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(54) **HETERODIMER MOLECULE BASED ON  
CH3 DOMAIN, AND PREPARATION  
METHOD AND USE THEREOF**

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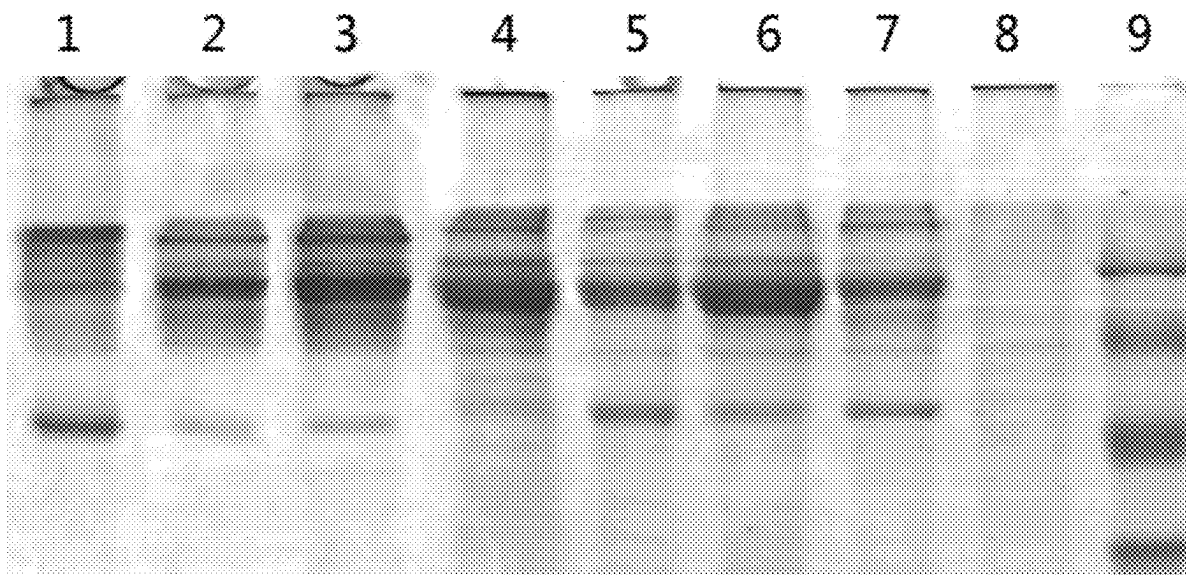
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(57)

**ABSTRACT**

A heterodimer molecule based on CH3, and a preparation method therefor and a use thereof. By comprehensively considering various interactions between molecules, for example, an ionic action, a hydrophobic interaction and a spatial action, a preferred Fc mutant sequence being more inclined to form a heterodimer rather than a homodimer is screened, and accordingly, the yield of the heterodimer molecule is greatly improved, thereby creating conditions for the preparation of bispecific molecules and the like.

**Specification includes a Sequence Listing.**



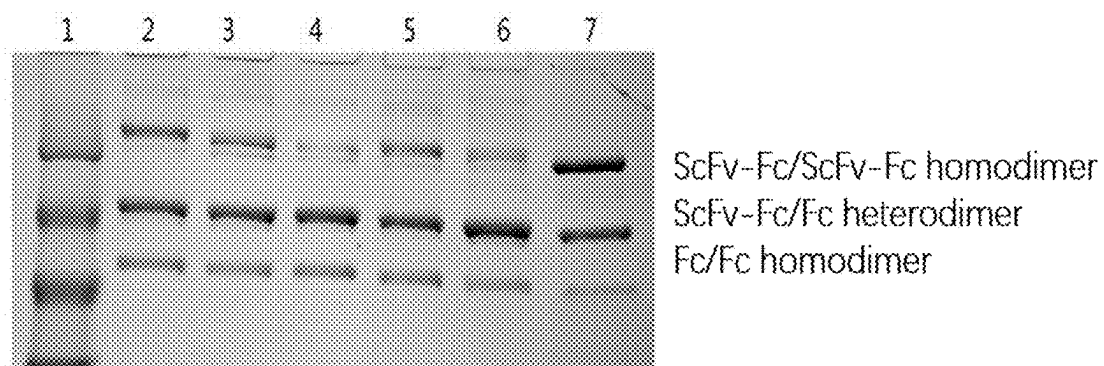


Fig. 1

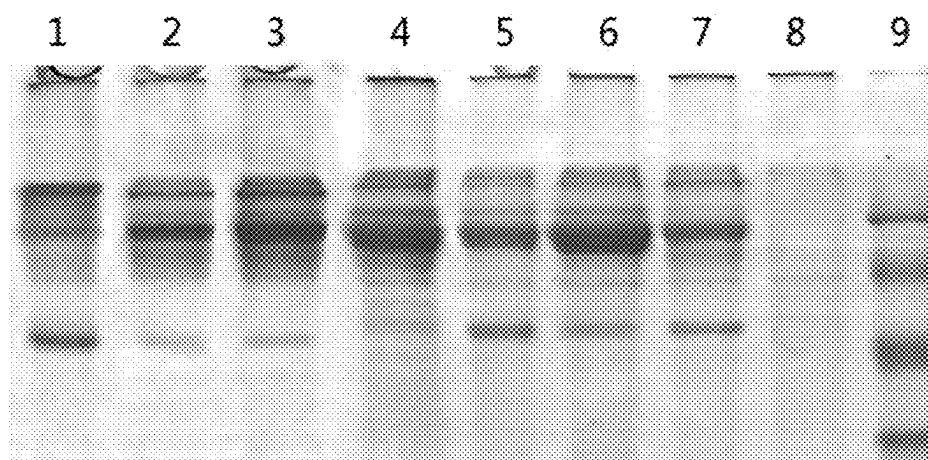


Fig. 2

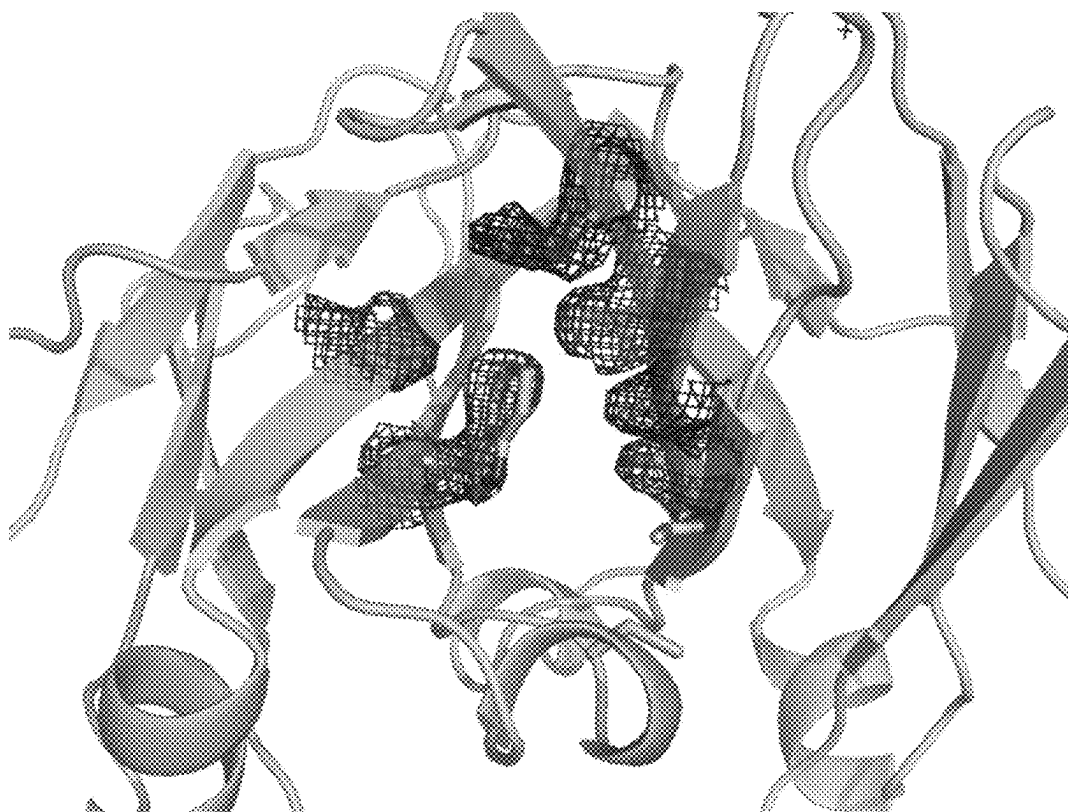
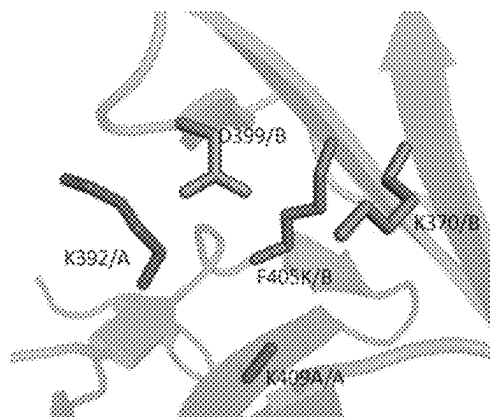
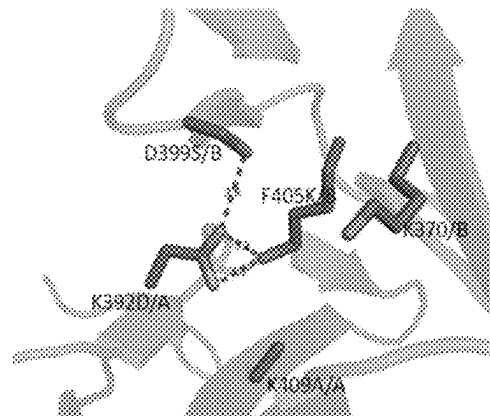


Fig. 3

A F405K-K409A mutation combination pair



B introduce new mutations



Figs. 4A-4B

## HETERODIMER MOLECULE BASED ON CH3 DOMAIN, AND PREPARATION METHOD AND USE THEREOF

### CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This is a continuation application of Ser. No. 17/496,240, filed on Oct. 7, 2021, which is a continuation application of Ser. No. 16/062,405, filed Jun. 14, 2018, which is a National Stage Application under 35 U.S.C. § 371 of International application PCT/CN2016/110252, filed Dec. 16, 2016, which claims priority under 35 USC § 119 to CN 201510938995.0, filed Dec. 16, 2015, the contents of which are incorporated herein by reference.

### INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[0002] The Sequence Listing in XML format, named as 36019ZY\_SequenceListing.xml of 60 KB, created on Mar. 19, 2025, and submitted to the United States Patent and Trademark Office via Patent Center, is incorporated herein by reference.

### TECHNICAL FIELD

[0003] The present disclosure relates to the field of antibody engineering, and in particular, provides a heterodimer molecule based on CH3 domain, and preparation method and use thereof.

### BACKGROUND

[0004] In recent 15 years, monoclonal antibody drugs have rapidly developed, becoming a growing point in the pharmaceutical industry. Since 1996, about 30 monoclonal antibody drugs have been approved to come into the market, among which 9 monoclonal antibody drugs obtain annual sales of more than USD 1 billion. In 2010, the total sales of monoclonal antibody drugs were more than USD 30 billion with the annual average growth rate of more than 10%. A monoclonal antibody exhibits strong target specificity, and therefore can only inhibit a single target site. However, for many diseases, including tumors, autoimmune diseases, etc., it is necessary to inhibit multiple signaling pathways to avoid compensatory effects. For viral infection diseases, due to high mutation rate of viruses, it is usually necessary to inhibit a plurality of antigen sites to avoid escape. In addition, bifunctional antibodies and proteins are used to specifically activate the human immune system (Wolf, Hofmeister et al. 2005).

[0005] It is well known that the crystallizable fragments (Fc) of an antibody form a homodimer, and plays a key role in maintaining the in vivo stability of the antibody and Fc fusion protein. Modifying Fc to form a heterodimer is an effective method to produce a multifunctional antibody, protein and maintain in vivo stability thereof.

[0006] A typical application example of a heterodimer is a bispecific antibody (BsAbs), which is an immunoglobulin molecule containing two different ligand binding sites. A bispecific antibody is active to at least two different antigens (Carter 2001). It replaces the form of two identical Fab arms in a classic antibody with the form of two Fab arms with different sequences. Therefore, two Y type arms can bind to different antigens. The application of bispecific antibodies in

treating cancers has been summarized by many literatures (Carter 2001; Chames and Baty 2009; Chames and Baty 2009).

[0007] There is no bispecific antibody in a natural state, which can be prepared only by a special method. The prior preparation methods of bispecific antibodies include chemical crosslinking, hybridizing F(ab')<sub>2</sub> molecules, murine hybridoma method, etc. For bispecific antibodies produced by the chemical crosslinking method, they exhibit heterogeneity, instability of products between batches, and the characteristic thereof that antibody specificity can be easily changed by some modifications or improper bindings, thus they are not suitable for use in vivo. A bispecific hybrid molecule produced from a mercapto-crosslinked proteinase digestion fragment F(ab') has a relatively homogeneous ingredient, but the preparation process is time-consuming and labor-consuming with a very low yield. Bispecific antibodies produced by the hybridoma method have a reliable source, but randomly pairing between light chains and heavy chains will produce a plurality of possible antibody forms, making the production and purification of bispecific antibodies very difficult as a result.

[0008] As early as in the 1990s, Carter et al. modified some amino acids in heavy chains of antibodies by a “knob into hole” model, and relatively successfully achieved the preparation of bispecific antibodies (Ridgway, Presta et al. 1996; Carter 2001). The “knob into hole” model was originally proposed by Crick to solve the problem of amino acid side chain folding between adjacent  $\alpha$ -spirals (Crick 1952). Carter et al. created a “knob” by mutating an amino acid with a short side chain in a CH3 region of a first heavy chain of the Fc region into an amino acid with a long side chain (e.g., T366Y), and created “holes” by mutating some amino acids in a CH3 region of a second heavy chain into amino acids with short side chains (Y407T, et al.) The principle of the “knob into hole” model is that the interaction of “knob into hole” supports the heterodimer formation, while the “knob-knob” model and “hole-hole” model hinder the homodimer formation. They further introduced a disulfide bond into the CH3 region on the basis of the “knob into hole” mutation to strengthen the binding capacity of the heterodimer. However, in their research results, the “hole-hole” model still did not have enough ability to hinder the homodimer formation. Later, the research group tried to further enhance the heterodimer content by random mutation-bacteriophage display and other methods, but still did not solve the essential issues. In order to enhance the proportion of heterodimer, some researchers prepared heterodimers by respectively preparing two antibodies and intermolecular disulfide bond reducing-repairing in vitro, but the preparation process is obviously too complex.

[0009] Therefore, it is still necessary to find suitable mutations in this field to further enhance the formation of heterodimer proteins and decrease the formation of homodimer proteins.

### SUMMARY OF INVENTION

[0010] By comprehensively considering various interactions between interfacial amino acids, for example, an ionic action, a hydrophobic interaction and a spatial action, the invention according to the present disclosure has obtained an optimal CH3 mutant sequence, which is more inclined to form a heterodimer rather than a homodimer, thereby greatly improving the yield of the heterodimer molecule.

**[0011]** In a respect, the present disclosure relates to a heterodimer molecule, comprising a first polypeptide chain and a second polypeptide chain, wherein said first polypeptide chain comprises a first CH3 domain of an antibody heavy chain constant region, said second polypeptide chain comprises a second CH3 domain of an antibody heavy chain constant region, and comparing to a corresponding wild-type CH3 domain of a human antibody heavy chain constant region, said first CH3 domain and said second CH3 domain comprise an amino acid mutation selected from the following groups (1) to (3):

**[0012]** (1) an amino acid mutation at Y349 and T366 of said first CH3 domain, and an amino acid mutation at D356, T366, L368 and Y407 of said second CH3 domain, and said first CH3 domain and/or said second CH3 domain further comprise an amino acid mutation at 1-3 residues selected from the group consisting of F405, K409, K360, Q347 and L368;

**[0013]** (2) an amino acid mutation at T366 and K409 of said first CH3 domain, and an amino acid mutation at T366, L368, Y407 and F405 of said second CH3 domain, and optionally said first CH3 domain and/or said second CH3 domain further comprise an amino acid mutation at 1-2 residues selected from the group consisting of K392, D399, Y349, S354 and E357; and

**[0014]** (3) an amino acid mutation at T366 and F405 of said first CH3 domain, and an amino acid mutation at T366, L368, Y407 and K409 of said second CH3 domain, and optionally said first CH3 domain and/or said second CH3 domain further comprise an amino acid mutation at 1-2 residues selected from the group consisting of K392, D399, Y349, S354 and E357;

**[0015]** wherein said amino acid is numbered according to the EU index of the KABAT numbering of the antibody Fc region.

**[0016]** In some embodiments, said first CH3 domain and said second CH3 domain comprise said amino acid mutation selected from said group (2) or (3), but do not comprise mutation Y349C or D356C.

**[0017]** In some embodiments, said first CH3 domain and/or said second CH3 domain further comprise a mutation selected from the following groups:

**[0018]** 1a) a mutation at F405 of said second CH3 domain;

**[0019]** 1b) a mutation at F405 of said first CH3 domain;

**[0020]** 1c) a mutation at K409 of said first CH3 domain, and a mutation at F405 of said second CH3 domain;

**[0021]** 1d) a mutation at F405, K360 and Q347 of said first CH3 domain, and a mutation at Q347 of said second CH3 domain;

**[0022]** 1e) a mutation at F405 and Q347 of said first CH3 domain, and a mutation at K360 and Q347 of said second CH3 domain;

**[0023]** 1f) a mutation at K409, K360 and Q347 of said first CH3 domain, and a mutation at F405 and Q347 of said second CH3 domain;

**[0024]** 1g) a mutation at K409 and Q347 of said first CH3 domain, and a mutation at F405, K360 and Q347 of said second CH3 domain; and

**[0025]** 1h) a mutation at K409 and L368 of said first CH3 domain, and a mutation at F405 of said second CH3 domain.

**[0026]** In some embodiments, said first CH3 domain and/or said second CH3 domain optionally further comprise a mutation selected from the following groups:

**[0027]** 2a) a mutation at K392 of said first CH3 domain and a mutation at D399 of said second CH3 domain;

**[0028]** 2b) a mutation at Y349 of said first CH3 domain and a mutation at E357 of said second CH3 domain; and

**[0029]** 2c) a mutation at Y349 and S354 of said first CH3 domain, and a mutation at E357 of said second CH3 domain.

**[0030]** In some embodiments, said first CH3 domain and/or said second CH3 domain optionally further comprise a mutation selected from the following groups:

**[0031]** 3a) a mutation at D399 of said first CH3 domain and a mutation at K392 of said second CH3 domain;

**[0032]** 3b) a mutation at Y349 of said first CH3 domain and a mutation at E357 of said second CH3 domain; and

**[0033]** 3c) a mutation at Y349 and S354D of said first CH3 domain and a mutation at E357 of said second CH3 domain.

**[0034]** In some embodiments, said amino acid mutations are independently selected from: a mutation from a non-charged amino acid to a charged amino acid, a mutation from a charged amino acid to a non-charged amino acid, or a mutation from a charged amino acid to an oppositely charged amino acid.

**[0035]** In some embodiments, said mutation in said first CH3 structural domain and/or said second CH3 structural domain comprises one or more mutations selected from the following group consisting of: Y349C, Y349D, D356C, T366W, T366S, L368A, L368E, L368G, F405K, Y407V, Y407A, K409E, K409A, K360E, Q347E, Q347R, K392D, D399S, E357A and S354D. For example, the mutation may be one or more mutations selected from the following mutations: Y349C, Y349D, D356C, T366W, T366S, L368A, L368E, L368G, F405K, Y407V, Y407A, K409E, K409A, K360E, Q347E, Q347R, K392D, D399S, E357A and S354D.

**[0036]** In some embodiments, said first CH3 domain comprises a mutation at one or more residues (e.g., at least one residue, at least two residues, at least three residues, at least four residues, at least five residues, at least six residues, at least seven residues, or at least eight residues) selected from the group consisting of: Y349, T366, F405, K409, L368, K392, S354 and D399.

**[0037]** In some embodiments, said second CH3 domain comprises a mutation at one or more residues (e.g., at least one residue, at least two residues, at least three residues, at least four residues, at least five residues, at least six residues, at least seven residues, at least eight residues or at least nine residues) selected from the group consisting of: D356, T366, L368, Y407, F405, D399, E357, K409 and K392.

**[0038]** In some embodiments, said first CH3 domain comprises a mutation at one or more residues (e.g., at least one residue, at least two residues, at least three residues, at least four residues, at least five residues, at least six residues, at least seven residues, or at least eight residues) selected from the group consisting of: Y349, T366, F405, K409, L368, K392, S354 and D399; and said second CH3 domain comprises a mutation at one or more residues (e.g., at least one residue, at least two residues, at least three residues, at least four residues, at least five residues, at least six residues, at

least seven residues, at least eight residues or at least nine residues) selected from the group consisting of: D356, T366, L368, Y407, F405, D399, E357, K409 and K392.

**[0039]** In some embodiments, said first CH3 domain comprises one or more mutations (e.g., at least one mutation, at least two mutations, at least three mutations, at least four mutations, at least five mutations, at least six mutations, at least seven mutations, at least eight mutations or at least nine mutations) selected from the group consisting of: Y349C, T366W, F405K, K409A, L368E, K392D, Y349D, S354D and D399S.

**[0040]** In some embodiments, said second CH3 domain comprises one or more mutations (e.g., at least one mutation, at least two mutations, at least three mutations, at least four mutations, at least five mutations, at least six mutations, at least seven mutations, at least eight mutations, at least nine mutations, at least ten mutations, or at least eleven mutations) selected from the group consisting of: D356C, T366S, L368A, Y407V, F405K, D399S, L368G, Y407A, E357A, K409A and K392D.

**[0041]** In some embodiments, said first CH3 domain comprises one or more mutations (e.g., at least one mutation, at least two mutations, at least three mutations, at least four mutations, at least five mutations, at least six mutations, at least seven mutations, at least eight mutations or at least nine mutations) selected from the group consisting of: Y349C, T366W, F405K, K409A, L368E, K392D, Y349D, S354D and D399S; and said second CH3 domain comprises one or more mutations (e.g., at least one mutation, at least two mutations, at least three mutations, at least four mutations, at least five mutations, at least six mutations, at least seven mutations, at least eight mutations or at least nine mutations) selected from the group consisting of: D356C, T366S, L368A, Y407V, F405K, D399S, L368G, Y407A, E357A, K409A and K392D.

**[0042]** In some embodiments, said first CH3 domain and said second CH3 domain comprise one group of mutations selected from the following groups:

**[0043]** 1) said first CH3 domain: Y349C+T366W, said second CH3 domain: D356C+T366S+L368A+Y407V+F405K;

**[0044]** 2) said first CH3 domain: Y349C+T366W+F405K, said second CH3 domain: D356C+T366S+L368A+Y407V;

**[0045]** 3) said first CH3 domain: Y349C+T366W+K409E, said second CH3 domain: D356C+T366S+L368A+Y407V+F405K;

**[0046]** 4) said first CH3 domain: Y349C+T366W+K409A, said second CH3 domain: D356C+T366S+L368A+Y407V+F405K;

**[0047]** 5) said first CH3 domain: Y349C+T366W+F405K+K360E+Q347E, said second CH3 domain: D356C+T366S+L368A+Y407V+Q347R;

**[0048]** 6) said first CH3 domain: Y349C+T366W+F405K+Q347R, said second CH3 domain: D356C+T366S+L368A+Y407V+K360E+Q347E;

**[0049]** 7) said first CH3 domain: Y349C+T366W+K409A+K360E+Q347E, said second CH3 domain: D356C+T366S+L368A+Y407V+F405K+Q347R;

**[0050]** 8) said first CH3 domain: Y349C+T366W+K409A+Q347R, said second CH3 domain: D356C+T366S+L368A+Y407V+F405K+K360E+Q347E;

**[0051]** 9) said first CH3 domain: Y349C+T366W+K409A+L368E, said second CH3 domain: D356C+T366S+L368A+Y407V+F405K;

**[0052]** 10) said first CH3 domain: T366W+K409A+K392D, said second CH3 domain: T366S+L368A+Y407V+D399S+F405K;

**[0053]** 11) said first CH3 domain: T366W+K409A, said second CH3 domain: T366S+L368G+Y407A+F405K;

**[0054]** 12) said first CH3 domain: T366W+K409A+Y349D, said second CH3 domain: T366S+L368A+Y407V+F405K+E357A;

**[0055]** 13) said first CH3 domain: T366W+K409A+Y349D+S354D, said second CH3 domain: T366S+L368A+Y407V+F405K+E357A;

**[0056]** 14) said first CH3 domain: T366W+F405K, said second CH3 domain: T366S+L368A+Y407V+K409A;

**[0057]** 15) said first CH3 domain: T366W+F405K+D399S, said second CH3 domain: T366S+L368A+Y407V+K409A+K392D;

**[0058]** 16) said first CH3 domain: T366W+F405K, said second CH3 domain: T366S+L368G+Y407A+K409A;

**[0059]** 17) said first CH3 domain: T366W+F405K+Y349D, said second CH3 domain: T366S+L368A+Y407V+K409A+E357A; and

**[0060]** 18) said first CH3 domain: T366W+F405K+Y349D+S354D, said second CH3 domain: T366S+L368A+Y407V+K409A+E357A.

**[0061]** In some embodiments, said first CH3 domain and said second CH3 domain contain one group of mutations selected from the following groups:

**[0062]** 2) said first CH3 domain: Y349C+T366W+F405K, said second CH3 domain: D356C+T366S+L368A+Y407V;

**[0063]** 4) said first CH3 domain: Y349C+T366W+K409A, said second CH3 domain: D356C+T366S+L368A+Y407V+F405K;

**[0064]** 9) said first CH3 domain: Y349C+T366W+K409A+L368E, said second CH3 domain: D356C+T366S+L368A+Y407V+F405K;

**[0065]** 10) said first CH3 domain: T366W+K409A+K392D, said second CH3 domain: T366S+L368A+Y407V+D399S+F405K;

**[0066]** 11) said first CH3 domain: T366W+K409A, said second CH3 domain: T366S+L368G+Y407A+F405K;

**[0067]** 13) said first CH3 domain: T366W+K409A+Y349D+S354D, said second CH3 domain: T366S+L368A+Y407V+F405K+E357A;

**[0068]** 15) said first CH3 domain: T366W+F405K+D399S, said second CH3 domain: T366S+L368A+Y407V+K409A+K392D;

**[0069]** 16) said first CH3 domain: T366W+F405K, said second CH3 domain: T366S+L368G+Y407A+K409A; and

**[0070]** 18) said first CH3 domain: T366W+F405K+Y349D+S354D, said second CH3 domain: T366S+L368A+Y407V+K409A+E357A.

**[0071]** In some embodiments, said first polypeptide chain and said second polypeptide chain further comprise a CH2 domain of an antibody heavy chain constant region, respectively. In some embodiments, the said CH2 domain is located at the N-terminal of the CH3 domain, and is linked directly or through a linker peptide to the N-terminal of the CH3 domain.

[0072] In some embodiments, said first polypeptide chain and said second polypeptide chain further comprise a hinge region of an antibody heavy chain constant region or a part thereof, respectively. In some embodiments, said part of said hinge region is D221-P230.

[0073] In some embodiments, said hinge region or a part thereof is located at the N-terminal of the CH3 domain. And when there is a said CH2 domain, said hinge region or a part thereof is further located at the N-terminal of the CH2 domain, and is linked directly or through a linker peptide to the CH2 or CH3 domain.

[0074] In some embodiments, said wild-type CH3 domain of the human antibody heavy chain constant region is selected from the group consisting of a CH3 domain of a human IgG (e.g., IgG1, IgG2, IgG3 or IgG4) heavy chain constant region, a CH3 domain of a human IgA (e.g., IgA1, IgA2) heavy chain constant region, a CH3 domain of a human IgD heavy chain constant region, a CH3 domain of a human IgE heavy chain constant region and a CH3 domain of a human IgM heavy chain constant region.

[0075] In some embodiments, said wild-type CH3 domain of the human antibody heavy chain constant region is a CH3 domain of a human IgG1 heavy chain constant region.

[0076] In some embodiments, said first polypeptide chain and/or said second polypeptide chain further comprise a molecule binding region, and said molecule binding region is selected from the group consisting of an antigen binding region, a receptor binding region and an enzyme binding region. In some embodiments, said antigen binding region comprises an antibody variable region.

[0077] In some embodiments, said heterodimer molecule is a bispecific antibody, a bispecific fusion protein or an antibody-fusion protein chimera.

[0078] In another respect, the present disclosure relates to a composition (e.g., a pharmaceutical composition), comprising the heterodimer molecule according to the present disclosure, and optionally a pharmaceutically acceptable carrier or excipient.

[0079] In another respect, the present disclosure provides a nucleic acid molecule, encoding said first polypeptide chain or said second polypeptide chain of the heterodimer molecule according to the present disclosure, or encoding said first polypeptide chain and said second polypeptide chain of the heterodimer molecule according to the present disclosure.

[0080] In another respect, the present disclosure provides a vector, comprising the nucleic acid molecule according to the present disclosure.

[0081] In another respect, the present disclosure provides a host cell, comprising the vector according to the present disclosure.

[0082] In another respect, the present disclosure provides a use of the heterodimer molecule, the composition, the nucleic acid, the vector or the host cell in the manufacture of a bispecific antibody, a bispecific fusion protein or an antibody-fusion protein chimera according to the present disclosure.

[0083] In another respect, the present disclosure provides a method for preparing a heterodimer molecule, comprising expressing the heterodimer molecule using the host cell according to the present disclosure.

[0084] In some embodiments of said method for preparing a heterodimer molecule, the host cell comprises a vector encoding said first polypeptide chain and said second poly-

peptide chain of the heterodimer molecule, and the method comprises expressing, recovering and obtaining the heterodimer molecule using the host cell.

[0085] In some embodiments of said method for preparing a heterodimer molecule, the host cell comprises a first group of cells comprising a vector encoding said first polypeptide chain of the heterodimer molecule, a second group of cells comprising a vector encoding said second polypeptide chain of the heterodimer molecule, and the method comprises expressing said first polypeptide chain in said first group of cells to form a homodimer of said first polypeptide chain, expressing said second polypeptide chain in said second group of cells to form a homodimer of said second polypeptide chain, and then mixing the homodimer of said first polypeptide chain with the homodimer of said second polypeptide chain under a condition to form the heterodimer molecule. In some embodiments, said method further comprises reducing the homodimer of said first polypeptide chain and the homodimer of said second polypeptide chain to monomers, mixing and oxidizing the monomers, and then purifying the obtained heterodimer molecule. In some embodiments, said host cell comprises a vector encoding said first polypeptide chain and said second polypeptide chain of the heterodimer molecule, said first polypeptide chain and said second polypeptide chain are expressed respectively in two said host cells to form a homodimer of said first polypeptide chain and a homodimer of said second polypeptide chain, and then reducing, mixing, oxidizing and purifying the homodimer of said first polypeptide chain and the homodimer of said second polypeptide chain under a proper condition to obtain said heterodimer molecule.

[0086] In some embodiments of said method for preparing a heterodimer molecule, said first group of cells and said second group of cells were transfected with a construct or vector which comprises said first polypeptide chain or said second polypeptide chain, respectively. Said transfection may be transient transfection. For said transfection, the molar ratio of said construct or vector comprising said first polypeptide chain to said construct or vector comprising said second polypeptide chain may be 1:4 to 4:1, for example, 1:2 to 2:1, for example, about 1:1.

[0087] Additional aspects and advantages of the present disclosure will become readily apparent to those skilled in this art from the following detailed description, wherein only illustrative embodiments of the present disclosure are shown and described. Numerous modifications of the embodiments of the disclosure described herein will now occur to those skilled in the art without departing from the disclosure. Accordingly, the drawings and description of the present disclosure are to be regarded as illustrative in nature, but not as restrictive.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0088] FIG. 1 shows the electrophoretic analysis result of transiently expressed ScFv-Fc/Fc heterodimer. 4% to 12% SDS-PAGE protein gel electrophoresis was used. Lanes 1 to 7 are successively: protein molecular mass markers; mutation combination KH, mutation combination 1, mutation combination 2, mutation combination 3, mutation combination 4 and a wild-type negative control combination. The homodimer and the heterodimer of products in each combination exhibit different migration distances in the gel

electrophoresis due to molecular weight differences. The sites of different homodimer and heterodimer proteins are indicated in the FIG. 1.

**[0089]** FIG. 2 shows the electrophoretic analysis result of transiently expressed ScFv-Fc/Fc heterodimer. 12% SDS-PAGE protein gel electrophoresis was used. Lanes 1 to 9 are successively: mutation combination 9, mutation combination 8, mutation combination 7, mutation combination 4, mutation combination 6, mutation combination 5, mutation combination 2, blank control (cell supernatant) and protein molecular mass markers. The homodimer and the heterodimer of products in each combination exhibit different migration distances in the gel electrophoresis due to molecular weight differences. Similar to FIG. 1, ScFv-Fc/ScFv-Fc homodimer, ScFv-Fc/Fc heterodimer and Fc/Fc homodimer are shown from top to bottom in FIG. 2.

**[0090]** FIG. 3 shows a partial view of a crystal structure of CH3-CH3 interface of a heterodimer Fc in the mutation combination 4. Mutated amino acid residues are shown by short sticks, and specifically contain the following mutually contacting mutated amino acid residue pairs: T366W/A chain-T366S, L368A, Y407V/B chain, K409A/A chain-F405K/B chain and S354C/A chain-Y349C/B chain. Chain A (the left chain in a slightly lighter color) is indicated in green, and chain B (the right chain in a slightly darker color) is indicated in light blue.

**[0091]** FIG. 4 shows that introducing a new pair of mutations D399S-K392D at a site near the amino acid residue pair of mutations F405K-K409A can further enhance the mutual attraction between heterodimers as well as mutual repulsion between homodimers. FIG. 4A shows the interaction between the mutation F405K-K409A and nearby interfacial amino acids when a new mutation F405K-K409A is introduced. FIG. 4B shows the interaction change caused by the introduction of a new mutation.

#### DETAILED DESCRIPTION

**[0092]** The embodiments of the invention are illustrated in conjunction with the specific embodiments below, and those skilled in the art can understand other advantages and functions of the invention through the contents disclosed in the description.

**[0093]** In the present disclosure, both of said first polypeptide chain and said second polypeptide chain comprise a CH3 domain of an antibody Fc region, and the said two polypeptide chains interact with each other through the CH3 domain or the Fc region which comprises the CH3 domain to form a dimer, especially a heterodimer. Two polypeptide chains of the heterodimer may be different combinations. For example, said first polypeptide chain is an antibody and said second polypeptide chain is a fusion protein, or both of the two polypeptide chains are fusion protein, or both of two polypeptide chains is an antibody (e.g., antibodies targeting to different antigen or antigen epitope). When the fusion protein comprises an antibody Fc region and an extracellular domain of a cell adhesion molecule, it is also known as an immune adhesin. Said cell adhesion molecule mainly refers to a molecule capable of identifying specific ligand cell surface receptor, for example, comprising cadherin, selectin, immunoglobulin superfamily, integrin and hyaladherin.

**[0094]** In the present disclosure, said CH3 domain is derived from a Fc region of an antibody, e.g., from a Fc region of human antibody (e.g., a Fc region of a human antibody heavy chain constant region). In some embodi-

ments, said CH3 domain is derived from a Fc region of a human immunoglobulin (Ig) heavy chain constant region, e.g., from a Fc region of a heavy chain constant region of IgM, IgG (e.g., IgG1, IgG2, IgG3, IgG4), IgA (e.g., IgA1, IgA2), IgE and/or IgD. In some embodiments, said CH3 domain (e.g., a wild-type CH3 domain of human antibody heavy chain constant region) is derived from wild-type IgG1 of human, e.g., a wild-type CH3 domain of a human IgG1 antibody heavy chain constant region. In general, said CH3 domain of the Fc region of human antibody is derived from a corresponding wild-type Fc region of human antibody. A wild-type human antibody Fc region refers to an antibody Fc region in a natural human population, e.g., a Fc region of human antibody that is not artificially induced or artificially modified. In some embodiments, the Fc region of human antibody according to the present disclosure also comprises particular amino acid mutation of a corresponding wild-type human antibody Fc sequence, e.g., comprising amino acid mutations at a glycosylation site or other nonsense mutations, and also comprising particular amino acid mutations including according to the “knob-hole” model. For example, for CH3 and CH2 domains, except for mutations mentioned in the present disclosure, there may also be other mutations that do not affect the functions of antibodies (especially Fc region).

**[0095]** In the present disclosure, when the first polypeptide chain and/or the second polypeptide chain comprise a hinge region, said hinge region is linked between the two polypeptide chains as a flexible chain to ensure the functions of each polypeptide chain. Those skilled in the art can select the length of the hinge region as required, for example, selecting a full-length sequence or a part of the sequence thereof.

**[0096]** In the present disclosure, said amino acid of said Fc region or CH2, CH3 domain or hinge region is numbered according to the EU index of the Kabat numbering. As is known to those skilled in the art, even if said amino acid insertions or deletions or other mutations lead to a change of the amino acid sequences in the above regions, site numbers of the amino acids determined according to the standard sequences of EU index of the Kabat numbering remain unchanged.

**[0097]** In the present disclosure, a human antibody heavy chain constant region may comprise a combination of two or more domains in CH1, CH2, CH3 and CH4 domain in heavy chains with an antibody hinge region. In some embodiments, said human antibody Fc region comprises at least one antibody hinge region, one CH2 domain and one CH3 domain. In some embodiments, said CH2 domain is a CH2 domain of a human IgG1 heavy chain constant region, which corresponds to amino acids 228-340 according to the EU index numbering system. In some embodiments, the CH2 domain corresponds to a corresponding region of any other isotypic antibody described in the present disclosure. In some embodiments, said CH3 domain is a CH3 domain of a human IgG1 heavy chain constant region, which corresponds to amino acids 341-447 according to the EU index numbering system. In some embodiments, the CH3 domain corresponds to a corresponding region of any other isotypic antibody described in the present disclosure.

**[0098]** In the present disclosure, the charged amino acid comprises arginine, lysine, aspartic acid and glutamic acid.

**[0099]** In the present disclosure, the heterodimer molecule can be purified from a host cell using a standard experimental method. For example, when a heterodimer protein com-



prises a Fc region of an antibody, it can be purified using protein A. The purification method comprises, but not limited to, a chromatographic technique, such as size exclusion, ion exchange, affinity chromatography and ultrafiltration, or an appropriate combination of the methods thereof.

[0100] In the present disclosure, the EU index is described in, e.g., Kabat, etc., *Sequences of Proteins of Immunological Interest, Public Health Service* 5th edition, National Institutes of Health, Bethesda, MD. (1991).

[0101] In the present disclosure, by comprehensively considering various interactions between interfacial amino acids, for example, an ionic action, a hydrophobic interaction and a spatial action, a preferred CH3 mutant sequence being more inclined to form a heterodimer rather than a homodimer was screened, thereby greatly enhancing the yield of the heterodimer molecule. Furthermore, in some embodiments of the present disclosure, a heterodimer protein crystal comprising a Fc region was prepared, and the crystal structure was analyzed and a three-dimensional structure model was established to further understand the direct interaction between interfacial amino acids, and the previous view that a stable disulfide bond is bound to be formed between two cysteines on Y349C and D356C was abandoned. A mutation combination formed on this basis is more inclined to form heterodimers, rather than homodimers, thus greatly reducing the proportion of homodimers while greatly enhancing the proportion of heterodimers.

[0102] The embodiments of the application according to the present disclosure will be described below in detail in conjunction with the examples, but those skilled in the art will understand that such examples are exemplary only and it is not intended that the invention be limited by the specific examples provided within the specification. The specific conditions which were not indicated in the specific examples, are in accordance with general conditions or conditions recommended by the manufacturer. The reagents or equipment which did not indicate manufacturers, are conventional products which can be obtained from the market.

Example 1: Acquisition of First Round Mutation Combination Candidate

1. Modeling of Fc Domain and Acquisition of an Interfacial Amino Acid

[0103] A total of 48 crystal structures of human IgG1 antibody comprising a Fc domain were acquired from Protein Database (PDB), and a structural similarity search algorithm (Reference: Yuzhen Ye and Adam Godzik. FAT-CAT: a web server for flexible structure comparison and structure similarity searching. *Nucleic Acids Res.*, 2004, 32 (Web Server issue): W582-585.) was used to conclude that the Fc regions of the 48 antibodies were derived from 1DN2 (PDB number).

[0104] Amino acid contact between CH3-CH3 domain of antibodies (PDB No.: 1DN2) was screened and identified based on amino acid interaction distance using CMA software which can identify contact amino acids of proteins. According to the amino acid contact regulation, an interfacial amino acid refers to an amino acid with the distance between a heavy atom on a side chain and a heavy atom of any one amino acid on the other chain less than a threshold value. In this example, the threshold value is 4.5 Å, and may also be 5.5 Å(e.g., manuscript: B. Erman, I. Bahar and R. L.

Jemigan. Equilibrium states of rigid bodies with multiple interaction sites. Application to protein helices. *J. Chem. Phys.* 1997, 107:2046-2059). The conservative conditions of the contact interface between human and mouse IgG subtype amino acids can be obtained through multiple sequence alignment. Table 1 shows 34 interfacial amino acids of an antibody 1DN2 screened by amino acid contact (that is, the distance between two amino acid molecules is less than 4.5 Å), where chain A and chain B represent a first chain and a second chain of the antibody 1DN2, respectively. The following amino acid was numbered according to the EU index of the KABAT numbering of the antibody Fc region.

TABLE 1	
List of CH3—CH3 Interfacial Amino Acids of Antibody 1DN2	
Contacting amino acids in chain A	Contacting amino acids in chain B
Gln347	Lys360
Val348	Glu356
Tyr349	Ser354, Glu356, Glu357, Lys360
Thr350	Ser354, Glu356
Leu351	Leu351, Pro352, Pro353, Ser354, Thr366
Pro352	Leu351, Pro352
Pro353	Leu351
Ser354	Tyr349, Thr350, Leu351
Glu356	Val348, Tyr349, Thr350, Lys439
Glu357	Tyr349, Leu368, Lys370
Lys360	Gln347, Tyr349, Lys370
Gln362	Lys370
Val363	Lys370
Ser364	Leu368, Lys370, Tyr407
Leu365	Tyr407
Thr366	Leu351, Leu368, Tyr407
Leu368	Glu357, Ser364, Thr366, Lys409
Lys370	Glu357, Lys360, Gln362, Ser364, Lys409, Thr411
Asn390	Ser400
Lys392	Val397, Leu398, Asp399, Ser400, Phe405
Thr393	Val397
Thr394	Thr394, Val397, Phe405, Tyr407
Pro395	Pro395, Val397
Val397	Lys392, Thr393, Thr394, Pro395
Leu398	Lys392
Asp399	Lys392, Lys409, Thr411
Ser400	Asn390, Lys392
Phe405	Lys392, Thr394, Tyr407, Lys409
Leu406	Thr394
Tyr407	Thr366, Thr394, Phe405, Tyr407, Lys409
Ser408	Tyr407
Lys409	Leu368, Lys370, Asp399, Phe405, Tyr407
Thr411	Lys370, Asp399
Lys439A	Glu356B

2. Mutating Amino Acids to Change Ionic Action

[0105] According to the results of Table 1, an amino acid pair containing a charged amino was selected from said contacting amino acid pairs, and one amino on one chain therein was mutated (a non-charged amino acid became a charged amino acid, or a charged amino acid became a non-charged amino acid, or a charged amino acid became oppositely charged), so that the ionic action between a Fc chain A and a Fc chain B was unbalanced, thus the probability of homodimer formation was decreased, and/or the heterodimer was increased.

[0106] As an example, e.g., Phe405 of chain A was mutated to Phe405Lys (may also be written as F405K), and

chain B remained unchanged. Because the contacting amino acid residue on the chain B, which was around 405th amino acid residue comprised two Lys, both of which were positively charged amino acids, when a chain A paired with a chain A, positive charges carried by an F405K mutation on the two chains would introduce a great repulsive force; while when a chain A paired with a chain B, only one chain (chain A) exhibited the repulsive force introduced by the F405K mutation, and the other chain (chain B) maintained as Phe405 without introducing a repulsive force. Under this condition, there was very significant mutual repulsion between two chains A, which is much greater than the mutual repulsion between the chain A and the chain B or between the two chains B, and therefore can effectively reduce the formation of AA homodimer.

[0107] If a F405K mutation was introduced into a chain A and the contacting amino acid residue Lys409 on a chain B corresponding to the F405K mutation residue on the chain A was mutated to K409E or K409A, then when a chain A paired with a chain A, positive charges introduced by the F405K mutation on the two chains A will still introduce a great repulsive force; when a chain A paired with a chain B, the F405k mutation on the chain A interacted with the K409E or K409A mutation on the chain B without a repulsive force, or even with an attractive force (K409E); and when a chain B paired with a chain B, neither repulsive force nor attractive force was introduced. Under this condition, there was very significant mutual repulsion between the two chains A, and the repulsive force between the chain A and the chain B was reduced or an attractive force was introduced between the chain A and the chain B, which can therefore effectively reduce the formation of AA homodimer, and promote the formation of AB heterodimer at the same time.

[0108] Similarly, mutation combinations obtained in this example are shown in the table below:

TABLE 2

List of Mutation Combinations of Heterodimers			
Combination	Fc chain	Mutation	Corresponding SEQ ID NO
KH	A	Y349C + T366W	6
	B	D356C + T366S + L368A + Y407V	7
1	A	Y349C + T366W	6
	B	D356C + T366S + L368A + Y407V + F405K	8
2	A	Y349C + T366W + F405K	9
	B	D356C + T366S + L368A + Y407V	7
3	A	Y349C + T366W + K409E	10
	B	D356C + T366S + L368A + Y407V + F405K	8
4	A	Y349C + T366W + K409A	11
	B	D356C + T366S + L368A + Y407V + F405K	8

Example 2: Preparation and Investigation of ScFv-Fc/Fc Heterodimer

1. Constructing a Recombinant Vector Expressing a Mutated Fc Region of Human IgG1 and a ScFv-Fc Fusion Protein

[0109] Based on an amino acid sequence (P01857) of a human immunoglobulin gamma1 (IgG1) constant region in

a UNIPROT™ protein database, an amino acid sequence (SEQ ID NO:1) of a human IgG1-Fc region was obtained. By reverse transcription PCR, a nucleic acid fragment (SEQ ID NO:2, named as Fc gene) which encodes human IgG1-Fc was obtained from total RNA of human PBMC. By overlapping PCR, adding a coding sequence (as shown in SEQ ID NO: 3) of a kappaIII signal peptide of mouse at the 5'-terminal, and then subcloning it into a vector pcDNA4 (Invitrogen, Cat V86220), a recombinant expression vector for expression of a human IgG1-Fc (Fc for short) protein in mammalian cells was obtained.

[0110] A ScFv-Fc fusion protein coding gene (ScFv therein refers to an anti-HER2 single chain antibody) as shown in SEQ ID NO: 5 was obtained by artificial synthesis. The gene encoding a ScFv-Fc fusion protein sequence is shown in SEQ ID: 4, and was then subcloned to a mammalian cell expression vector pcDNA4 (Invitrogen, cat V86220) to obtain a recombinant expression vector for expressing the ScFv-Fc fusion protein in mammalian cells.

[0111] According to Table 2 of Example 1, a mutation combination of ScFv-Fc and Fc coding genes was performed by overlapping PCR, where the mutation of the chain A was located on the ScFv-Fc fusion protein, and the mutation of the chain B was located on the Fc protein. The mutant gene was subcloned to pcDNA4 (Invitrogen, cat V86220) to finally obtain the ScFv-Fc fusion protein to express mutations in mammalian cells and a recombinant expression vector of mutant Fc protein, respectively.

2. Transient Expression of a ScFv-Fc/Fc Heterodimer and Detection of the Influence of Different Mutation Combinations on Heterodimer Content

[0112] Corresponding expression vectors of 4 mutation combinations in step 1, KH combination (as a reference group) and a wild-type combination (i.e., unmutated ScFv-Fc fusion protein and Fc protein as negative control group) were transfected into 293H suspension-culture cell (American Type Culture Collection (or "ATCC") catalog #ATCC CRL-1573). Each mutation combination included cotransfection of recombinant expression vectors of the corresponding chain A (referring to ScFv-Fc fusion protein chain) and the chain B (referring to Fc protein chain), and the cotransfection ratio of recombinant expression vectors of the chain A to that of chain B was 1:1. After cultivation for 5-6 days, the transiently expressed culture supernatant was collected, and preliminarily purified transient transfection products of 4 groups of mutation combinations, KH mutation combination and wild-type negative control group were obtained by ProteinA affinity chromatography. Each of these transient transfection products contained different proportions of homodimer proteins (ScFv-Fc/ScFv-Fc, Fc/Fc) and heterodimer protein (ScFv-Fc/Fc). As the three proteins (ScFv-Fc/ScFv-Fc, Fc/Fc, and ScFv-Fc/Fc) had different molecular weights, the compositions of the homodimer protein (ScFv-Fc/ScFv-Fc, Fc/Fc) and the heterodimer protein (ScFv-Fc/Fc) in the product of each group could be detected by SDS-PAGE electrophoresis under non-reducing conditions, and the proportion of the homodimer protein (ScFv-Fc/ScFv-Fc, Fc/Fc) to the heterodimer protein (ScFv-Fc/Fc) was analyzed with Imagelab professional image analysis software provided by BioRad company. The electrophoresis test results are shown in FIG. 1 and Table 3.

TABLE 3

Ratio of Homodimer to Heterodimer in Transient Transfection Product for Various Mutation Combinations					
Mutation combination	Mutant amino acids on chain A (ScFv-Fc protein)	Mutant amino acids on chain B (Fc protein)	ScFv-Fc homodimer (%)	ScFv-Fc/Fc heterodimer (%)	Proportion of Fc homodimer (%)
Wild-type control group	N.A.	N.A.	59	35	6
1	Y349C + T366W	D356C + T366S + L368A + Y407V + F405K	24	58	18
2	Y349C + T366W + F405K	D356C + T366S + L368A + Y407V	10	70	20
3	Y349C + T366W + K409E	D356C + T366S + L368A + Y407V + F405K	25	57	18
4	Y349C + T366W + K409A	D356C + T366S + L368A + Y407V + F405K	10	77	13
KH	Y349C + T366W	D356C + T366S + L368A + Y407V	29	51	20

[0113] Compared with the wild-type negative control combination, the proportions of heterodimer (ScFv-Fc/Fc) in 4 groups of candidate mutation combinations and the KH combination were increased significantly. At the same time, on the basis of KH, after introducing new mutations, the proportion of heterodimers had also changed with the proportion of some heterodimers significantly increased (e.g., combinations 2,4) and the proportion of other heterodimers modestly improved (e.g., combinations 1,3). Here, it should be noted that new mutation combinations of the groups comprised regulation of two major interactions (spatial effect and ionic action) on interfacial side chain groups, and therefore, their impact on heterodimer contents cannot be simply considered as superposition of the two interactions. For example, combination 1 and combination 2 both introduced the F405K mutation to increase the repulsive force between homodimers, but combination 2 showed better effect than combination 1 in enhancing the heterodimer content (heterodimer content in the mutation combination 2 is about 70%, while combination 1 is about 58%). In addition, for the mutation introduced into K409, the heterodimer content was increased (77%) resulted from non-charged mutation in the mutation combination 4, which was more significant than that resulted from oppositely charged mutation in the mutation combination 3 (57%). However, similar effects should be resulted from the two mutation combination 1 and 4 if simply considering the superposition of the two interactions in theory.

[0114] In order to further investigate the influence of the cotransfection ratio of recombinant expression vectors of the chain A to those of the chain B on the ratio of homodimers to heterodimers, the cotransfection expression vectors used in two superior mutation combinations (2 and 4) and the KH combination were transfected with PEI to 293H suspension-culture cells (catalog #ATCC CRL-1573) at a ratio of 4:1 and 1:4, respectively, and the cell culture supernatant was collected after 5-6 days of cultivation. The respective transient transfection products were obtained through Protein A

affinity chromatography. The compositions of homodimer proteins (ScFv-Fc/ScFv-Fc, Fc/Fc) and heterodimer proteins (ScFv-Fc/Fc) were detected by SDS-PAGE electrophoresis under non-reducing conditions. The specific results are shown in Table 4. As can be seen from the results, the cotransfection ratio of recombinant expression vectors had a significant influence on the ratio of homodimers and heterodimers in the product. The content of heterodimers in the product is significantly reduced at a cotransfection ratio of 4:1 and 1:4. The result shows that when expressions of the chain A and the chain B were relatively balanced, the three combinations could greatly enhance the proportion of heterodimers in the product and reduced the proportion of homodimers, but when expressions of the chain A and the chain B in the product are imbalanced, resultant excessive chain A or chain B would enhance the proportion of homodimers while reduced the heterodimer. In the KH combination, no matter which chain was excessive, the heterodimer content would be significantly reduced. Excessive chain B (Fc) in the mutation combination 2 had greater effect; while excessive chain A (ScFv-Fc) in the mutation combination 4 had greater effect. However, even if the chain B or chain A in the mutation combination 2 or the mutation combination 4 was excessive, the proportion of heterodimers formed thereof was still significantly higher than those in the KH combination of the control group. As can be seen through further analysis on the results, among the three mutation combinations, while the interaction between the chain A and the chain B has been significantly enhanced, the weakening degree of the interaction between the chain A and the chain A or between the chain B and the chain B was still insufficient, which further resulted in a fact that when one component thereof was excessively expressed, the balance between homodimers and heterodimers was broken, and more homodimers were produced. The new mutation combinations 2 and 4 therein had obvious optimization in preventing the formation of homodimers, compared to the KH combination.

TABLE 4

Influence of Different Cotransfection Ratios on the Ratio of Homodimers to Heterodimers				
Combination	Cotransfection ratio of recombinant expression vector of chain A (ScFv-Fc) to that of chain B (Fc) in	ScFv-Fc homodimer (%)	ScFv-Fc/Fc heterodimer (%)	Fc-Fc homodimer (%)
2	4:1	23	66	11
	1:4	<1	49	51
4	4:1	46	53	1
	1:4	4	66	30
KH	4:1	52	42	6
	1:4	10	44	46

#### Example 3: Acquisition of Second Round Candidate Mutation Combination

**[0115]** On the basis of the preferred Fc mutation combinations (mutation combination 2 and mutation combination 4) mentioned in Examples 1 and 2, interfacial amino acid mutation was further introduced according to the disclosed three-dimensional crystal structure of wild-type Fc, so as to further reduce the mutual attraction between the chain A and the chain A and between the chain B and the chain B, and inhibit the formation of homodimer proteins.

**[0116]** According to the results in Table 1, an amino acid paired with a charged amino acid was further selected from contacting amino acids near mutation sites in the mutation combination 2 or mutation combination 4, and one amino acid on one chain therein (a non-charged amino acid was mutated to a charged amino acid, or a charged amino acid was mutated to a non-charged amino acid, or a charged amino acid was mutated to oppositely charged) was mutated in order to further improve the unbalance property of the ionic action between the chain A and the chain B, as well as to decrease the probability of homodimers formation or to increase the probability of formation of heterodimers at the same time.

**[0117]** For example, the contacting amino acid pair of Lys360 on chain A and Gln347 on chain B was mutated to change the ionic action therebetween. The two amino acid residues on one chain (e.g., chain A) thereof were mutated to negatively charged amino acid residues by, e.g., introducing mutations K360E and Q347E; non-charged amino acid residues on the other chain (e.g., chain B) were mutated to positively charged amino acid residues by, e.g., introducing mutation Q347R. Under the condition, when the chain A interacts with the chain A, the negative charge carried at 360th and 347th site would be mutually repulsive; when the chain B interacts with the chain B, the positive charge on the two sites would be mutually repulsive; and only when the chain A interacts with the chain B, the respective positive and negative charges thereof would attract each other. It was expected that such mutation would increase the mutual repulsion between the chain A and the chain A and between the chain B and the chain B, whilst increasing the mutual attraction between the chain A and the chain B.

**[0118]** Moreover, the amino acid residue of Leu368 was also investigated. The residue was surrounded by two charged amino acid residues: Glu357 and Lys409. Considering that the K409A mutation was introduced into the foregoing mutation combination 4, Leu368 on the Fc chain

into which the K409A mutation was introduced (according to Example 2, here referred as the chain A) was further mutated to a negatively charged amino acid residue (e.g., 368E). Under the condition, when the chain A paired with the chain A, the negative charge carried by L368E on the two chains would interact with the negative charge carried by E357 to introduce a repulsive force; when chain A paired with chain B, the negative charge carried by L368E on chain A would not only repel the negative charge carried by E357 on chain B, but also attracted K409 on the chain B. Comprehensively, not too much repulsive force or attractive force was introduced. It was expected that such mutation would increase the mutual repulsion between the chain A and the chain A, but would not affect the interaction between the chain A and the chain B or between the chain B and the chain B.

**[0119]** Based on the preferred Fc mutation combinations (mutation combination 2 and mutation combination 4) mentioned in Examples 1 and 2, as well as the newly introduced mutation combination, the resulting mutation combinations are shown in Table 5:

TABLE 5

List of Mutation Combinations of Heterodimers-2			
Combination	Fc chain	Mutation	Corresponding SEQ ID NO
5	A	Y349C + T366W + F405K + K360E + Q347E	12
	B	D356C + T366S + L368A + Y407V + Q347R	13
6	A	Y349C + T366W + F405K + Q347R	14
	B	D356C + T366S + L368A + Y407V + K360E + Q347E	15
7	A	Y349C + T366W + K409A + K360E + Q347E	16
	B	D356C + T366S + L368A + Y407V + F405K + Q347R	17
8	A	Y349C + T366W + K409A + Q347R	18
	B	D356C + T366S + L368A + Y407V + F405K + K360E + Q347E	19
9	A	Y349C + T366W + K409A + L368E	20
	B	D356C + T366S + L368A + Y407V + F405K	8

#### Example 4: Preparation and Investigation of a New Round of ScFv-Fc/Fc Heterodimer Mutation Combination

##### 1. Constructing a Recombinant Vector Expressing a Mutated Fc Region of Human IgG1 and a ScFv-Fc Fusion Protein

**[0120]** According to Table 5 of Example 3, a recombined mutation of ScFv-Fc and Fc encoding genes was performed by overlapping PCR with the recombinant expression vector of the wild-type ScFv-Fc and Fc proteins constructed in Example 2 as the template, where the mutation of the chain A was located on the ScFv-Fc fusion protein, and the mutation of the chain B was located on the FC protein. The mutant gene was subcloned to pcDNA4 (Invitrogen, cat V86220) to finally obtain the ScFv-Fc fusion protein to

express a new round of mutations in mammalian cells and a recombinant expression vector of the mutant Fc protein.

2. Transient Expression of a ScFv-Fc/Fc Heterodimer and Detection of the Influence of Different Mutation Combinations on Heterodimer Content

[0121] According to the method in Example 2-2, the 5 new mutation combinations (5 to 9) and the first round of preferred mutation combinations (2 and 4) was transiently expressed using 293H cells (catalog #ATCC CRL-1573). The cotransfection ratio of recombinant expression vector of the chain A to that of the chain B was 1:1. After 5-6 days of cultivation, the transiently expressed culture supernatant was collected, and 5 groups of preliminarily purified new mutation combinations and 2 groups of the first round of preferred combinations of transient transfection products were obtained by Protein A affinity chromatography. Each of these transient transfection products contained different proportions of homodimer proteins (ScFv-Fc/ScFv-Fc, Fc/Fc) and heterodimer protein (ScFv-Fc/Fc). As the three proteins (ScFv-Fc/ScFv-Fc, Fc/Fc, and ScFv-Fc/Fc) have different molecular weights, the compositions of the homodimer protein (ScFv-Fc/ScFv-Fc, Fc/Fc) and the heterodimer protein (ScFv-Fc/Fc) in the product of each group can be detected by SDS-PAGE electrophoresis under non-reducing conditions, and the proportion of the homodimer protein (ScFv-Fc/ScFv-Fc, Fc/Fc) to the heterodimer protein (ScFv-Fc/Fc) was analyzed with ImageLab professional image analysis software provided by Biorad company. The electrophoresis test results are shown in FIG. 2 and Table 6.

8 over the combination 4. In addition, after new mutations were introduced into the combination 9, it contrarily greatly reduced the proportion of formation of heterodimers, presumably because the mutual repulsion between the negative charge carried by L368E newly introduced into the chain A and the negative charge carried by E357 on the chain B was more than the mutual attraction between the negative charge carried by L368E on the chain A and K409 on the chain B, resulting in an unstable heterodimer. In general, some groups of the newly introduced mutations appropriately contributed to the formation of heterodimers, but did not bring significant improvements.

[0123] In order to further investigate the influence of the newly introduced mutations on AA homodimer and BB homodimers, proteins of the chain A or proteins of the chain B were separately transiently expressed, and the tendency of forming homodimers was investigated by comparing the homodimer protein expression level under equivalent transient transfection conditions. The recombinant expression vector was transfected with PEI into suspension-cultured 293H cells (catalog #ATCC CRL-1573), and the cell supernatant was collected after 5-6 days of cultivation. The respective transient transfection product was obtained through Protein A affinity chromatography, and the expression levels thereof were detected by OD280. The results are shown in Table 7. As can be seen from the expression level, some mutations (combination 8, combination 9) introduced into the chain A of the combination 4 could reduce the tendency of forming homodimers thereof; some mutations (combination 5) introduced into the chain B of the combi-

TABLE 6

Ratio of Homodimers and Heterodimers in Transient Transfection Product of Each Mutation Combination-2					
Mutation combination	Mutant amino acids on chain A (ScFv-Fc protein)	Mutant amino acids on chain B (Fc protein)	ScFv-Fc homodimer (%)	ScFv-Fc/Fc heterodimer (%)	Proportion of Fc homodimer (%)
2	Y349C + T366W + F405K	D356C + T366S + L368A + Y407V	17	60	23
5	Y349C + T366W + F405K + K360E + Q347E	D356C + T366S + L368A + Y407V + Q347R	14	72	14
6	Y349C + T366W + F405K + Q347R	D356C + T366S + L368A + Y407V + K360E + Q347E	14	62	24
4	Y349C + T366W + K409A	D356C + T366S + L368A + Y407V + F405K	21	69	10
7	Y349C + T366W + K409A + K360E + Q347E	D356C + T366S + L368A + Y407V + F405K + Q347R	24	64	12
8	Y349C + T366W + K409A + Q347R	D356C + T366S + L368A + Y407V + F405K + K360E + Q347E	21	71	8
9	Y349C + T366W + K409A + L368E	D356C + T366S + L368A + Y407V + F405K	39	30	31

[0122] Compared with the first round of the preferred mutation combinations, some newly introduced mutations had slightly increased the proportion of formation of heterodimers, such as the combination 5 over the combination 2; but a few groups had small changes, such as the combination 6 over the combination 2, and the combinations 7 and

nation 2 could reduce the tendency of forming homodimers; and the remaining new mutations had little effect on the formation of homodimers. Moreover, as can be further seen from the results, the combination 2 and its derivative combinations (5, 6) exhibited a smaller tendency of forming the homodimers of the chain A compared with the mutation

combination 4 and its derivative combinations (7, 8, 9); and the latter exhibited a smaller tendency of forming the homodimers of chain B compared with the former. The results were consistent with the results obtained in Example 2, and further proved the feasibility of preliminary investigation of the tendency of forming homodimer using the method. In addition, the expression levels of all the B chains were far lower than those of the chain A. It was found through separate transient expression of the wild-type chain A and the wild-type chain B that, when not any mutation was introduced, the expression level of homodimers of the wild-type chain B was lower than that of the wild-type chain A (the former was about half of the latter). Therefore, it was inferred that the N-terminal of the Fc sequence in the chain A was fused with the ScFv sequence, which helped to enhance its expression level. However, the difference between the expression level of the chain A and that of the chain B cannot directly reflect the difference of tendency between forming AA homodimers and forming BB homodimers.

TABLE 7

Comparison of the Expression Levels of Homodimers in Case of Separate Transient Transfection of Chain A or Chain B in Each Mutation Combination				
Mutation combination	Mutant amino acids on chain A (ScFv-Fc protein)	Expression levels of AA homodimers in case of separate expression	Mutant amino acids on chain B (Fc protein)	Expression levels of BB homodimers in case of separate expression
2	Y349C + T366W + F405K	48 mg/L	D356C + T366S + L368A + Y407V	55 mg/L
5	Y349C + T366W + F405K + K360E + Q347E	46 mg/L	D356C + T366S + L368A + Y407V + Q347R	36 mg/L
6	Y349C + T366W + F405K + Q347R	40 mg/L	D356C + T366S + L368A + Y407V + K360E + Q347E	58 mg/L
4	Y349C + T366W + K409A	111 mg/L	D356C + T366S + L368A + Y407V + F405K	21 mg/L
7	Y349C + T366W + K409A + K360E + Q347E	110 mg/L	D356C + T366S + L368A + Y407V + F405K + Q347R	18 mg/L
8	Y349C + T366W + K409A + Q347R	96 mg/L	D356C + T366S + L368A + Y407V + F405K + K360E + Q347E	21 mg/L
9	Y349C + T366W + K409A + L368E	10 mg/L	D356C + T366S + L368A + Y407V + F405K	20 mg/L

#### Example 5: Acquisition of Third Round Candidate Mutation Combination

**[0124]** 1, According to the crystal structure of the mutation combination 4, in conjunction with structural modeling, candidate amino acid mutation sequence on a new contact interface was found out to further inhibit the formation of homodimer proteins or promote the formation of heterodimer proteins on the basis of the original mutation combinations (e.g., mutation combination 2 or 4).

#### Crystallographic Structure Analysis on Heterodimer Proteins of the Mutation Combination 4

**[0125]** Mutation combination 4 was selected to obtain a heterodimer protein of mutation combination 4 by transient expression in 293H cells (catalog #ATCC CRL-1573) and purification, and the crystal structure was analyzed. Here, a fragment of His-tag sequence was inserted into the C-terminal of the chain B of the mutation combination 4 using molecular cloning, so as to obtain a pure AB heterodimer protein using the IMAC method for crystallization after Protein A affinity chromatography.

#### Crystallographic Structure was Analyzed as Follows:

**[0126]** The heterodimer Fc crystal was formed under the following conditions: mixing 2  $\mu$ L of a crystallization buffer (15% PEG3350, 1 M LiCl and 0.1 M MES at pH6.0) with 2  $\mu$ L of a protein solution (10 mg/mL target protein, 10 mM TRIS® buffer and 150 mM NaCl at pH 7.4), and was left to stand for crystallization at 22° C. The crystal grew about 3 days later. The crystal was then placed in the following solution: 17% PEG3350, 1M LiCl, 0.1M MES and 20% glycerol at pH6.0; and then quickly infiltrated and frozen in liquid nitrogen. The X-diffraction data was collected by SSRF BL17U. The structure of the wild-type Fc (PDB landing number: 3AVE) was used as the framework to analyze the molecular replacement structure.

**[0127]** The crystal structure showed that the overall structure of the mutant Fc heterodimer was similar to that of the wild-type Fc, but was changed on some degree on the CH3 interface into which mutations were introduced due to the interaction between different side chain groups. The specific crystal structure of the CH3 interface is shown in FIG. 3.

#### 2. Acquisition of New Candidate Mutation Combinations

**[0128]** A new candidate mutation was further screened according to the result of the crystal structure of the mutation combination 4.

**[0129]** First of all, it was found through the three-dimensional crystal structure that Y349C on chain A and D356C on chain B could not form a disulfide bond because of the directions of two side chain groups of Cys, but formed a pair of free sulfhydryls. According to this result, this pair of mutations would not be performed in the third round of mutation, and restored to the wild-type amino acid sequence before the mutation.

**[0130]** Secondly, by comparing the three-dimensional structural modeling, mutations were further introduced near a pair of mutated amino acid residues F405K-K409A to change the ionic bonding and hydrogen bonding. If K409A was on chain A, and F405K was on chain B, then K392D mutation would be introduced into the chain A, and D399S mutation would be introduced into the chain B. As shown in

FIG. 4, for the interaction between the chain A and the chain B, the ionic bond between K392D and F405K and the hydrogen bond between K392D and D399S were added to the newly introduced mutation pair, which was expected to improve the tendency of forming heterodimers. The electrostatic repulsion between K329D and D399 was introduced into the interaction between the chain A and the chain A to inhibit the formation of AA homodimers. In the interaction between the chain B and the chain B, the ionic bond between the original K409 and D399 disappeared due to the introduction of the D399S mutation, thereby reducing the trend of forming BB homodimers.

[0131] Thirdly, comparison of the crystal structure of the mutation combination 4 with the wild-type Fc protein crystal structure showed that, the chain A of the mutation combination 4 had outward shift (away from the chain B), presumably because largened side chain groups in the T366W mutation of the chain A brought certain spatial steric hindrance. On this basis, amino acid residues of the chain B which contacted with the T366W residue on the chain A were further mutated to amino acid residues with smaller side chain groups. For example, the original Y407V and L368A mutations on the chain B were replaced with Y407A and L386G mutations to leave enough space for the T366W mutation, which may further stabilize the heterodimer structure.

[0132] Fourthly, in the peripheral of the mutant amino acid residue pair F405K-K409A, other amino acids on the contact interface were mutated to change the interfacial electrostatic interaction. Here, the contacting amino acid pair Y349 and E357 was investigated. The mutation Y349D was introduced into the chain A, and E357A was introduced into the chain B. The electrostatic repulsion introduced between Y349D and E357A of the chain A and the chain A would hinder the formation of AA homodimers. No new interaction was introduced between the chain A and the chain B and between the chain B and the chain B. On this basis, S354D mutation was further introduced into the chain A to strengthen the electrostatic repulsion thereof with E357A so as to further hinder the formation of AA homodimers.

[0133] A mutation combination as shown in Table 8 was obtained by introducing the above mutation on the basis of the mutation combination 4:

TABLE 8

List of Mutation Combinations of Heterodimers-3			
Combination	Fc chain	Mutation	Corresponding SEQ ID NO
10	A	T366W + K409A + K392D	21
	B	T366S + L368A + Y407V + D399S + F405K	22
11	A	T366W + K409A	23
	B	T366S + L368G + Y407A + F405K	24
12	A	T366W + K409A + Y349D	21
	B	T366S + L368A + Y407V + F405K + E357A	25
13	A	T366W + K409A + Y349D + S354D	26
	B	T366S + L368A + Y407V + F405K + E357A	25

[0134] Then, a mutation combination as shown in Table 9 was obtained by introducing the above mutation on the basis of the mutation combination 2 whilst referring to the mutation combination 4.

TABLE 9

List of Mutation Combinations of Heterodimers-4			
Combination	Fc chain	Mutation	Corresponding SEQ ID NO
14	A	T366W + F405K	27
	B	T366S + L368A + Y407V + K409A	28
15	A	T366W + F405K + D399S	29
	B	T366S + L368A + Y407V + K409A + K392D	30
16	A	T366W + F405K	27
	B	T366S + L368G + Y407A + K409A	31
17	A	T366W + F405K + Y349D	32
	B	T366S + L368A + Y407V + K409A + E357A	33
18	A	T366W + F405K + Y349D + S354D	34
	B	T366S + L368A + Y407V + K409A + E357A	33

#### Example 6: Preparation and Investigation of a Third Round of ScFv-Fc/VhH-Fc Heterodimer Mutation Combination

##### 1. Constructing a Recombinant Vector Expressing a Mutated Fc Region of Human IgG1 and a ScFv-Fc Fusion Protein

[0135] Considering that the expression level of pure Fc regions was lower than that of ScFv-Fc, in order to better grasp the expression ratio of the two chains, a variable region sequence (labeled as VhH) of a single domain antibody of a camel was fused at the N terminal of the original B chain (simple Fc chain). A gene encoding VhH-Fc fusion protein is shown in SEQ ID NO: 36 obtained by artificial synthesis. This gene encodes a VhH-Fc fusion protein which has a sequence shown in SEQ ID:35, and the gene was then subcloned to a mammalian cell expression vector pcDNA4 (Invitrogen, cat V86220) to obtain a recombinant expression vector for expressing the VhH-Fc fusion protein in mammalian cells.

[0136] According to Table 8 of Example 5, a combined mutation of ScFv-Fc and VhH-Fc encoding genes (SEQ ID NO:5 and SEQ ID NO:36) was performed by overlapping PCR with the recombinant expression vector of the wild-type ScFv-Fc protein constructed in Example 2 and the recombinant expression vector of the VhH-Fc fusion protein as the templates, where the mutation of the chain A was located on the ScFv-Fc fusion protein, and the mutation of the chain B was located on the VhH-Fc protein. The mutant gene was subcloned to pcDNA4 (Invitrogen, cat V86220) to finally obtain the ScFv-Fc fusion protein to express a third round of mutations in mammalian cells and a recombinant expression vector of the mutant VhH-Fc protein (SEQ ID NO:4 to SEQ ID NO:35).

##### 2. Transient Expression of a ScFv-Fc/VhH-Fc Heterodimer and Detection of the Influence of Different Mutation Combinations on Heterodimer Content

[0137] According to the method in Example 2-2, the 4 mutation combinations (10 to 13) in Table 8 and the mutation combination 4 were transiently expressed using 293H cells (catalog #ATCC CRL-1573). The cotransfection ratio of recombinant expression vector of the chain A to that of the chain B was 4:1, 1:1 and 1:4. After 5-6 days of cultivation, the transiently expressed culture supernatant was collected, and 4 groups of preliminarily purified new mutation of

combinations and transient transfection products of the mutation combination 4 were obtained by Protein A affinity chromatography. Each of these transient transfection products contained different proportions of homodimer proteins (SCFv-Fc/ScFv-Fc, VhH-Fc/VhH-Fc) and heterodimer protein (ScFv-Fc/VhH-Fc). As the three proteins (SCFv-Fc/ScFv-Fc, VhH-Fc/VhH-Fc and SCFv-Fc/VhH-Fc) had different molecular weights, the compositions of the homodimer protein (ScFv-Fc/ScFv-Fc, VhH-Fc/VhH-Fc) and the heterodimer protein (ScFv-Fc/VhH-Fc) in the product of each group could be detected by SDS-PAGE electrophoresis under non-reducing conditions, and the proportions of the homodimer protein (ScFv-Fc/ScFv-Fc, VhH-Fc/VhH-Fc) and the heterodimer protein (SCFv-Fc/VhH-Fc) were analyzed with ImageLab professional image analysis software provided by Biorad company at the same time. The electrophoresis test results were shown in Table 10.

TABLE 10

Ratio of Homodimers to Heterodimers in Transient Transfection Products of Each Mutation Combination-3				
Combination	Cotransfection ratio of vector of chain A (ScFv-Fc) to that of chain B (VhH-Fc) in	ScFv-Fc/VhH-Fc		
		ScFv-Fc homodimer (%)	VhH-Fc heterodimer (%)	VhH-Fc homodimer (%)
4	4:1	46	54	<1
	1:1	13	68	19
	1:4	<1	47	53
10	4:1	39	61	<1
	1:1	7	80	13
	1:4	<1	73	27
11	4:1	52	52	0
	1:1	15	85	0
	1:4	11	89	0
12	4:1	48	49	3
	1:1	14	83	3
	1:4	9	60	31
13	4:1	37	61	2
	1:1	10	84	6
	1:4	2	64	34

**[0138]** In order to further investigate the influence of the newly introduced mutations on AA homodimer and BB homodimers, proteins of the chain A or proteins of the chain B were separately transiently expressed, and the tendency of forming homodimers was investigated by comparing the expression levels of homodimer protein under equivalent transient transfection conditions. The recombinant expression vector was transfected with PEI into suspension-cultured 293H cells (catalog #ATCC CRL-1573), and the cell supernatant was collected after 5-6 days of cultivation. The respective transient transfection products were obtained through Protein A affinity chromatography, and the expression levels thereof were detected by OD280. The results were shown in Table 11.

TABLE 11

Comparison of the Expression Levels of AA Homodimers and BB Homodimers in Each Mutation Combination-2				
Mutation combination	Mutant amino acids on chain A (ScFv-Fc protein)	Expression levels of AA homodimers in case of separate expression	Mutant amino acids on chain B (Fc protein)	Expression levels of BB homodimers in case of separate expression
4	Y349C + T366W + K409A	365 mg/L	D356C + T366S + L368A + Y407V + F405K	293 mg/L
10	T366W + K409A + K392D	370 mg/L	T366S + L368A + Y407V + D399S + F405K	76 mg/L
11	T366W + K409A	342 mg/L	T366S + L368G + Y407A + F405K	<6 mg/L
12	T366W + K409A + Y349D	354 mg/L	T366S + L368A + Y407V + F405K + E357A	66 mg/L
13	T366W + K409A + Y349D + S354D	308 mg/L	T366S + L368A + Y407V + F405K + E357A	66 mg/L

**[0139]** Through comprehensively considering the above results, it was found that after introducing the third round of mutation into the mutation combination 4, it did not show significant effect in inhibiting the formation of AA homodimers, but significantly hindered the formation of BB homodimers, and effectively promoted the formation of heterodimers. When the expressions of the two chains were close to equilibrium (1:1), each of the contents of heterodimers in groups of new mutation combinations reached more than 80%, and was significantly improved compared with that in the mutation combination 4. In the mutation combination 11, new mutations for the chain B could almost completely hinder the formation of BB homodimers. It can be seen that even at the transient transfection ratio of 1:4 (A:B), the BB homodimer was still not observed, and the heterodimer content reached 89%.

**[0140]** According to the results of the combinations 10 to 13, mutation combination 15, 16 and 18 were further selected to investigate their influence on the formation of heterodimers by transient expression.

**[0141]** According to the method in Example 2-2, the 3 mutation combinations (15, 16 and 18) in Table 9 and the mutation combination 2 were transiently expressed using 293H cells (catalog #ATCC CRL-1573). The cotransfection ratio of recombinant expression vector of the chain A to that of the chain B was 4:1, 1:1 and 1:4. The transiently expressed culture supernatant was collected after 5-6 days of cultivation. And transient transfection products of 3 new preliminarily purified mutation combinations and the mutation combination 2 were obtained by Protein A affinity chromatography. Each of these transient transfection products contained different proportions of homodimer proteins (ScFv-Fc/ScFv-Fc, VhH-Fc/VhH-Fc) and heterodimer protein (ScFv-Fc/VhH-Fc). As the three proteins (ScFv-Fc/ScFv-Fc, VhH-Fc/VhH-Fc and ScFv-Fc/VhH-Fc) had different molecular weights, the compositions of the homodimer protein (ScFv-Fc/ScFv-Fc, VhH-Fc/VhH-Fc)



and the heterodimer protein (ScFv-Fc/VhH-Fc) in the product of each group could be detected by SDS-PAGE electrophoresis under non-reducing conditions, and the proportion of the homodimer protein (ScFv-Fc/ScFv-Fc, VhH-Fc/VhH-Fc) and the heterodimer protein (ScFv-Fc/VhH-Fc) was analyzed with ImageLab professional image analysis software provided by Biorad company at the same time, and the electrophoresis test results were shown in Table 12. It can be seen that after introducing the third round of mutation into the mutation combination 2, it also showed significant effect in hindering the formation of BB homodimers, and enhanced the formation of heterodimers. When the expressions of the two chains were close to equilibrium (1:1), each of the contents of heterodimers in new combinations could reach more than 80% which was significantly enhanced compared with the mutation combination 4. When the proportion of the transient transfection vector was appropriately changed in the mutation combinations 16 and 18 (plasmid of chain B was excessive or plasmids of the two chains were balanced), the proportion of heterodimers thereof was still more than 80%.

TABLE 12

Ratio of Homodimers to Heterodimers in Transient Transfection Products of Each Mutation Combination-4				
Combination	Cotransfection ratio of vector of chain A		ScFv-Fc/ VhH-Fc	
	chain B (VhH-Fc)	ScFv-Fc (%)	homodimer (%)	heterodimer (%)
2	4:1	28	55	17
	1:1	9	64	27
	1:4	<1	39	61
15	4:1	28	66	6
	1:1	6	81	13
	1:4	<1	69	31
16	4:1	23	77	<1
	1:1	5	88	7
	1:4	3	93	4

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TABLE 12-continued

Ratio of Homodimers to Heterodimers in Transient Transfection Products of Each Mutation Combination-4				
Combination	Cotransfection ratio of vector of chain A		ScFv-Fc/ VhH-Fc	
	chain B (VhH-Fc)	ScFv-Fc (%)	heterodimer (%)	homodimer (%)
18	4:1	29	61	10
	1:1	9	84	7
	1:4	5	86	9

Example 7: Assessment on Other Features of Heterodimers

1. Accelerated Stability Test of Heterodimers

[0142] Heterodimers of the mutation combinations 4, 11 and 16 were selected for accelerated stability test with PBS as the buffer at a temperature of 45° C. with an experimental period of 31 days. The heterodimers were tested with non-reducing CE-SDS on 0th day, 8th day, 18th day and 31st day, which were compared with the corresponding wild-type Fc protein. The SDS-PAGE results of the 31-day accelerated stability test showed that each of the main peak contents of the three mutation samples and wild-type control samples decreased by no more than 2% until the 31st day. It can be concluded that the heterodimer exhibited same thermal stability as the wild-type one.

[0143] While the specific embodiments of the invention according to the present disclosure have been described in detail, and will be understood by those skilled in the art, but the details can be modified and substituted according to all disclosed inspirations, and all of these changes fall within the protection scope of the invention according to the present disclosure. It is intended that the appended claims and other equivalents thereof define the entire scope of the invention

-continued

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                                organism = Homo sapiens
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ttcttcaacg cctacgactt ctggggccag ggcaccctgg tgaccgtgag cagcgccagc 420
accaagggcc ccagcgtggg cggcgccggc agcgcgccgc gcggcagcga gatcgtgatg 480
accagagacc ccgccacct gagcgtgagc cccggcgagc gcgccacct gagctgccgc 540
gccagccaga cgctgcgcag caacctggcc tggtaaccag agaagcccg ccaggccccc 600
cgctgcttga tctacgccgc cagcacccgc gccaccggca tccccggccg cttcagcgcc 660
agcggcagcg gccaccagtt caccctgacc atcagcagcc tgcagagcga ggacttcgcc 720
gtgtactact gccagcagta caacagtggt ttccgcacca gcggccaggg caccaagggt 780
gagatcaagc gcgacaagac ccacacctgc cccccctgcc ccgccccga gctgctgggc 840

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ggccccagcg tgttctgtt ccccccaag cccaaggaca ccctgatgat cagccgcacc 900
cccgaggtga cctcgctggt ggtggacgtg agccacgaga accccgaggt gaagttaaac 960
tggtacgtgg acggcgtgga ggtgcacaac gccaaagacca agcccccgga ggagcagtac 1020
aacagcacct acccgctggt gagcgtgctg accgtgtgtc accaggactg gctgaacggc 1080
aaggagtaca agtgcaaggt gagcaacaag gccctgcccc ccccatcgga gaagaccatc 1140
agcaaggcca agggccagcc ccgcgagccc caggtgtaca ccctgcccc cagcccgagc 1200
gagctgacca agaaccaggt gagcctgacc tgcctggtga agggcttcta ccccgagcag 1260
atcgccgtgg agtgggagag caacggccag cccgagaaca actacaagac ccccccccc 1320
gtgctggaca gcgacggcag cttcttctg tacagcaagc tgaccgtgga caagagccgc 1380
tggcagcagg gcaacgtggt cagctgcagc gtgatgcagc aggcctcgca caaccactac 1440
accagaaga gctgagcct gagccccgc aag 1473

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SEQ ID NO: 6      moltype = AA length = 471
FEATURE          Location/Qualifiers
REGION          1..471
                note = Y349C+T366W
source          1..471
                mol_type = protein
                organism = synthetic construct

```

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SEQUENCE: 6
EVQLLESGGG VVQGRSLRL SCIASGFTFS SYPMTWVRQA PGKGLEWVAS ISYDGSYKYK 60
ADSMKGRLLTI SRDNSKNTLY LEMNSLTAED TAVYYCARTA FFNAYDFWQG GTLVTVSSAS 120
TKGPSVGGGG SGGGGSEIVM TQSPATLSVS PGERATLSCR ASQSVRSNLA WYQKPGQAP 180
RLLIYAASR ATGIPARFSG SGSGTEFTLT ISSLQSEDFA VYYCQYNEW FRTSGQGTKV 240
EIKRDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHENPEVKFN 300
WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI 360
SKAKGQPREP QVCTLPPSRD ELTKNQVSLW CLVKGFPYPSD IAVEWESNGQ PENNYKTTTP 420
VLDSGGSFPL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K 471

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SEQ ID NO: 7      moltype = AA length = 227
FEATURE          Location/Qualifiers
REGION          1..227
                note = D356C+T366S+L368A+Y407V
source          1..227
                mol_type = protein
                organism = synthetic construct

```

```

SEQUENCE: 7
DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD 60
GVEVHNATK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK 120
GQPREPQVYT LPPSRCELTK NQVSLSCAVK GFYPSDIAVE WESNGQPENN YKTTTPVLDS 180
DGSFPLVSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK 227

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SEQ ID NO: 8      moltype = AA length = 227
FEATURE          Location/Qualifiers
REGION          1..227
                note = D356C+T366S+L368A+Y407V+F405K
source          1..227
                mol_type = protein
                organism = synthetic construct

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SEQUENCE: 8
DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD 60
GVEVHNATK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK 120
GQPREPQVYT LPPSRCELTK NQVSLSCAVK GFYPSDIAVE WESNGQPENN YKTTTPVLDS 180
DGSFPLVSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK 227

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SEQ ID NO: 9      moltype = AA length = 471
FEATURE          Location/Qualifiers
REGION          1..471
                note = Y349C+T366W+F405K
source          1..471
                mol_type = protein
                organism = synthetic construct

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SEQUENCE: 9
EVQLLESGGG VVQGRSLRL SCIASGFTFS SYPMTWVRQA PGKGLEWVAS ISYDGSYKYK 60
ADSMKGRLLTI SRDNSKNTLY LEMNSLTAED TAVYYCARTA FFNAYDFWQG GTLVTVSSAS 120
TKGPSVGGGG SGGGGSEIVM TQSPATLSVS PGERATLSCR ASQSVRSNLA WYQKPGQAP 180
RLLIYAASR ATGIPARFSG SGSGTEFTLT ISSLQSEDFA VYYCQYNEW FRTSGQGTKV 240
EIKRDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHENPEVKFN 300
WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI 360
SKAKGQPREP QVCTLPPSRD ELTKNQVSLW CLVKGFPYPSD IAVEWESNGQ PENNYKTTTP 420
VLDSGGSFPL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K 471

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SEQ ID NO: 10     moltype = AA length = 471
FEATURE          Location/Qualifiers
REGION          1..471
                note = Y349C+T366W+K409E

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source                1..471
                      mol_type = protein
                      organism = synthetic construct

SEQUENCE: 10
EVQLLESGGG VVQPGRLRL SCIASGFTFS SYPMTWVRQA PGKGLEWVAS ISYDGSYKYK 60
ADSMKGRLLTI SRDNSKNTLY LEMNSLTAED TAVYYCARTA FFNAYDFWQG GTLVTVSSAS 120
TKGPSVGGGG SGGGSEIVM TQSPATLSVS PGERATLSCR ASQSVRSNLA WYQKPGQAP 180
RLLIYAASR ATGIPARFSG SGSGTEFTLT ISSLQSEDFA VYYCQYNEW FRTSGQGTV 240
EIKRDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHENPEVKFN 300
WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI 360
SKAKGQPREP QVCTLPPSRD ELTKNQVSLW CLVKGFYPSD IAVEWESNGQ PENNYKTTTP 420
VLDSGGSFPL YSELTVDKSR WQQGNVFSCS VMHEALHNYH TQKSLSLSPG K 471

SEQ ID NO: 11         moltype = AA length = 471
FEATURE              Location/Qualifiers
REGION               1..471
                      note = Y349C+T366W+K409A

source                1..471
                      mol_type = protein
                      organism = synthetic construct

SEQUENCE: 11
EVQLLESGGG VVQPGRLRL SCIASGFTFS SYPMTWVRQA PGKGLEWVAS ISYDGSYKYK 60
ADSMKGRLLTI SRDNSKNTLY LEMNSLTAED TAVYYCARTA FFNAYDFWQG GTLVTVSSAS 120
TKGPSVGGGG SGGGSEIVM TQSPATLSVS PGERATLSCR ASQSVRSNLA WYQKPGQAP 180
RLLIYAASR ATGIPARFSG SGSGTEFTLT ISSLQSEDFA VYYCQYNEW FRTSGQGTV 240
EIKRDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHENPEVKFN 300
WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI 360
SKAKGQPREP QVCTLPPSRD ELTKNQVSLW CLVKGFYPSD IAVEWESNGQ PENNYKTTTP 420
VLDSGGSFPL YSALTVDKSR WQQGNVFSCS VMHEALHNYH TQKSLSLSPG K 471

SEQ ID NO: 12         moltype = AA length = 471
FEATURE              Location/Qualifiers
REGION               1..471
                      note = Y349C+T366W+F405K+K360E+Q347E

source                1..471
                      mol_type = protein
                      organism = synthetic construct

SEQUENCE: 12
EVQLLESGGG VVQPGRLRL SCIASGFTFS SYPMTWVRQA PGKGLEWVAS ISYDGSYKYK 60
ADSMKGRLLTI SRDNSKNTLY LEMNSLTAED TAVYYCARTA FFNAYDFWQG GTLVTVSSAS 120
TKGPSVGGGG SGGGSEIVM TQSPATLSVS PGERATLSCR ASQSVRSNLA WYQKPGQAP 180
RLLIYAASR ATGIPARFSG SGSGTEFTLT ISSLQSEDFA VYYCQYNEW FRTSGQGTV 240
EIKRDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHENPEVKFN 300
WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI 360
SKAKGQPREP EVCTLPPSRD ELTENQVSLW CLVKGFYPSD IAVEWESNGQ PENNYKTTTP 420
VLDSGGSFPL YSKLTVDKSR WQQGNVFSCS VMHEALHNYH TQKSLSLSPG K 471

SEQ ID NO: 13         moltype = AA length = 227
FEATURE              Location/Qualifiers
REGION               1..227
                      note = D356C+T366S+L368A+Y407V+Q347R

source                1..227
                      mol_type = protein
                      organism = synthetic construct

SEQUENCE: 13
DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD 60
GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK 120
GQPREPRVYT LPPSRCELTK NQVSLSCAVK GFYPSDIAVE WESNGQPENN YKTTTPVLDS 180
DGSFPLVSKL TVDKSRWQQG NVFSCSVMHE ALHNYHTQKS LSLSPGK 227

SEQ ID NO: 14         moltype = AA length = 471
FEATURE              Location/Qualifiers
REGION               1..471
                      note = Y349C+T366W+F405K+Q347R

source                1..471
                      mol_type = protein
                      organism = synthetic construct

SEQUENCE: 14
EVQLLESGGG VVQPGRLRL SCIASGFTFS SYPMTWVRQA PGKGLEWVAS ISYDGSYKYK 60
ADSMKGRLLTI SRDNSKNTLY LEMNSLTAED TAVYYCARTA FFNAYDFWQG GTLVTVSSAS 120
TKGPSVGGGG SGGGSEIVM TQSPATLSVS PGERATLSCR ASQSVRSNLA WYQKPGQAP 180
RLLIYAASR ATGIPARFSG SGSGTEFTLT ISSLQSEDFA VYYCQYNEW FRTSGQGTV 240
EIKRDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHENPEVKFN 300
WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI 360
SKAKGQPREP EVCTLPPSRD ELTKNQVSLW CLVKGFYPSD IAVEWESNGQ PENNYKTTTP 420
VLDSGGSFPL YSKLTVDKSR WQQGNVFSCS VMHEALHNYH TQKSLSLSPG K 471

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SEQ ID NO: 15                   moltype = AA   length = 227  
FEATURE                       Location/Qualifiers  
REGION                       1..227  
                              note = D356C+T366S+L368A+Y407V+K360E+Q347E  
source                       1..227  
                              mol\_type = protein  
                              organism = synthetic construct

SEQUENCE: 15  
DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD   60  
GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK   120  
GQPREPEVYT LPPSRCELTE NQVSLSCAVK GFYPSPDIAVE WESNGQPENN YKTPPVLD   180  
DGSFFLVSKL TVDKSRWQQG NVFSCSVME ALHNHYTQKS LSLSPGK                   227

SEQ ID NO: 16                   moltype = AA   length = 471  
FEATURE                       Location/Qualifiers  
REGION                       1..471  
                              note = Y349C+T366W+K409A+K360E+Q347E  
source                       1..471  
                              mol\_type = protein  
                              organism = synthetic construct

SEQUENCE: 16  
EVQLLESGGG VVQPGSRSLRL SCIASGFTFS SYPMTWVRQA PGKGLEWVAS ISYDGSYK   60  
ADSMKGRLLTI SRDNSKNTLY LEMNSLTAED TAVYYCARTA FFNAYDFWQQ GTLVTVSSAS   120  
TKGPSVGGGG SGGGSEIVM TQSPATLSVS PGERATLSCR ASQSVRSNLA WYQOKPGQAP   180  
RLLIYAASR ATGIPARFSG SGSGTEFTLT ISSLQSEDFA VYYCQYNEW FRTSGQGTV   240  
ETKDKTHTC PPCAPELLG GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHENPEVKFN   300  
WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI   360  
SKAKGQPREP EVCTLPSSRD ELTENQVSLW CLVKGFPYPSD IAVEWESNGQ PENNYKTPP   420  
VLDSGSGFFL YSALTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K           471

SEQ ID NO: 17                   moltype = AA   length = 227  
FEATURE                       Location/Qualifiers  
REGION                       1..227  
                              note = D356C+T366S+L368A+Y407V+F405K+Q347R  
source                       1..227  
                              mol\_type = protein  
                              organism = synthetic construct

SEQUENCE: 17  
DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD   60  
GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK   120  
GQPREPRVYT LPPSRCELTK NQVSLSCAVK GFYPSPDIAVE WESNGQPENN YKTPPVLD   180  
DGSFKLVSKL TVDKSRWQQG NVFSCSVME ALHNHYTQKS LSLSPGK                   227

SEQ ID NO: 18                   moltype = AA   length = 471  
FEATURE                       Location/Qualifiers  
REGION                       1..471  
                              note = Y349C+T366W+K409A+Q347R  
source                       1..471  
                              mol\_type = protein  
                              organism = synthetic construct

SEQUENCE: 18  
EVQLLESGGG VVQPGSRSLRL SCIASGFTFS SYPMTWVRQA PGKGLEWVAS ISYDGSYK   60  
ADSMKGRLLTI SRDNSKNTLY LEMNSLTAED TAVYYCARTA FFNAYDFWQQ GTLVTVSSAS   120  
TKGPSVGGGG SGGGSEIVM TQSPATLSVS PGERATLSCR ASQSVRSNLA WYQOKPGQAP   180  
RLLIYAASR ATGIPARFSG SGSGTEFTLT ISSLQSEDFA VYYCQYNEW FRTSGQGTV   240  
ETKDKTHTC PPCAPELLG GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHENPEVKFN   300  
WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI   360  
SKAKGQPREP RVCTLPSSRD ELTKNQVSLW CLVKGFPYPSD IAVEWESNGQ PENNYKTPP   420  
VLDSGSGFFL YSALTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K           471

SEQ ID NO: 19                   moltype = AA   length = 227  
FEATURE                       Location/Qualifiers  
REGION                       1..227  
                              note = D356C+T366S+L368A+Y407V+F405K+K360E+Q347E  
source                       1..227  
                              mol\_type = protein  
                              organism = synthetic construct

SEQUENCE: 19  
DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD   60  
GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK   120  
GQPREPEVYT LPPSRCELTE NQVSLSCAVK GFYPSPDIAVE WESNGQPENN YKTPPVLD   180  
DGSFKLVSKL TVDKSRWQQG NVFSCSVME ALHNHYTQKS LSLSPGK                   227

SEQ ID NO: 20                   moltype = AA   length = 471  
FEATURE                       Location/Qualifiers

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REGION 1..471  
note = Y349C+T366W+K409A+L368E

source 1..471  
mol\_type = protein  
organism = synthetic construct

SEQUENCE: 20

EVQLLES	GGG	VVQ	PGRSLRL	SCIASGFTFS	SYPMTWVRQA	PGKGLEWVAS	ISYDGSYKYK	60
ADSMKGR	LTI	SRD	NSKNTLY	LEMNSLTAED	TAVYYCARTA	FFNAYDFWGQ	GTLVTVSSAS	120
TKGPSV	GGGG	SGGGG	SEIVM	TQSPATLSVS	PGERATLSCR	ASQSVRSNLA	WYQQKPGQAP	180
RLLIYA	ASTR	ATGIPAR	FSG	SGSGTEFTLT	ISSLQSEDA	VYYCQYNEW	FRTSGGQTKV	240
EIKRDK	THTC	PPCPAPE	LLG	GPSVFLFPPK	PKDTLMISRT	PEVTCVVVDV	SHENPEVKFN	300
WYVDG	VEVHN	AKTKPRE	EQY	NSTYRVVSVL	TVLHQDWLNG	KEYKCKVSNK	ALPAPIEKTI	360
SKAKGQ	PREP	QVCTLPP	SRD	ELTKNQVSLW	CEVKGFPSPD	IAVEWESNGQ	PENNYKTTTP	420
VLDSDG	SFPL	YSALTVD	KSR	WQQGNVFSCS	VMHEALHNHY	TQKSLSLSPG	K	471

SEQ ID NO: 21 moltype = AA length = 471  
FEATURE Location/Qualifiers  
REGION 1..471  
note = T366W+K409A+K392D  
source 1..471  
mol\_type = protein  
organism = synthetic construct

SEQUENCE: 21

EVQLLES	GGG	VVQ	PGRSLRL	SCIASGFTFS	SYPMTWVRQA	PGKGLEWVAS	ISYDGSYKYK	60
ADSMKGR	LTI	SRD	NSKNTLY	LEMNSLTAED	TAVYYCARTA	FFNAYDFWGQ	GTLVTVSSAS	120
TKGPSV	GGGG	SGGGG	SEIVM	TQSPATLSVS	PGERATLSCR	ASQSVRSNLA	WYQQKPGQAP	180
RLLIYA	ASTR	ATGIPAR	FSG	SGSGTEFTLT	ISSLQSEDA	VYYCQYNEW	FRTSGGQTKV	240
EIKRDK	THTC	PPCPAPE	LLG	GPSVFLFPPK	PKDTLMISRT	PEVTCVVVDV	SHENPEVKFN	300
WYVDG	VEVHN	AKTKPRE	EQY	NSTYRVVSVL	TVLHQDWLNG	KEYKCKVSNK	ALPAPIEKTI	360
SKAKGQ	PREP	QVYTLPP	SRD	ELTKNQVSLW	CLVKGFPSPD	IAVEWESNGQ	PENNYDTTTP	420
VLDSDG	SFPL	YSALTVD	KSR	WQQGNVFSCS	VMHEALHNHY	TQKSLSLSPG	K	471

SEQ ID NO: 22 moltype = AA length = 227  
FEATURE Location/Qualifiers  
REGION 1..227  
note = T366S+L368A+Y407V+D399S+F405K  
source 1..227  
mol\_type = protein  
organism = synthetic construct

SEQUENCE: 22

DKTHTC	PPCP	APELLGG	PSV	FLFPPKPKDT	LMISRTPEVT	CVVVDVSHED	PEVKFNWYVD	60
GVEVHN	AKTK	PREEQYN	STY	RVVSVLTVLH	QDWLNGKEYK	CKVSNKALPA	PIEKTISKAK	120
GQPREP	QVYT	LPPSRDEL	TK	NQVSLSCAVK	GFYPSDIAVE	WESNGQPENN	YKTTTPVLSS	180
DGSFKL	VSKL	TVDKSRW	QQG	NVFSCSVMHE	ALHNHYTQKS	LSLSPGK		227

SEQ ID NO: 23 moltype = AA length = 471  
FEATURE Location/Qualifiers  
REGION 1..471  
note = T366W+K409A  
source 1..471  
mol\_type = protein  
organism = synthetic construct

SEQUENCE: 23

EVQLLES	GGG	VVQ	PGRSLRL	SCIASGFTFS	SYPMTWVRQA	PGKGLEWVAS	ISYDGSYKYK	60
ADSMKGR	LTI	SRD	NSKNTLY	LEMNSLTAED	TAVYYCARTA	FFNAYDFWGQ	GTLVTVSSAS	120
TKGPSV	GGGG	SGGGG	SEIVM	TQSPATLSVS	PGERATLSCR	ASQSVRSNLA	WYQQKPGQAP	180
RLLIYA	ASTR	ATGIPAR	FSG	SGSGTEFTLT	ISSLQSEDA	VYYCQYNEW	FRTSGGQTKV	240
EIKRDK	THTC	PPCPAPE	LLG	GPSVFLFPPK	PKDTLMISRT	PEVTCVVVDV	SHENPEVKFN	300
WYVDG	VEVHN	AKTKPRE	EQY	NSTYRVVSVL	TVLHQDWLNG	KEYKCKVSNK	ALPAPIEKTI	360
SKAKGQ	PREP	QVYTLPP	SRD	ELTKNQVSLW	CLVKGFPSPD	IAVEWESNGQ	PENNYKTTTP	420
VLDSDG	SFPL	YSALTVD	KSR	WQQGNVFSCS	VMHEALHNHY	TQKSLSLSPG	K	471

SEQ ID NO: 24 moltype = AA length = 227  
FEATURE Location/Qualifiers  
REGION 1..227  
note = T366S+L368G+Y407A+F405K  
source 1..227  
mol\_type = protein  
organism = synthetic construct

SEQUENCE: 24

DKTHTC	PPCP	APELLGG	PSV	FLFPPKPKDT	LMISRTPEVT	CVVVDVSHED	PEVKFNWYVD	60
GVEVHN	AKTK	PREEQYN	STY	RVVSVLTVLH	QDWLNGKEYK	CKVSNKALPA	PIEKTISKAK	120
GQPREP	QVYT	LPPSRDEL	TK	NQVSLSCGVK	GFYPSDIAVE	WESNGQPENN	YKTTTPVLDS	180
DGSFKL	ASKL	TVDKSRW	QQG	NVFSCSVMHE	ALHNHYTQKS	LSLSPGK		227

SEQ ID NO: 25 moltype = AA length = 227

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FEATURE	Location/Qualifiers	
REGION	1..227	
	note = T366S+L368A+Y407V+F405K+E357A	
source	1..227	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 25		
DKTHTCPCPC	APELLGGPSV	FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD 60
GVEVHNAKTK	PREEQYNSTY	RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK 120
GQPREPQVYT	LPPSRDALTK	NQVSLSCAVK GFYPSDIAVE WESNGQPENN YKTTPPVLD 180
DGSFKLVSKL	TVDKSRWQGG	NVFSKSCVMHE ALHNHYTQKS LSLSPGK 227
SEQ ID NO: 26		
	moltype = AA length = 471	
FEATURE	Location/Qualifiers	
REGION	1..471	
	note = T366W+K409A+Y349D+S354D	
source	1..471	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 26		
EVQLLESGGG	VVQGRSLRL	SCIASGFTFS SYPMTWVRQA PGKGLEWVAS ISYDGSYK 60
ADSMKGRLLT	SRDNSKNTLY	LEMNSLTAED TAVYYCARTA FFNAYDFWGQ GTLVTVSSAS 120
TKGPSVGGGG	SGGGGSEIVM	TQSPATLSVS PGERATLSR ASQSVRSNLA WYQKPGQAP 180
RLLIYAASR	ATGIPARFSG	SGSGTEFTLT ISSLQSEDF VYYCQYNEW FRTSGGQTKV 240
EIKRDKTHTC	PPCPAPELLG	GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHENPEVKFN 300
WYVDGVEVHN	AKTKPREEQY	NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI 360
SKAKGQPREP	QVDTLPPDRD	ELTKNQVSLW CLVKGFPYPSD IAVEWESNGQ PENNYKTPP 420
VLDSDGSFPL	YSALTVDKSR	WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K 471
SEQ ID NO: 27		
	moltype = AA length = 471	
FEATURE	Location/Qualifiers	
REGION	1..471	
	note = T366W+F405K	
source	1..471	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 27		
EVQLLESGGG	VVQGRSLRL	SCIASGFTFS SYPMTWVRQA PGKGLEWVAS ISYDGSYK 60
ADSMKGRLLT	SRDNSKNTLY	LEMNSLTAED TAVYYCARTA FFNAYDFWGQ GTLVTVSSAS 120
TKGPSVGGGG	SGGGGSEIVM	TQSPATLSVS PGERATLSR ASQSVRSNLA WYQKPGQAP 180
RLLIYAASR	ATGIPARFSG	SGSGTEFTLT ISSLQSEDF VYYCQYNEW FRTSGGQTKV 240
EIKRDKTHTC	PPCPAPELLG	GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHENPEVKFN 300
WYVDGVEVHN	AKTKPREEQY	NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI 360
SKAKGQPREP	QVYTLPPSRD	ELTKNQVSLW CLVKGFPYPSD IAVEWESNGQ PENNYKTPP 420
VLDSDGSFPL	YSKLTVDKSR	WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K 471
SEQ ID NO: 28		
	moltype = AA length = 227	
FEATURE	Location/Qualifiers	
REGION	1..227	
	note = T366S+L368A+Y407V+K409A	
source	1..227	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 28		
DKTHTCPCPC	APELLGGPSV	FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD 60
GVEVHNAKTK	PREEQYNSTY	RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK 120
GQPREPQVYT	LPPSRDELTK	NQVSLSCAVK GFYPSDIAVE WESNGQPENN YKTTPPVLD 180
DGSFPLVSAL	TVDKSRWQGG	NVFSKSCVMHE ALHNHYTQKS LSLSPGK 227
SEQ ID NO: 29		
	moltype = AA length = 471	
FEATURE	Location/Qualifiers	
REGION	1..471	
	note = T366W+F405K+D399S	
source	1..471	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 29		
EVQLLESGGG	VVQGRSLRL	SCIASGFTFS SYPMTWVRQA PGKGLEWVAS ISYDGSYK 60
ADSMKGRLLT	SRDNSKNTLY	LEMNSLTAED TAVYYCARTA FFNAYDFWGQ GTLVTVSSAS 120
TKGPSVGGGG	SGGGGSEIVM	TQSPATLSVS PGERATLSR ASQSVRSNLA WYQKPGQAP 180
RLLIYAASR	ATGIPARFSG	SGSGTEFTLT ISSLQSEDF VYYCQYNEW FRTSGGQTKV 240
EIKRDKTHTC	PPCPAPELLG	GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHENPEVKFN 300
WYVDGVEVHN	AKTKPREEQY	NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI 360
SKAKGQPREP	QVYTLPPSRD	ELTKNQVSLW CLVKGFPYPSD IAVEWESNGQ PENNYKTPP 420
VLDSDGSFPL	YSKLTVDKSR	WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K 471

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SEQ ID NO: 30                   moltype = AA   length = 227  
 FEATURE                       Location/Qualifiers  
 REGION                        1..227  
                               note = T366S+L368A+Y407V+K409A+K392D  
 source                        1..227  
                               mol\_type = protein  
                               organism = synthetic construct

SEQUENCE: 30  
 DKHTTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD   60  
 GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK   120  
 GQPREPQVYT LPPSRDELTK NQVSLSCAVK GFYPSTIAVE WESNGQPENN YDTPPVVLD   180  
 DGSFPLVSAL TVDKSRWQGG NVFSCSVMHE ALHNHYTQKS LSLSPGK                   227

SEQ ID NO: 31                   moltype = AA   length = 227  
 FEATURE                       Location/Qualifiers  
 REGION                        1..227  
                               note = T366S+L368G+Y407A+K409A  
 source                        1..227  
                               mol\_type = protein  
                               organism = synthetic construct

SEQUENCE: 31  
 DKHTTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD   60  
 GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK   120  
 GQPREPQVYT LPPSRDELTK NQVSLSCGVK GFYPSTIAVE WESNGQPENN YKTPPVVLD   180  
 DGSFPLASAL TVDKSRWQGG NVFSCSVMHE ALHNHYTQKS LSLSPGK                   227

SEQ ID NO: 32                   moltype = AA   length = 471  
 FEATURE                       Location/Qualifiers  
 REGION                        1..471  
                               note = T366W +F405K +Y349D  
 source                        1..471  
                               mol\_type = protein  
                               organism = synthetic construct

SEQUENCE: 32  
 EVQLLESGGG VVQPGRSLRL SCIASGFTFS SYPMTWVRQA PGKGLEWVAS ISYDGSYKYK   60  
 ADMSKGRLLTI SRDNSKNTLY LEMNSLTAED TAVYYCARTA FFNAYDFWQG GTLVTVSSAS   120  
 TKGPSVGGGG SGGGGSEIVM TQSPATLSVS PGERATLSCR ASQSVRSNLA WYQQKPGQAP   180  
 RLLIYAASR ATGIPARFSG SSGGTEFTLT ISSLQSEDFA VYYCQYNEW FRTSGQGTVK   240  
 EIKRDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHENPEVKFN   300  
 WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI   360  
 SKAKGQPREP QVDTLPPSRD ELTKNQVSLW CLVKGFPYPSD IAVEWESNGQ PENNYKTTTP   420  
 VLDSGSGFKL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K           471

SEQ ID NO: 33                   moltype = AA   length = 227  
 FEATURE                       Location/Qualifiers  
 REGION                        1..227  
                               note = T366S+L368A+Y407V +K409A +E357A  
 source                        1..227  
                               mol\_type = protein  
                               organism = synthetic construct

SEQUENCE: 33  
 DKHTTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD   60  
 GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK   120  
 GQPREPQVYT LPPSRDALTK NQVSLSCAVK GFYPSTIAVE WESNGQPENN YKTPPVVLD   180  
 DGSFPLVSAL TVDKSRWQGG NVFSCSVMHE ALHNHYTQKS LSLSPGK                   227

SEQ ID NO: 34                   moltype = AA   length = 471  
 FEATURE                       Location/Qualifiers  
 REGION                        1..471  
                               note = T366W+F405K+Y349D+S354D  
 source                        1..471  
                               mol\_type = protein  
                               organism = synthetic construct

SEQUENCE: 34  
 EVQLLESGGG VVQPGRSLRL SCIASGFTFS SYPMTWVRQA PGKGLEWVAS ISYDGSYKYK   60  
 ADMSKGRLLTI SRDNSKNTLY LEMNSLTAED TAVYYCARTA FFNAYDFWQG GTLVTVSSAS   120  
 TKGPSVGGGG SGGGGSEIVM TQSPATLSVS PGERATLSCR ASQSVRSNLA WYQQKPGQAP   180  
 RLLIYAASR ATGIPARFSG SSGGTEFTLT ISSLQSEDFA VYYCQYNEW FRTSGQGTVK   240  
 EIKRDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHENPEVKFN   300  
 WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI   360  
 SKAKGQPREP QVDTLPPDRD ELTKNQVSLW CLVKGFPYPSD IAVEWESNGQ PENNYKTTTP   420  
 VLDSGSGFKL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K           471



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SEQ ID NO: 35      moltype = AA  length = 354
FEATURE           Location/Qualifiers
REGION            1..354
                  note = VhH-Fc fusion protein
source            1..354
                  mol_type = protein
                  organism = synthetic construct

SEQUENCE: 35
QVQLQESGGG SVQAGGSLRL SCAASEYIYS SYCMAWFRQA PGKEREGVAV IGSDGSTSYA 60
DSVKGGRFTIS KDNAKNTLYL QMNSLKPEDT AMYYCAAIGG YCYQPPYEQ YWQGQTQVTV 120
SSEPKSSDKT HTCPCCPAPE LLGGPSVFLF PPKPKDTLMI SRTPEVTCVV VDVSHEDPEV 180
KFNWYVDGVE VHNATKPRE EQYNSTYRVV SVLTVLHQDW LNGKEYKCKV SNKALPAPIE 240
KTISKAKGQP REPQVYTLPP SRDELTKNQV SLTCLVKGFY PSDIAVEWES NGQPENNYKT 300
TPPVLDSDGS FFLYSLKTV D KSRWQQGNVF SCSVMHEALH NHYTQKSLSL SPGK 354

SEQ ID NO: 36      moltype = DNA  length = 1121
FEATURE           Location/Qualifiers
misc_feature       1..1121
                  note = VhH-Fc encoding genes
source            1..1121
                  mol_type = other DNA
                  organism = synthetic construct

SEQUENCE: 36
atggagaccg acaccctgct gctgtgggtg ctgctgctgt gggtgcccg cagcacccgc 60
cagggtgcagc tgcaggagtc tgggggaggc tcgggtgcagg ctggagggtc tctgagactc 120
tctctgtgcag cctctgaata catctacagt agctactgca tggcctggtt ccgccaggct 180
ccagggaagg agcgcgaggg ggtcgcagtt attgggagtg atggtagcac aagctacgca 240
gactccgtga aaggccgatt caccatctcc aaagacaacg ccaagaacac tctgtatctg 300
caaatgaaca gcctgaaacc tgaggacact gccatgtact actgtgcggc catcggtggt 360
tactgtacc aaccacccta tgagtaccag tactggggcc aggggaccca ggtaaccgtc 420
tcccagaacc gaaaagcagc gacaagaccc acacctgccc cccctgccc gcccccgcgc 480
tgctggggcg cccagcggtg ttctgttccc cccccaagcc caaggacacc ctgatgatca 540
gccgcacccc cgaggtgacc tgcgtggtgg tggacgtgag ccacgagaac cccgaggtga 600
agttcaactg gtacgtggac ggcgtggagg tgcacaacgc caagaccaag cccgcgagg 660
agcagtacaa cagcacctac cgcgtggtga gcgtgctgac cgtgctgcac caggactggc 720
tgaaaggcaa ggagtacaag tgcaagggtga gcaacaaggc cctgcccgcc cccatcgaga 780
agaccatcag caaggccaag ggcagcccc gcgagcccca ggtgtacacc ctgcccccca 840
gccgcgacga gctgaccaag aaccagggtga gcctgacctg cctgggtgaag ggcttctacc 900
ccagcgacat cgccgtggag tgggagagca acggccagcc cgagaacaac tacaagacca 960
cccccccggt gctggacagc gacggcagct tcttctctga cagcaagctg accgtggaca 1020
agagccgctg gcagcagggc aacgtgttca gctgcagcgt gatgcacgag gccctgcaca 1080
accactacac ccagaagagc ctgagcctga gccccggcaa g 1121

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1. A heterodimer molecule, comprising a first polypeptide chain and a second polypeptide chain, wherein said first polypeptide chain comprises a first CH3 domain of an antibody heavy chain constant region, said second polypeptide chain comprises a second CH3 domain of an antibody heavy chain constant region, and comparing to a corresponding wild-type CH3 domain of a human antibody heavy chain constant region, said first CH3 domain and said second CH3 domain comprise an amino acid mutation as following:

an amino acid mutation at Y349 and T366 of said first CH3 domain, and an amino acid mutation at D356, T366, L368 and Y407 of said second CH3 domain, and said first CH3 domain and/or said second CH3 domain further comprise an amino acid mutation at 1-3 residues selected from the group consisting of F405, K409, K360, Q347 and L368;

wherein said amino acid mutations are independently selected from: a mutation from a non-charged amino acid to a charged amino acid, a mutation from a charged amino acid to a non-charged amino acid, or a mutation from a charged amino acid to an oppositely charged amino acid,

wherein said amino acid is numbered according to the EU index of the KABAT numbering of the antibody Fc region.

2. The heterodimer molecule according to claim 1, wherein said first CH3 domain and/or said second CH3 domain further comprise a mutation selected from the following groups:

- (a) amino acid mutations in said first CH3 domain: Y349+T366, amino acid mutations in said second CH3 domain: D356+T366+L368+Y407+F405;
- (b) amino acid mutations in said first CH3 domain: Y349+T366+F405, amino acid mutations in said second CH3 domain: D356+T366+L368+Y407;
- (c) amino acid mutations in said first CH3 domain: Y349+T366+K409, amino acid mutations in said second CH3 domain: D356+T366+L368+Y407+F405;
- (d) amino acid mutations in said first CH3 domain: Y349+T366+F405+K360+Q347, amino acid mutations in said second CH3 domain: D356+T366+L368+Y407+Q347;
- (e) amino acid mutations in said first CH3 domain: Y349+T366+F405+Q347, amino acid mutations in said second CH3 domain: D356+T366+L368+Y407+K360+Q347;
- (f) amino acid mutations in said first CH3 domain: Y349+T366+K409+K360+Q347, amino acid mutations in said second CH3 domain: D356+T366+L368+Y407+F405+Q347;

- (g) amino acid mutations in said first CH3 domain: Y349+T366+K409+Q347, amino acid mutations in said second CH3 domain: D356+T366+L368+Y407+F405+K360+Q347;
3. The heterodimer molecule according to claim 1, wherein said mutation in said first CH3 domain and/or said second CH3 domain comprises one or more mutations selected from the group consisting of: Y349C, D356C, T366W, T366S, L368A, F405K, Y407V, K409E, K409A, K360E, Q347E, and Q347R.
4. The heterodimer molecule according to claim 2, wherein in said first CH3 domain:  
 said amino acid mutation at Y349 is Y349C;  
 said amino acid mutation at T366 is T366W;  
 said amino acid mutation at F405 is F405K;  
 said amino acid mutation at K409 is K409E or K409A;  
 said amino acid mutation at K360 is K360E;  
 said amino acid mutation at Q347 is Q347E or Q347R;  
 and,  
 in said second CH3 domain:  
 said amino acid mutation at D356 is D356C;  
 said amino acid mutation at T366 is T366S;  
 said amino acid mutation at L368 is L368A;  
 said amino acid mutation at Y407 is Y407V;  
 said amino acid mutation at F405 is F405K;  
 said amino acid mutation at Q347 is Q347R or Q347E;  
 said amino acid mutation at K360 is K360E.
5. The heterodimer molecule according to claim 1, wherein said first CH3 domain and said second CH3 domain comprise one group of mutations selected from the following groups:
- 1) said first CH3 domain: Y349C+T366W, said second CH3 domain: D356C+T366S+L368A+Y407V+F405K;
  - 2) said first CH3 domain: Y349C+T366W+F405K, said second CH3 domain: D356C+T366S+L368A+Y407V;
  - 3) said first CH3 domain: Y349C+T366W+K409E, said second CH3 domain: D356C+T366S+L368A+Y407V+F405K;
  - 4) said first CH3 domain: Y349C+T366W+K409A, said second CH3 domain: D356C+T366S+L368A+Y407V+F405K;
  - 5) said first CH3 domain: Y349C+T366W+F405K+K360E+Q347E, said second CH3 domain: D356C+T366S+L368A+Y407V+Q347R;
  - 6) said first CH3 domain: Y349C+T366W+F405K+Q347R, said second CH3 domain: D356C+T366S+L368A+Y407V+K360E+Q347E;
  - 7) said first CH3 domain: Y349C+T366W+K409A+K360E+Q347E, said second CH3 domain: D356C+T366S+L368A+Y407V+F405K+Q347R;
  - 8) said first CH3 domain: Y349C+T366W+K409A+Q347R, said second CH3 domain: D356C+T366S+L368A+Y407V+F405K+K360E+Q347E.
6. The heterodimer molecule according to claim 1, wherein said first polypeptide chain and said second polypeptide chain further comprise a CH2 domain of an antibody heavy chain constant region, respectively.
7. The heterodimer molecule according to claim 1, wherein said first polypeptide chain and said second polypeptide chain further comprise a hinge region of an antibody heavy chain constant region or a part thereof, respectively.
8. The heterodimer molecule according to claim 1, wherein said wild-type CH3 domain of the human antibody heavy chain constant region is selected from the group consisting of a CH3 domain of a human IgG heavy chain constant region, a CH3 domain of a human IgA heavy chain constant region, a CH3 domain of a human IgD heavy chain constant region, a CH3 domain of a human IgE heavy chain constant region and a CH3 domain of a human IgM heavy chain constant region.
9. The heterodimer molecule according to claim 1, wherein said wild-type CH3 domain of the human antibody heavy chain constant region is a CH3 domain of a human IgG1 heavy chain constant region.
10. The heterodimer molecule according to claim 1, wherein said first polypeptide chain and/or said second polypeptide chain further comprise a molecule binding region, and said molecule binding region comprises an antigen binding region.
11. The heterodimer molecule according to claim 10, wherein said antigen binding region comprises an antibody variable region.
12. The heterodimer molecule according to claim 1, wherein said heterodimer molecule is a bispecific antibody, a bispecific fusion protein or an antibody-fusion protein chimera.
13. A composition, comprising the heterodimer molecule according to claim 1, and a pharmaceutically acceptable carrier or excipient.
14. A nucleic acid molecule, encoding said first polypeptide chain and/or said second polypeptide chain of the heterodimer molecule according to claim 1.
15. A vector, comprising the nucleic acid molecule according to claim 14.
16. A host cell, comprising the vector according to claim 15.
17. A method for preparing a heterodimer molecule, comprising expressing the heterodimer molecule using the host cell according to claim 16.

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