 | 505 | |
| | 2w4°C | | 0.001 | 1.0 | 6.3 | 337 | | | |
| | 2w40°C | >2000** | 0.003 | 0.9 | 6.3 | 337 | | | |
| | T0 | | 0.002 | 1.1 | 6.3 | 337 | | | |
| | 5FT | >2000** | 0.003 | 1.2 | 6.0 | 337 | | | |
| Combi. # 5 | 2d4°C | >2000** | 0.001 | 1.1 | 4.9 | 337 | | | |
| | 2d50°C | >10000** | 0.001 | 0.9 | 5.0 | 337 | | | |
| Combi. # 6 | 2d4°C | 10-20 | 0 | 0.7 | 4.3 | 337 | | | |
| | 2d50°C | 20-30 | 0.001 | 0.7 | 4.3 | 337 | | | |
| Combi. # 9 | 2d4°C | 30-50 | 0 | 1.0 | 5.5 | 337 | 7.5 | 507 | |
| | 2d50°C | 50-100 | 0 | 1.0 | 5.5 | 337 | 8.1 | 500 | 7 |
| | 2w4°C | >2000** | 0 | 0.9 | 5.1 | 337 | | | |
| | 2w40°C | >2000** | 0 | 0.9 | 5.2 | 337 | | | |
| | T0 | | 0.002 | 0.8 | 5.1 | 337 | | | |
| | 5FT | >2000** | 0.007 | 1.4 | 4.9 | 337 | | | |
| Combi. # 10 | 2d4°C | 30-50 | 0.002 | 1.0 | 5.6 | 337 | 7.0 | 505 | |
| | 2d50°C | 150-200 | 0.001 | 1.1 | 5.9 | 337 | 8.7 | 499 | 6 |
| | 2w4°C | >2000** | 0 | 0.9 | 5.2 | 337 | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2w40°C | >2000** | 0 | 0.9 | 5.4 | 337 | | | |
| | T0 | | 0.005 | 1.0 | 5.3 | 337 | | | |
| | 5FT | 20-30 | 0.004 | 1.1 | 5.4 | 337 | | | |
| Combi. # 11 | 2d4°C | 20-30 | 0 | 0.9 | 4.9 | 337 | | | |
| | 2d50°C | 30-50 | 0.002 | 0.9 | 5.1 | 337 | | | |
| | 2w4°C | >2000** | 0 | 0.8 | 5.0 | 337 | | | |
| | 2w40°C | >2000** | 0 | 0.8 | 5.1 | 337 | | | |
| | T0 | | 0.004 | 1.1 | 5.0 | 337 | | | |
| | 5FT | >2000** | 0.002 | 1.2 | 5.0 | 337 | | | |
| Combi. # 12 | 2d4°C | 10-20 | 0.001 | 0.8 | 3.8 | 337 | 6.2 | 511 | |
| | 2d50°C | 10-20 | 0.002 | 0.7 | 3.8 | 337 | 6.5 | 508 | -3 |
| | 2w4°C | >2000** | 0.003 | 0.6 | 3.6 | 337 | | | |
| | 2w40°C | >2000** | 0.001 | 0.5 | 3.5 | 337 | | | |
| | T0 | | 0.005 | 0.6 | 3.7 | 337 | | | |
| | 5FT | | 0.004 | 0.7 | 3.6 | 337 | | | |

Taken together, these data indicate that the various IgG samples are remarkably stable. Severe stress conditions (e.g. 2 days at 50°C) were needed to generate measurable differences between the tested samples. Under these conditions, samples of combinations # 9 and # 10 seem to aggregate more than other samples.

The most discriminating factors for stability between the proteins are the freeze-thaw cycles and increased temperature. Taking into account the very stringent stress factor of incubating at 50°C, the T366K/L351E,Y349E (combi.#4) and T366K,L351K/L351D,Y349E (combi.#11) variants are the two most stable proteins within panel, closely followed by T366K,L351K/L351D,Y349D (combi.#10) and T366K,L351K/L351D,L368E (combi.#12).

**Example 21: Native MS on ratio experiments; transfection ratio's from 1:5 to 5:1**

To become more knowledgeable about the behavior of the CH3 mutated IgGs in skewed transfection mixtures, in particular about the T366K:L351K/L351D':L368E' combination (from now on dubbed KK/DE or DEKK), a more elaborate ratio experiment was conducted.

Previously used antibody VH regions with known ability to pair with the common light chain IGKV1-39 were used for recloning into constructs 1, 2, 68 and 69, resulting in vectors I-V of Table 17. Vectors I-V, each containing nucleic acid sequences encoding the common human light chain as well as an Ig heavy chain with different CH3 region and different antigen specificity, were subsequently transfected into cells with different transfection ratios as indicated in Table 18. Results are shown in figure 23.

Table 17:

| **Vector** | **VH gene** | **Antigen specificity** | **VH mass (Da)** | **Merus designation** | **Cloned in construct #** |
|---|---|---|---|---|---|
| I | IGHV 3.30 | Fibrinogen (A) | 12794 | MF1122 | 69 (L351D, L368E) |
| II | IGHV 3.23 | RSV (C) | 13941 | MF2729 | 69 (L351D, L368E) |
| III | IGHV 1.08 | Tetanus (B) | 13703 | MF1337 | 68 (T366K, L351K) |
| IV | IGHV 3.30 | Fibrinogen (A) | 12794 | MF1122 | 1 (E356K, D399K) |
| V | IGHV 1.08 | Tetanus (B) | 13703 | MF1337 | 2 (K392D, K409D) |

Table 18:

| **Transfection nr** | **vectors** | **ratio** |
|---|---|---|
| 1 | I and III | 5:1 |
| 2 | I and III | 3:1 |
| 3 | I and III | 1:1 |
| 4 | I and III | 1:3 |

| | | |
|---|---|---|
| 5 | I and III | 1:5 |
| 6 | II and III | 5:1 |
| 7 | II and III | 3:1* |
| 8 | II and III | 1:1 |
| 9 | II and III | 1:3 |
| 10 | II and III | 1:5 |
| 11 | IV and V | 5:1 |
| 12 | IV and V | 3:1 |
| 13 | IV and V | 1:1 |
| 14 | IV and V | 1:3 |
| 15 | IV and V | 1:5 |

*due to a technical error, this sample has not been measured.

Figures 23A and B show that for the DEKK combination of mutations, when an excess of A or C is present (A or C are on the 'DE side' and B is on the 'KK side'), AB or BC is formed but the surplus of A or C is present as a mixture of both homodimers and half bodies in all cases. However, when an excess of B is present (B is on the 'KK side' and A or C are on the 'DE side'), there is a clear difference. AB or BC is still formed but the surplus of B is essentially absent as homodimer and only half bodies are formed. Percentages were again measured by peak height Nota bene: peaks detected in the range of 2% or lower are below the threshold of what the nMS technology as applied can accurately measure. Measurements of <2% are therefore regarded to be within the noise level of analysis and therefore ignored. It is striking that the excess of B results in high percentages of half body B only. Especially at the 1:3 and 1:5 ratios of A:B, high percentages of half body B were observed (Fig. 23A and 23B) in the absence of homodimer BB, indicating that the CH3 mutations of the KK-side disfavour homodimerization. The absence of homodimers offers a crucial advantage, as this 'KK side' of the DEKK combination can be chosen to incorporate a specificity that may have known adverse effects when present as a homodimer (for example cMET or CD3 antibodies are known to have undesired adverse side effects when present as bivalent homodimers in therapeutic compositions).

The observed findings for the different ratio's of DE:KK are in contrast to the control charge reversal CH3 mutations in vectors IV and V. Figure 23C shows that for the E356K:D399K/K392D':K409D' combination of mutations when an excess of A is present (A is on the 'K392D:K409D side'), the surplus of A is present as a mixture of both homodimers and half bodies in all cases, but also when an excess of B is present (B is on the 'E356K:D399K side'), the surplus of B is present as a mixture of both homodimers and half bodies in all cases. Even at the higher ratios 1:3 and 1:5 no half bodies B are observed although homodimers are present, indicating that the E356K:D399K side does not disfavour homodimerization as much as the KK-side of the DEKK combination.

Taken together, the DEKK combination of mutations offers a clear benefit over the charge reversal CH3 mutations, in that one of the chains of the heterodimer does not form homodimers.

**Example 22: varieties of mixtures using the DEKK combination**

As it was demonstrated that the DEKK combination of mutations drives the formation of bispecific IgG molecules ('AB') with high purity, we next explored the feasibility of controlled production of more complex antibody mixtures from one cell, such as 'AB and AA' or 'AB and AC' mixtures. Previously used model Fabs were incorporated in vectors that contain either the 'DE construct' or the 'KK construct' and various combinations of these vectors were co-expressed to create mixtures, to demonstrate the versatility of the technology. Model Fabs MF1337 (tetanus toxoid), MF1122 (fibrinogen) and MF1025 (thyroglobulin) were chosen based on their overall stable behaviour, good expression levels and mass differences between the IgGs containing these Fabs (see Table 19)

Table 19:

| **Specificity** | **Fab name** | **IgG mass** | **Δ-mass MF1122** |
|---|---|---|---|
| Tetanus (A) | (MF)*1337 | 146747.03 | +1842.05 |
| Fibrinogen (B) | (MF)1122 | 144904.98 | 0 |
| Thyroglobulin (C) | (MF)1025 | 144259.87 | -645.11 |

* MF=Merus Fab, designations such as MF1337 and 1337 are both used interchangeably.

Table 20: Transfection schedule:

| **Tr. #** | **Heavy chain 1** | **Heavy chain 2** | **Heavy chain 3** | **Tr. ratio** | **Expected species (%)** | **Observed species (%)** |
|---|---|---|---|---|---|---|
| 1 | 1337-KK | 1122-DE | 1025-DE | 2:1:1 | AB (50%) AC (50%) | AB (43%) AC (57%) |
| 2 | 1337-DE | 1122-KK | 1025-KK | 2:1:1 | AB (50%) AC (50%) | AB (40%) AC (54%) AA (6%) |
| 3 | 1337-KK | 1122-DE | 1025-KK | 1:2:1 | AB (50%) BC (50%) | AB (54%) BC (46%) |
| 4 | 1337-KK | 1122-KK | 1025-DE | 1:1:2 | AC (50%) BC (50%) | AC (66%) BC (33%) CC (1%) |
| 5 | 1337-KK | 1337-DE | 1122-DE | 2:1:1 | AA (50%) AB (50%) | AA (57%) AB (43%) |
| 6 | 1337-KK | 1122-KK | 1122-DE | 1:1:2 | AB (50%) BB (50%) | AB (75%) BB (25%) |
| 7 | 1337-KK | 1337-DE | 1025-DE | 2:1:1 | AA (50%) AC (50%) | AA (46%) AC (54%) |
| 8 | 1337-KK | 1025-KK | 1025-DE | 1:1:2 | AC (50%) CC (50%) | AC (60%) CC (40%) |
| 9 | 1337-KK | 1122-DE | | 1:1 | AB (100%) | AB (>98%) |
| 10 | 1337-KK | 1025-DE | | 1:1 | AC (100%) | AC (>98%) |
| 11 | 1122-KK | 1025-DE | | 1:1 | BC (100%) | AC (>98%) |

SDS-PAGE analysis demonstrated that most samples consisted of predominantly full IgGs and in some cases half bodies were present at small percentages. Furthermore, many of the samples showed two bands at ca. 150 kDa on non-reduced gels, reflecting the presence of two distinct IgG species in the sample. Also on the reduced gels, two heavy chain bands were visible in some samples (data not shown).

Native MS was performed on all samples and the percentages of observed species were calculated based on peak height (% of observed species in Table 20). Results are presented in Figure 24. In all eight samples where three heavy chains were co-expressed, two main peaks were observed which corresponded to the expected species. In two of these samples (transfections 2 and 4), and in transfection 11, a small amount of contaminating DE-DE homodimer was observed. Half bodies were detected in very small amounts in most of the samples (less than 2%), which are not

problematic as they can be easily separated from the full length IgG fraction as discussed previously. After nMS it was discovered that the observed mass of the IgG in sample 11 corresponded to a different species than expected, and it was concluded that this was due to an transfection error, i.e. in sample 11 apparently 1025-DE was co-transfected with 1337-KK instead of 1122-KK.

The IgG samples were further tested in a sandwich ELISA to confirm the functional presence of the desired specificities. Coating of ELISA plates was done with fibrinogen or thyroglobulin and detection was performed with fluorescein-labelled thyroglobulin or - tetanus toxoid. The detection antigens were labelled with fluorescein (Pierce NHS-fluorescin Antibody Labeling kit, cat. # 53029) according to the manufacturer's instructions. Fluorescein-labeled antigens could subsequently be detected by a FITC-conjugated anti-fluorescein antibody (Roche diagnostics, cat. # 11426346910).

Results of the bispecific ELISA (OD450 values) are summarized in Table 21. The greyed cells indicate the expected species for each transfection. Generally, the results meet the expected outcome with view exceptions as indicated in italic or bold. In transfections 1-3, the supposed 'negative' well for species BC (tr. #1 and 2) or AC (tr.# 3) demonstrated a significant background signal. It is known from previous studies that bispecific ELISAs may suffer from high background levels. These background levels may also be caused by the potential presence of half-bodies in the sample. Of note is that the results of bispecific ELISA indeed confirmed that an error had occurred in transfection #11, as the species AC (bold value) was detected rather than BC.

Table 21: OD450 values from bispecific ELISA

| | Detected IgG Species | | |
|---|---|---|---|
| Tr. # | AB (Tet-Fib) | AC (Tet-Thyr) | BC (Fib-thyr) |
| 1 | 0.989 | 1.792 | *0.438* |
| 2 | 1.085 | 1.852 | *0.418* |
| 3 | 1.419 | *0.775* | 1.547 |
| 4 | 0.205 | 1.795 | 1.22 |
| 5 | 1.367 | 0.047 | 0.057 |

| | | | |
|---|---|---|---|
| 6 | 1.359 | 0.043 | 0.06 |
| 7 | 0.054 | 1.779 | 0054 |
| 8 | 0.04 | 1.338 | 0.052 |
| 9 | 1.588 | 0.048 | 0.051 |
| 10 | 0.044 | 1.805 | 0.055 |
| 11 | 0.043 | **1.821** | 0.056 |

**Example 23: improved mixtures of two bispecific antibodies recognizing 4 different epitopes (AB and CD) from a single cell**

In example 12 it was hypothesized that mixtures resulting from transfections ZA or ZB are expected to become problematic when transferred to larger scale production, as knob-into-hole variants are reported to be unstable and it cannot be excluded that CH3 domains comprising a 'knob' or a 'hole' will dimerize with charge-engineered CH3 domains. As it was demonstrated in the above examples that novel charge pair mutants have been found that preferentially drive heterodimerization with virtually no formation of homodimers, CH3 domain-comprising polypeptide chains comprising these novel charge pair mutants can be expressed in cells together with previously known charge-engineered CH3 domain-comprising polypeptide chains or potentially with SEED bodies, and are likely to result in the preferential formation of two bispecific molecules only.

From the above examples it was clear that the DEKK combination of mutations is excellent for the production of one bispecific (AB) or two bispecifics (AB plus AC) by clonal cells where dimerization of the heavy chains is driven by the CH3 domains. However, using only one vector set of complementary CH3 mutations limits the number of possibilities of mixture-varieties that can be produced. It would be possible to produce more complex mixtures of IgGs and/or bispecifics, such as 'AB and CD' or 'AB and CC' mixtures if a second 'orthogonal' vector set could be used in combination with DEKK. When combining two vector sets, an important requirement is that the heavy chains expressed from the two different sets of CH3 engineered vectors cannot make 'crossed' dimers, which is that the heavy chains produced by one of the vector sets dimerize into full IgG with heavy chains expressed by the other vector set.

To test for such potential formation of 'crossed' dimers, an *in silico* analysis was performed using HADDOCK to obtain further insights whether possible pairing between wildtype CH3 domains and CH3 domains containing DE- or KK-mutations would occur. Similarly, potential pairings between wildtype CH3 domains and CH3 domains containing E356K,D399K or K392D,K409D mutations were analyzed, as well as potential pairings between wildtype CH3 domains and CH3 domains containing knob-into-hole mutations and any combination of the above. Combinations of CH3-

mutants that were analyzed in HADDOCK are listed in Table 22 and the resulting HADDOCK scores are summarized in Figure 25.

Table 22: CH3 variants analyzed in HADDOCK, with one letter codes for assigned for each CH3-variant carrying heavy chain. *Wildtype chains are designated 'C' and 'D' for matters of consistency; **The charge reversal variants are designated 'A and B' when combined with knob-into-hole variants, and are designated 'C and D' when combined with DE/KK variants.

| **CH3 combination** | **Mutations** | **One letter code in HADDOCK** |
|---|---|---|
| DEKK | Chain 1: T366K,L351K | A |
| | Chain 2: L351D,L368E | B |
| Wildtype (WT) | Chain 1: none | C* |
| | Chain 2: none | D* |
| Charge reversal (CR) | Chain 1: K392D,K409D | A/C** |
| | Chain 2: E356K,D399K | B/D** |
| Knob-into-hole (KIH) | Chain 1: T366W | C |
| | Chain 2: T366S,L368A,Y407V | D |

Figure 25 shows that, based on these HADDOCK predictions, combining the CH3 combinations of DEKK with charge reversal CH3 combinations is most likely to be successful in forming the desired combination of two bispecifics (AB and CD) without contaminating by-products (especially AC, AD, BC, BD) when co-transfected in a single cell. As can be seen from figure 25, these undesired bispecific species AC, AD, BC, and BD have relatively high HADDOCK scores, whereas the desired AB and CD species have the lowest HADDOCK scores. Of course, when either the CH3 combinations of DEKK or charge reversal will be put into a construct carrying the same specificity (e.g. 'C' on the DE-side, 'C' on the KK-side, 'A' on the E356K,D399K-side and 'B' on the E356K,D399K-side, or 'A' on the DE-side, 'B' on the KK-side, 'C' on

the E356K,D399K-side and 'C' on the E356K,D399K-side) this will result in the production of predominantly CC and AB upon co-expression in a cell.

In contrast, when looking at the predictions for co-expressing DEKK with wildtype, it can be seen that the HADDOCK scores for AC and AD are lower than the HADDOCK score for CD, which indicates that AC and AD are very likely contaminants when trying to produce a mixture of AB and CD by co-expression of vectors encoding for CH3 combinations of DEKK together with vectors encoding wildtype CH3. Lastly, the predictions for co-expressing either DEKK or charge reversal variants together with the knob-into-hole variants results in undesired bispecific variants with relatively low HADDOCK scores, i.e. a high likelihood that these undesired species will be produced upon co-expression.

It is thus concluded that combining the CH3 combinations of DEKK with charge reversal CH3 combinations (E356K,D399K/K392'D,K409D') is ideally suited for obtaining essentially pure 'AB and CD' and/or 'AB and CC' mixtures of antibodies.

Next, mixtures of 2 bispecifics recognizing 4 targets/epitopes (AB and CD) and mixtures of one bispecific and 1 monospecific antibody recognizing 3 targets/epitopes (AB and CC) were created by putting the above into practice. These mixtures were created using 4 different VHs that are all capable of pairing with the common light chain IGVK1-39, but the individual VH/VL combinations all have different specificities. To enable native MS analysis, the mass difference between the (expected) species has to be sufficient, i.e. > 190 Da. Four individual VHs have been selected and the masses of these were such that the expected species upon co-transfection could be identified and separated by nMS. Furthermore, the mass differences between the 4 selected VHs are also large enough to identify most of the possible contaminants in the mixtures, in addition to the two desired species. Selected VHs are listed in Table 23.

Table 23:

| **VH (target)** | **Mass as wt IgG** |
|---|---|
| A (RTK1) | 146736.78 |
| B (Tetanus toxoid) | 146106.20 |

| | |
|---|---|
| C (Fibrinogen) | 144904.98 |
| D (RTK2) | 145421.37 |

The 4 different VHs were cloned into vectors containing the 'DE' or 'KK' constructs or the charge reversal constructs, and several co-transfections were performed as indicated in Table 24. NB: as always, all vectors also contained the nucleic acid encoding the common light chain IGKV1-39. As previously indicated, when combining two vector sets, an important requirement is that the heavy chains expressed from the two different sets of CH3 engineered vectors cannot make 'crossed' dimers, which is that the heavy chains produced by one of the vector sets dimerize into full IgG with heavy chains expressed by the other vector set. To test for such potential formation of 'crossed' dimers between heavy chains containing charge reversal mutations and heavy chains containing DE or KK mutations, control transfections were performed.

Table 24:

| **Tr. #** | **1st VH / construct #** | **2nd VH / construct #** | **Expected species** |
|---|---|---|---|
| 1 | D / 68 | A / 68 | mismatch 'KK' with 'KK'; Mostly half-bodies expected |
| 2 | D / 68 | A / 69 | match 'KK' with 'DE'; AD product expected |
| 3 | D / 68 | A / 1 | Expected mismatch 'KK' with 'E356K:D399K' |
| 4 | D / 68 | A / 2 | Expected mismatch 'KK' with 'K392D:K409D' |
| 5 | D / 69 | A / 68 | match 'DE' with 'KK'; AD product expected |
| 6 | D / 69 | A / 69 | mismatch 'DE' with 'DE'; mixture of half-bodies, AA, AD and DD expected |
| 7 | D / 69 | A / 1 | Expected mismatch 'DE' with 'E356K:D399K' |
| 8 | D / 69 | A / 2 | Expected mismatch 'DE' with 'K392D:K409D' |

| **Tr. #** | **1st VH / construct #** | **2nd VH / construct #** | **3rd VH / construct #** | **4th VH / construct #** | **Expected species** |
|---|---|---|---|---|---|
| 9 | A / 68 | B / 69 | C / 1 | D / 2 | AB and CD |
| 10 | A / 68 | A / 69 | C / 1 | D / 2 | AA and CD |
| 11 | A / 68 | B / 69 | C / 1 | C / 2 | AB and CC |

Table 25 provides a further overview of masses of the expected species, and the possible contaminants, of transfections # 9-11 of Table 24.

Table 25: For each of transfections # 9-11, the species are sorted by mass, mass difference is calculated with the mass above. Grey cells: expected (and desired) species; italics: mass difference too small to separate in nMS analysis. *Species: single letters represent half-bodies; two-letter code intact IgG.

| **Transfection # 9** | | |
|---|---|---|
| **Species*** | **Mass** | **Mass difference** |
| C | 72464,62 | |
| D | 72684,53 | 219,90 |
| B | 73070,99 | 386,47 |
| A | 73410,46 | 339,47 |
| CC | 144929,2 | 71518,78 |
| CD | 145149,2 | 219,90 |
| DD | 145369,1 | 219,90 |
| BC | 145535,6 | *166,56* |
| BD | 145755,5 | 219,90 |
| AC | 145875,1 | *119,57* |
| AD | 146095 | 219,90 |
| BB | 146142 | *47,00* |
| AB | 146481,5 | 339,47 |
| AA | 146820,9 | 339,47 |
| **Transfection # 10** | | |
| **Species** | **Mass** | **Mass difference** |
| C | 72464,62 | |
| D | 72684,53 | 219,90 |
| A | 73410,46 | 725,94 |
| CC | 144929,2 | 71518,78 |
| CD | 145149,2 | 219,90 |
| DD | 145369,1 | 219,90 |
| AC | 145875,1 | 506,03 |
| AD | 146095 | 219,90 |
| AA | 146820,9 | 725,94 |
| **Transfection # 11** | | |
| **Species** | **Mass** | **Mass difference** |
| C | 72464,62 | |
| B | 73070,99 | 606,37 |
| A | 73410,46 | 339,47 |
| CC | 144890,95 | 71480,49 |
| BC | 145535,61 | 644,66 |
| AC | 145875,08 | 339,47 |
| BB | 146141,98 | 266,90 |
| AB | 146481,45 | 339,47 |
| AA | 146820,92 | 339,47 |

All purified protein samples obtained from transfections # 1-11 were analyzed on SDS-PAGE, and three control samples were included (Figure 26). In addition, nMS analysis was performed on protein samples from transfections # 9-11 to identify all species in the samples. As can be seen from Figure 26, transfections # 3 and # 4 resulted in the expected mismatch between 'KK' constructs and either 'E356K:D399K' or 'K392D:K409D' and the amount of half bodies in protein samples from these transfections exceeded the amount of full IgG molecules. Transfections # 7 and # 8 resulted in protein samples wherein both half bodies and full IgG is present in about equal amounts. However, from SDS-PAGE it cannot be deduced whether the full IgG represents a DE/DE dimer, a DE/E356K:D399K dimer or a DE/K392D:K409D dimer. Remarkably, virtually no half bodies were observed in samples from transfections # 9-11.

In Figure 27, the nMS analysis of transfections # 9 and # 11 are presented. Percentages of expected species and contaminating species were calculated by peak height. It was demonstrated that, for transfection # 9, the expected species 'AB and CD' are represented for 97% in the mixture (30% AB and 67% CD) whereas only as little of about 3% of contaminating BD is present (Figure 27A). For transfection # 11, the expected species 'AB and CC' are represented for 94% in the mixture (33% AB and 61% CC) whereas only as little of about 6% of contaminating BC (4.1%) and AC (1.8%) is present (Figure 27B). These data show that it is indeed possible to produce more complex mixtures of IgGs and/or bispecifics, such as 'AB and CD' or 'AB and CC' mixtures when a second 'orthogonal' vector set is used in combination with DEKK. Combination of the charge reversal constructs together with the DEKK constructs results in only very limited formation of 'crossed' dimers. By adjusting the transfection ratio's it is expected that the low percentages of these contaminating by-products can be even further reduced.

**Example 24: single dose pharmacokinetic study in mice**

To study the pharmacokinetic (pK) behavior of bispecific antibodies carrying the DEKK combination of mutations in their CH3 regions, in this study the pK parameters for three different IgG batches were determined and compared.

The three IgG batches included 1) wildtype anti-tetanus toxoid parental antibody 1337:1337 (two MF1337 Fabs on a wildtype Fc backbone); 2) wildtype anti-tetanus toxoid parental antibody 1516:1516 (two MF1516 Fabs on a wildtype Fc backbone); 3) CH3 engineered bispecific anti-tetanus toxoid antibody 1516:1337 that carries the DEKK combination of mutations in its Fc region (MF1516 Fab on DE-side, MF1337 Fab on KK-side).

The parental antibodies 1337:1337 and 1516:1516 were chosen as specificities to be included in the DEKK-bispecific product, as it was known based on previous studies that no pre-dose serum response against these antibodies was present in several mice strains. NB: the presence of a pre-dose serum response would of course invalidate the study. In addition, there is sufficient mass difference between the parental antibodies to enable the identification of 1337:1337 (wt Fc), 1516:1337 (DEKK Fc) and 1516:1516 (wt Fc) species by nMS. The three IgG batches were prepared as previously described, but the DNA used for transfection was made using an endo-free maxiprep kit to ensure that the amount of endotoxins is as low as possible. The batches were subsequently tested for protein concentration, aggregate levels, endotoxin levels and percentage bispecific product. It was demonstrated that the acceptance criteria for subsequent use of the IgG batches in a pK study were met, i.e. the IgG concentration after gel filtration was >0.3 mg/ml, aggregate levels were <5%, endotoxin levels were <3 EU/mg protein and the DEKK batch contained > 90% bispecific IgG.
Native mass spectrometry of the gel filtrated samples showed that the expected species were present in high percentages. In sample 1516:1337 a small amount of the DE:DE homodimer is detected, which is estimated to be ca. 2% (Figure 28). It was concluded that the 3 IgG batches are qualified to be used in the pK study.

For comparison of pK parameters between the three batches, 3 groups of female C57BL/6J mice (Harlan, The Netherlands) were dosed at 1 mg/kg human lgG (5 ml/kg immunoglobulin solution/kg body weight). At dosing time, the animals were between 7-8 weeks of age and had a body weight of about 18-20 grams. Blood samples were collected pre-dose and at 15, 60 minutes, and 2, 4, 8, 24, 48, 96, 168, 268 and 336 h after dosing. Serum samples were prepared and stored at < -20 °C until analysis. Each group consisted of 3 subgroups of 4 mice, i.e. 12 mice/group. From each mice 6

time points were sampled. The welfare of the animals was maintained in accordance with the general principles governing the use of animals in experiments of the European Communities (Directive 86/609/EEC) and Dutch legislation (The Experiments on Animals Act, 1997). This study was also performed in compliance with the Standards for Humane Care and Use of Laboratory Animals, as issued by the Office of Laboratory Animal Welfare of the U.S. National Institutes of Health under identification number 45859-01 (expiration date: 30 April 2015).

Mice of Group 1 received the full length monospecific IgG 1516:1516 antibody (triangles); Mice of Group 2 received the full length monospecific IgG 1337:1337 antibody (squares); Mice of Group 3 received the full length bispecific IgG 1516:1337 antibody (diamonds), with DEKK engineered CH3 regions (1516 on the DE-side and 1337 on the KK-side).

An ELISA assay was applied for the quantitative analysis of monoclonal human antibodies in mouse serum using a quantitative human lgG ELISA (ZeptoMetrix, NY USA; ELISA kit nr. 0801182). Briefly, the ELISA assay is based on the principle that the human monoclonal antibody binds to anti-human lgG coated in a 96-wells ELISA plate. Bound antibody was subsequently visualized using a polyclonal antihuman lgG antibody conjugated with horseradish peroxidase (HRP). The optical density (OD) of each well is directly proportional to the amount of antibody in the serum sample. Results are shown in Figure 29, and it was observed that serum levels of both the bispecific full length IgG antibody carrying the DEKK combination of mutations and its parental monospecific antibodies are strikingly similar. It is concluded that the CH3 mutations as present in the DEKK-bispecific antibody does not alter stability nor half life, and the DEKK variant is behaving as wildtype IgG.

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Claims

1. A method for producing at least two different Ig-like molecules from a single host cell, wherein each of said two Ig-like molecules comprises two CH3 domains that are capable of forming an interface, said method comprising providing in said cell

a. A first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain,

b. A second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,

c. A third nucleic acid molecule encoding a 3rd CH3 domain-comprising polypeptide chain, and

d. A fourth nucleic acid molecule encoding a 4th CH3 domain-comprising polypeptide chain,

wherein at least two of said nucleic acid molecules are provided with means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides and said 3rd and 4th CH3-domain comprising polypeptides, said method further comprising the step of culturing said host cell and allowing for expression of said at least four nucleic acid molecules and harvesting said at least two different Ig-like molecules from the culture.

2. The method of claim 1, further comprising providing said host cell with a nucleic acid molecule encoding a common light chain.

3. The method of claim 1 or 2, wherein said first CH3-domain comprising polypeptide chain comprises the amino acid substitution T366K, and said second CH3-domain comprising polypeptide chain comprises the amino acid substitution L351D.

4. The method of claim 3, wherein said first CH3-domain comprising polypeptide chain further comprises the amino acid substitution L351K.

5. The method of claim 3 or 4, wherein said second CH3-domain comprising polypeptide chain further comprises an amino acid substitution selected from the group consisting of Y349E, Y349D and L368E.

6. The method of claim 5, wherein said second CH3-domain comprising polypeptide chain further comprises the amino acid substitution L368E.

7. The method of any one of claims 1-6, wherein said third CH3-domain comprising polypeptide chain comprises the amino acid substitutions E356K and D399K, and said fourth CH3-domain comprising polypeptide chain comprises the amino acid substitutions K392D and K409D.

8. The method of any one of claims 1-7, wherein each of the CH3-domain comprising polypeptide chains further comprises a variable region recognizing a target epitope.

9. The method of claim 8, wherein each of the 4 variable regions of the 4 CH3-domain comprising polypeptide chains recognizes different target epitopes.

10. The method of claim 8, wherein the variable regions of the 1st and the 2nd CH3-domain comprising polypeptide chains recognize different target epitopes, whereas the variable regions of the 3rd and the 4th CH3-domain comprising polypeptide chains recognize the same target epitopes.

11. The method of claim 10 wherein the target epitope recognized by the variable regions of the 3rd and 4th CH3 domain comprising polypeptide chain is the same as the target epitope recognized by the variable region of the 1st or the 2nd CH3-domain comprising polypeptide chain.

12. The method of claim 10 wherein the target epitope recognized by the variable regions of the 3rd and 4th CH3 domain comprising polypeptide chain is different from the target epitope recognized by the variable region of the 1st or the 2nd CH3-domain comprising polypeptide chain.

13. The method of claim 8, wherein the variable regions of the 1st and the 2nd CH3-domain comprising polypeptide chains recognize the same target epitope, whereas the variable regions of the 3rd and the 4th CH3-domain comprising polypeptide chains recognize a second target epitope which differs from the target epitope recognized by said 1st and 2nd variable regions.

14. The method of any one of claims 8-13 wherein the target epitopes are located on the same target molecule.

15. The method of claim 14 wherein the target molecule is a soluble molecule.

16. The method of claim 14 wherein the target molecule is a membrane-bound molecule.

17. The method of any one of claims 8-13 wherein the target epitopes are located on different target molecules.

18. The method of claim 17, wherein the different target molecules are expressed on the same cells.

19. The method of claim 17, wherein the different target molecules are expressed on different cells.

20. The method of claim 17, wherein the different target molecules are soluble molecules.

21. The method of claim 17, wherein one target molecule is a soluble molecule whereas the second target molecule is a membrane bound molecule.

22. The method according to any one of claims 1-21, wherein said at least two different Ig-like molecules are antibodies.

23. The method of claim 1, wherein said means for preferential pairing comprises engineered complementary knob-into-hole mutations, disulfide bridges, charge mutations or combinations thereof.

24. The method of claim 23, wherein said means for preferential pairing are selected from Table B.

25. The method of claim 1, wherein all 4 of said nucleic acid molecules are provided with means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides and said 3rd and 4th CH3-domain comprising polypeptides, wherein said means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides are different from those means for preferential pairing of said 3rd and 4th CH3-domain comprising polypeptides.

26. The method of any one of claims 8-13, 17-19 or 21, wherein at least one of said target epitopes is located on a tumor cell.

27. The method of any one of claims 8-13, 17-19 or 21, wherein at least one of said target epitopes is located on an effector cell.

28. The method of claim 27, wherein said effector cell is an NK cell, a T cell, a B cell, a monocyte, a macrophage, a dendritic cell or a neutrophilic granulocyte.

29. The method of claims 27 or 28, wherein said target epitope is located on a CD3, CD16, CD25, CD28, CD64, CD89, NKG2D or a NKp46 molecule.

30. A mixture of at least two different Ig-like molecules obtainable by the methods according to any one of claims 1-29.

31. A mixture according to claim 30, wherein said at least two different Ig-like molecules bind to different epitopes on the same antigen and/or to different epitopes on different antigens.

32. A mixture according to claim 30 or 31, wherein said at least two different Ig-like molecules comprises at least one heterodimeric Ig-like molecule.

33. A mixture according to any one of claims 30-32, wherein two of said at least two different Ig-like molecules are heterodimeric Ig-like molecules.

34. A recombinant host cell comprising nucleic acid sequences encoding at least a first, a second, a third and a fourth CH3-domain comprising polypeptide chain, wherein at least two of said nucleic acid sequences are provided with means for preferential pairing of said first and second CH3-domain comprising polypeptides and said third and fourth CH3-domain comprising polypeptides.

35. A recombinant host cell according to claim 34, wherein said host cell further comprises a nucleic acid sequence encoding a common light chain.

36. A pharmaceutical composition comprising the at least two different Ig-like molecules of any one of claims 30-33, and a pharmaceutically acceptable carrier.

37. A pharmaceutical composition according to claim 36, wherein said at least two different Ig-like molecules have been produced by recombinant host cells according to claims 34 or 35.

38. A method for making a host cell for production of at least two different Ig-like molecules, the method comprising the step of introducing into said host cell nucleic acid sequences encoding at least a first, a second, a third and a fourth CH3-domain comprising polypeptide chain, wherein at least two of said nucleic acid sequences are provided with means for preferential pairing of said first and second CH3-domain comprising polypeptides and said third and fourth CH3-domain comprising polypeptides, wherein said nucleic acid sequences are introduced consecutively or concomitantly.

39. A method according to claim 38, further comprising the step of introducing into said host cell a nucleic acid sequence encoding a common light chain.

40. A culture of recombinant host cells of claims 34 or 35, or of recombinant host cells obtained by the method according to claims 38 or 39, producing at least two different Ig-like molecules.