Description

Title of Invention: METHODS FOR PRODUCING AND/OR ENRICHING RECOMBINANT ANTIGEN-BINDING MOLECULES

Technical Field

[0001] The present invention relates to antigen-binding molecules containing a first antigen-binding domain and a second antigen-binding domain which are capable of being linked with each other via at least one disulfide bond formed between the two antigen-binding domains, and methods for producing such antigen-binding molecules. More particularly, the invention relates to methods for increasing or enriching a preferred form of antibody proteins, and methods for eliminating disulfide heterogeneity of recombinant antibody proteins.

Background Art

[0002] Antibodies are proteins which specifically bind to an antigen with high affinity. It is known that various molecules ranging from low-molecular compounds to proteins can be antigens. Since the technique for producing monoclonal antibodies was developed, antibody modification techniques have advanced, making it easy to obtain antibodies that recognize a particular molecule. Now the antibody modification techniques are not only for modifying proteins themselves, but have also expanded into a field that aims at addition of new functions where conjugation with low molecular compounds is contemplated. For example, cysteine-engineered antibodies, which contain a free cysteine amino acid in the heavy chain or light chain, are used as antibody-drug conjugates (ADCs) for medical purposes (PTL 1).

[0003] Antibodies are drawing attention as pharmaceuticals because they are highly stable in blood plasma and have less side effects. Not only do antibodies bind to an antigen and exhibit agonistic or antagonistic effects, but they also induce cytotoxic activity mediated by effector cells (also referred to as effector functions) including ADCC (Antibody Dependent Cell Cytotoxicity), ADCP (Antibody Dependent Cell Phagocytosis), and CDC (Complement Dependent Cytotoxicity). Taking advantage of these antibody functions, pharmaceuticals for cancer, immune diseases, chronic disease, infections, etc. have been developed (NPL 1).

[0004] For example, pharmaceuticals utilizing an agonist antibody against a costimulatory molecule promoting activation of cytotoxic T cells have been developed as anti-cancer agents (NPL 2). Recently, immune checkpoint-inhibiting antibodies with antagonist activity on co-inhibitory molecules were found to be useful as anticancer agents. This finding led to the launch of a series of antibody pharmaceuticals inhibiting the in-

teraction of CTLA4/CD80 or PD-1/PD-L1: Ipilimumab, Nivolumab, Pembrolizumab, and Atezolizumab (NPL 1).

[0005] However, such antibodies sometimes do not sufficiently exert expected effects in their original native IgG form. Therefore, second generation antibody pharmaceuticals, in which the functions of the native IgG antibody have been artificially enhanced or added, or diminished or deleted, depending on the purpose of use, have been developed. The second generation antibody pharmaceuticals include, for example, antibodies with enhanced or deleted effector functions (NPL 3), antibodies binding to an antigen in an pH-dependent manner (NPL 4), and antibodies binding to two or more different antigens per molecule (antibodies binding to two different antigens are generally referred to as "bispecific antibodies") (NPL 5).

[0006] Bispecific antibodies are expected to be more effective pharmaceuticals. For example, antibodies with enhanced antitumor activity which crosslink a cytotoxic T cell with a cancer cell by binding to a protein expressed on the cell membrane of the T cell as one antigen and to a cancer antigen as the other antigen have been developed (NPL 7, NPL 8, and PTL 2). The previously reported bispecific antibodies include molecules with two antibody Fab domains each having a different sequence (common light chain bispecific antibodies and hybrid hybridomas), molecules with an additional antigen-binding site attached to the N or C terminus of antibody (DVD-Ig and scFv-IgG), molecules with one Fab domain binding to two antigens (Two-in-one IgG), molecules in which the loop regions of the CH3 domain have been engineered to form new antigen-binding sites (Fcab) (NPL 9), and molecules with tandem Fab-Fab (NPL 10).

[0007] Meanwhile, antibodies with effector functions readily cause side effects by acting even on normal cells that express a target antigen at low levels. Thus, efforts have been made to allow antibody pharmaceuticals to exert their effector functions specifically on target tissue. Previously reported examples are antibodies whose binding activity changes upon binding to a cell metabolite (PTL 3), antibodies which become capable of binding to an antigen upon protease cleavage (PTL 4), and a technology that regulates antibody-mediated crosslinking between chimeric antigen receptor T cells and cancer cells by addition of a compound (ABT-737) (NPL 11).

[0008] Agonist antibodies may be difficult to obtain depending on the target. In particular, for membrane proteins such as G-protein-coupled receptors, many different techniques have been developed (NPL 12). Thus, there is a demand for simple methods for enhancing the agonistic effect of antibodies on such targets. Known existing methods include, for example, a method of crosslinking an anti-DR4 (Death Receptor 4) or anti-DR5 (Death Receptor 5) antibody (NPL13), a method of multimerizing nanobodies of anti-DR5 (Death Receptor 5) antibody (NPL 14), a method of converting an anti-

thrombopoietin receptor antibody into a covalent diabody, sc(Fv)$_2$ (NPL 15), a method of changing the IgG subclass of anti-CD40 antibody (NPL 16), a method of hexamerizing an anti-CD20 antibody (NPL 17), and a method of producing a circular, antibody-like molecule (PTL 5). In addition, reported methods using bispecific antibodies include, for example, a method of using a combination of two appropriate anti-erythropoietin antibodies against different epitopes as a bispecific antibody (NPL 18), a method of using a combination of an antibody for guide functions and an antibody for effector functions as a bispecific antibody (NPL 19), and a method of introducing Cys residues into multiple antibody fragments specific for different epitopes and conjugating them (NPL 20, NPL 21, and PTL 6).

Citation List

Patent Literature

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[PTL 2] WO 2008/157379
[PTL 3] WO 2013/180200
[PTL 4] WO 2009/025846
[PTL 5] WO 2017/191101
[PTL 6] WO 2018/027204

Non Patent Literature

[0010] [NPL 1] Nature Reviews Drug Discovery (2018) 17, 197-223
[NPL 2] Clinical and Experimental Immunology (2009) 157, 9-19
[NPL 3] Current Pharmaceutical Biotechnology (2016) 17, 1298-1314
[NPL 4] Nature Biotechnology (2010) 28, 1203-1208
[NPL 5] MAbs (2012) 4, 182-197
[NPL 6] Nature Reviews Immunology (2010) 10, 301-316
[NPL 7] Sci Transl Med (2017) 9(410), eaal4291
[NPL 8] Blood (2011) 117(17): 4403-4404
[NPL 9] Protein Eng Des Sel (2010) 23(4), 289-297
[NPL 10] J Immunol (2016) 196(7): 3199-3211
[NPL 11] Nature Chemical Biology (2018) 14, 112-117
[NPL 12] Exp Mol Med (2016) 48(2): e207
[NPL 13] Nature Reviews Drug Discovery (2008) 7, 1001-1012
[NPL 14] MAbs (2014) 6(6): 1560-1570
[NPL 15] Blood (2005) 105(2): 562-566
[NPL 16] J Biol Chem (2008) 283(23): 16206-16215
[NPL 17] PLoS Biol (2016) 14(1): e1002344
[NPL 18] Proc Natl Acad Sci U S A (2012) 109(39): 15728-15733

[NPL 19] Scientific Reports (2018) 8, Article number: 766
[NPL 20] PLoS One (2012) 7(12): e51817
[NPL 21] Nucleic Acids Res (2010) 38(22): 8188-8195

Summary of Invention

Technical Problem

[0011] An objective of the present invention is to provide novel antigen-binding molecules (for example, an IgG antibody) that have activity of regulating interaction between two or more antigen molecules, and/or methods for producing or using such antigen-binding molecules. More particularly, the present invention solves the issues that conventional antibody (e.g. wild type IgG) has uncontrolled flexibility of the two antigen-binding domains (e.g. two Fab arms) by means of introducing one or more engineered disulfide bond(s) between the two antigen-binding domains (two Fabs) of the antibody through introducing mutation in the heavy and/or light chain. Specifically, by introducing one or more thiol-containing amino acid (e.g. cysteine and methionine) at each of the two antigen-binding domains (two Fabs) of the antibody, such antibody is capable of forming one or more disulfide bond between the two antigen-binding domains (two Fabs).

Solution to Problem

[0012] An antigen-binding molecule of the present invention contains a first antigen-binding domain and a second antigen-binding domain which are "capable of being linked" with each other via at least one disulfide bond between the two antigen-binding domains. The at least one disulfide bond is "capable of being formed" between the two antigen-binding domains, e.g., between amino acid residues which are not in a hinge region. The terms "capable of being linked" and "capable of being formed" include cases where the disulfide bond has already been formed, and cases where the disulfide bond has not been formed but will be formed later under suitable conditions.

[0013] In one non-limiting aspect, the one or more engineered disulfide bond(s) between the two Fabs of the IgG antibody enables controls of the flexibility, the distance, and/or the cell binding orientation (i.e. cis or trans) of the two Fab arms, thereby improving activity, and/or safety of the IgG antibody compared to corresponding wild type IgG antibody without the one or more engineered disulfide bond(s). In one non-limiting aspect, the one or more engineered disulfide bond(s) between the two Fabs of the IgG improves the agonistic activity of the IgG antibody compared to corresponding wild type IgG antibody without the one or more engineered disulfide bond(s). In addition, in another non-limiting aspect, the one or more engineered disulfide bond(s) between the two Fabs of the IgG improves the resistance of the IgG antibody to protease digestion, compared to corresponding wild type IgG antibody without the one or more engineered

disulfide bond(s).

[0014] While preparing the antibody capable of forming one or more engineered disulfide bond(s) between the two Fabs of the antibody, the inventors further found the several conformational isoforms of the same antibody (same sequence) but with different disulfide structures, in particular the isoform having the "paired cysteines" and the isoform having the "free or unpaired cysteines" (i.e., two structural isoforms), can be generated during recombinant antibody production in mammalian cell. Therefore, another aspect of the present invention is directed to providing efficient and facile production, purification and analysis of the antibody having one or more engineered disulfide bond(s) between the two Fabs of the antibody. More particularly, the invention describes methods for increasing structural homogeneity and relative abundance of the antibody in the "paired cysteines" form, i.e. having one or more engineered disulfide bond(s) formed between the two Fabs of the antibody. In other words, the invention describes methods for decreasing relative abundance of the antibody in the "free or unpaired cysteines" form, i.e. having no engineered disulfide bond formed between the two Fabs of the antibody.

[0015] As described in further detail hereinbelow, in some embodiments of the invention, the addition of reducing agent can facilitate the formation of one or more engineered disulfide bond(s) in the antibody and thus produce structurally homogeneous of the molecule.

[0016] More specifically, the present invention provides the following:

[1] A method for (i) producing an antibody preparation, (ii) purifying an antibody having a desired conformation, or (iii) improving homogeneity of an antibody preparation;

said method comprising contacting an antibody preparation with a reducing reagent, wherein the antibody comprises a first antigen-binding domain and a second antigen-binding domain which are capable of being linked with each other via at least one disulfide bond, wherein said at least one disulfide bond is capable of being formed between amino acid residues which are not in a hinge region.

[2] A method for (i) producing an antibody preparation, (ii) purifying an antibody preparation, or (iii) improving homogeneity of an antibody preparation;

comprising isolating a fraction of the antibody having a desired conformation via one or more chromatography steps selected from the group consisting of: reversed-phase chromatography, size-exclusion chromatography, ion-exchange chromatography, hydrophobic interaction chromatography, affinity chromatography, and electrophoresis; wherein said antibody having a desired conformation is characterized by having at least one disulfide bond formed between amino acid residues which are not in a hinge region.

[2A] The method of [2], wherein one or more chromatography steps is ion exchange chromatography (IEC) and/or hydrophobic interaction chromatography (HIC), or mixed-mode chromatography of IEC and HIC.

[3] The method of any one of [1]-[2A], wherein said antibody preparation comprises two or more structural isoforms which differ by at least one disulfide bond formed between amino acid residues which are not in a hinge region.

[3A] The method of [3], wherein said antibody preparation comprises two structural isoforms which differ by at least one disulfide bond formed between amino acid residues which are not in a hinge region.

[3B] The method of any one of [1] to [3A], wherein said method preferentially enriches or increases the population of an antibody structural isoform having at least one disulfide bond formed between amino acid residues which is not in a hinge region.

[3C] The method of any one of [1] to [3B], wherein said method produces a homogenous antibody preparation having at least 50%, 60%, 70%, 80%, 90%, preferably at least 95% molar ratio of said antibody having at least one disulfide bond formed between amino acid residues which are not in a hinge region.

[3D] The method of any one of [1] to [3C], wherein each of said first antigen-binding domain and second antigen-binding domain comprises a hinge region, or does not comprise a hinge region.

[3E] The method of any of [1] to [3D], wherein said amino acid residues which are not in a hinge region are introduced or engineered cysteines.

[3F] The method of any of [1] to [3E], wherein said at least one disulfide bond is an interchain disulfide bond.

[3I] The method of any of [1] to [3F], wherein said at least one disulfide bond is an engineered disulfide bond which is not present in a wild type IgG.

[4] The method of any of [1] to [3J],

wherein said at least one disulfide bond is formed between a CH1 region, a CL region, a VL region, a VH region and/or a VHH region of the first antigen-binding domain and the second antigen-binding domain.

[5] The method of any of [1] to [4],

wherein said at least one disulfide bond is formed between a CH1 region of the first antigen-binding domain and a CH1 region of the second antigen-binding domain.

[5.1] The method of [5], wherein said at least one disulfide bond is formed between the antigen-binding domains at any one of positions 119 to 123, 131 to 140, 148 to 150, 155 to 167, 174 to 178, 188 to 197, and 201 to 214, according to EU numbering, in the CH1 region.

[5.2] The method of [5], wherein said at least one disulfide bond is formed between the antigen-binding domains at any one of positions 119, 122, 123, 131, 132, 133, 134,

135, 136, 137, 138, 139, 140, 148, 150, 155, 156, 157, 159, 160, 161, 162, 163, 164, 165, 167, 174, 176, 177, 178, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 201, 203, 205, 206, 207, 208, 211, 212, 213, 214, according to EU numbering, in the CH1 region.

[5.3] The method of [5], wherein said at least one disulfide bond is formed between the antigen-binding domains at any one of positions 134, 135, 136, 137, 191, 192, 193, 194, 195, or 196, according to EU numbering, in the CH1 region.

[5.4] The method of [5], wherein said at least one disulfide bond is formed between the antigen-binding domains at any one of positions 135, 136, or 191, according to EU numbering, in the CH1 region.

[5.5] The method of [5], wherein said at least one disulfide bond is formed between the amino acid residues in the first antigen-binding domain and the second antigen-binding domain, selected from the group consisting of positions 119, 120, 121, 122, and 123 according to EU numbering.

[5.6] The method of [5], wherein said at least one disulfide bond is formed between the amino acid residues in the first antigen-binding domain and the second antigen-binding domain, selected from the group consisting of positions 131, 132, 133, 134, 135, 136, 137, 138, 139, and 140 according to EU numbering.

[5.7] The method of [5], wherein said at least one disulfide bond is formed between the amino acid residues in the first antigen-binding domain and the second antigen-binding domain, selected from the group consisting of positions 148, 149, and 150 according to EU numbering.

[5.8] The method of [5], wherein said at least one disulfide bond is formed between the amino acid residues in the first antigen-binding domain and the second antigen-binding domain, selected from the group consisting of positions 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, and 167 according to EU numbering.

[5.9] The method of [5], wherein said at least one disulfide bond is formed between the amino acid residues in the first antigen-binding domain and the second antigen-binding domain, selected from the group consisting of positions 174, 175, 176, 177, and 178 according to EU numbering.

[5.10] The method of [5], wherein said at least one disulfide bond is formed between the amino acid residues in the first antigen-binding domain and the second antigen-binding domain, selected from the group consisting of positions 188, 189, 190, 191, 192, 193, 194, 195, 196, and 197 according to EU numbering.

[5.11] The method of [5], wherein said at least one disulfide bond is formed between the amino acid residues in the first antigen-binding domain and the second antigen-binding domain, selected from the group consisting of positions 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, and 214 according to EU numbering.

[5.12] The method of [5], wherein the difference between the positions of the amino acid residues in the first antigen-binding domain and the second antigen-binding domain is three amino acids or less.

[5.13] The method of [5], wherein said at least one of the disulfide bonds linking the two antigen-binding domains is formed by linking an amino acid residue at position 135 according to EU numbering in the CH1 region of the first antigen-binding domain with an amino acid residue at any one of positions 132 to 138 according to EU numbering in the CH1 region of the second antigen-binding domain.

[5.14] The method of [5], wherein said at least one of the disulfide bonds linking the two antigen-binding domains is formed by linking an amino acid residue at position 136 according to EU numbering in the CH1 region of the first antigen-binding domain with an amino acid residue at any one of positions 133 to 139 according to EU numbering in the CH1 region of the second antigen-binding domain.

[5.15] The method of [5], wherein said at least one of the disulfide bonds linking the two antigen-binding domains is formed by linking an amino acid residue at position 191 according to EU numbering in the CH1 region of the first antigen-binding domain with an amino acid residue at any one of positions 188 to 194 according to EU numbering in the CH1 region of the second antigen-binding domain.

[5.16] The method of [5], wherein one disulfide bond is formed between the two antigen-binding domains at position 135, according to EU numbering, in the CH1 region.

[5.17] The method of [5], wherein one disulfide bond is formed between the two antigen-binding domains at position 136, according to EU numbering, in the CH1 region.

[5.18] The method of [5], wherein one disulfide bond is formed between the two antigen-binding domains at position 191, according to EU numbering, in the CH1 region.

[5A] The method of [5], wherein the subclass of the CH1 region is gamma 1, gamma 2, gamma 3, gamma 4, alpha 1, alpha 2, mu, delta, or epsilon.

[6] The method of [5]-[5A], wherein one disulfide bond is formed between the amino acid residues at position 191 according to EU numbering in the respective CH1 regions of the first antigen-binding domain and the second antigen-binding domain.

[6A] The method of [6], wherein additional one, two or more disulfide bond(s) is/are formed between the first antigen-binding domain and the second antigen-binding domain via the amino acid residues at the following positions according to EU numbering in each of the respective CH1 regions of the first antigen-binding domain and the second antigen-binding domain:

(a) between amino acid residues at any position of 131 to 138, 194 and 195 in each of

the two antigen-binding domains;
(b) between the amino acid residues at position 131 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;
(c) between the amino acid residues at position 132 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;
(d) between the amino acid residues at position 133 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;
(e) between the amino acid residues at position 134 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;
(f) between the amino acid residues at position 135 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;
(g) between the amino acid residues at position 136 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;
(h) between the amino acid residues at position 137 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;
(i) between the amino acid residues at position 138 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;
(j) between the amino acid residues at position 131 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;
(k) between the amino acid residues at position 132 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;
(l) between the amino acid residues at position 133 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;
(m) between the amino acid residues at position 134 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;
(n) between the amino acid residues at position 135 in each of the two antigen-binding

domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;
(o) between the amino acid residues at position 136 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;
(p) between the amino acid residues at position 137 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains; and
(q) between the amino acid residues at position 138 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains.
[6B] The method of [6] or [6A], wherein any one of the first and second antigen-binding domains comprises one, two or more charged amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain of the first and second antigen-binding domains comprises one, two or more oppositely charged amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.
[6C] The method of [6] or [6A], wherein any one of the first and second antigen-binding domains comprises one, two or more positively charged amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more negatively charged amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.
[6D] The method of [6] or [6A], wherein any one of the first and second antigen-binding domains comprises one, two or more negatively charged amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more positively charged amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.
[6E] The method of [6] or [6A], wherein any one of the first and second antigen-binding domains comprises one, two or more of the following amino acid residues in the respective CH1 region (according to EU numbering):
(a) the amino acid residue at position 136 is glutamic acid (E) or aspartic acid (D);
(b) the amino acid residue at position 137 is glutamic acid (E) or aspartic acid (D);
(c) the amino acid residue at position 138 is glutamic acid (E) or aspartic acid (D); and the other antigen-binding domain of the first and second antigen-binding domains comprises one, two or more of the following amino acid residues in the respective CH1 region (according to EU numbering):

(d) the amino acid residue at position 193 is lysine (K), arginine (R), or histidine (H);
(e) the amino acid residue at position 194 is lysine (K), arginine (R), or histidine (H); and
(f) the amino acid residue at position 195 is lysine (K), arginine (R), or histidine (H).

[6F-1] The method of [6] or [6A], wherein any one of the first and second antigen-binding domains comprises one or more of the following amino acid residues in the respective CH1 region (according to EU numbering):
(a) the amino acid residue at position 136 is lysine (K), arginine (R), or histidine (H);
(b) the amino acid residue at position 137 is lysine (K), arginine (R), or histidine (H);
(c) the amino acid residue at position 138 is lysine (K), arginine (R), or histidine (H); and
the other antigen-binding domain out of the first and second antigen-binding domains comprises one or more of the following amino acid residues in the respective CH1 region (according to EU numbering):
(d) the amino acid residue at position 193 is glutamic acid (E) or aspartic acid (D);
(e) the amino acid residue at position 194 is glutamic acid (E) or aspartic acid (D); and
(f) the amino acid residue at position 195 is glutamic acid (E) or aspartic acid (D).

[6F-2] The method of [6] or [6A], wherein each of the first and second antigen-binding domains comprises any of the specific charged mutation combination in the respective CH1 region (according to EU numbering) as listed in Tables 7, Table 82 or Table 85.

[6G] The method of [6] or [6A], wherein any one of the first and second antigen-binding domains comprises one, two or more hydrophobic amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more hydrophobic amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

[6H] The method of [6G], wherein said hydrophobic amino acid residue(s) is/are alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), and/or tryptophan (Trp).

[6I] The method of [6] or [6A], wherein any one of the first and second antigen-binding domains comprises one "knob" amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more "hole" amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

[6J] The method of [6] or [6A], wherein any one of the first and second antigen-binding domains comprises one, two or more "hole" amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other

antigen-binding domain out of the first and second antigen-binding domains comprises one "knob" amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

[6K] The method of [6I] or [6J], wherein said "knob" amino acid residue(s) is/are selected from the group consisting of tryptophan (Trp) and phenylalanine (Phe); and said "hole" amino acid residue(s) is/are selected from the group consisting of alanine (Ala), valine (Val), threonine (T) or serine (S).

[6L] The method of [6] or [6A], wherein any one of the first and second antigen-binding domains comprises one, two or more aromatic amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more positively charged amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

[6M] The method of [6] or [6A], wherein any one of the first and second antigen-binding domains comprises one, two or more positively charged amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more aromatic amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

[6N-1] The method of [6L] or [6M], wherein said aromatic amino acid residue(s) is/are selected from the group consisting of tryptophan (Trp), tyrosine (Tyr), histidine (His), and phenylalanine (Phe); and said positively charged amino acid residue(s) is/are selected from the group consisting of lysine (K), arginine (R), or histidine (H).

[6N-2] The method of [6] or [6A], wherein each of the first and second antigen-binding domains comprises any of the specific hydrophobic amino acid mutation combination in the respective CH1 region (according to EU numbering) as listed in Table 10.

[7] The method of any one of [1] to [4], wherein said at least one disulfide bond is formed between a CL region of the first antigen-binding domain and a CL region of the second antigen-binding domain.

[7.1] The method of [7], wherein the amino acid residue from which the at least one disulfide bond between the two antigen-binding domains is formed is present at any one of positions 108 to 112, 121 to 128, 151 to 156, 184 to 190, 195 to 196, 200 to 203, and 208 to 213, according to Kabat numbering, in the CL region.

[7.2] The method of [7], wherein the amino acid residue from which the at least one disulfide bond between the two antigen-binding domains is formed is present at a position selected from the group consisting of positions 108, 109, 112, 121, 123, 126, 128, 151, 152, 153, 156, 184, 186, 188, 189, 190, 195, 196, 200, 201, 202, 203, 208,

210, 211, 212, and 213 according to Kabat numbering in the CL region.

[7.3] The method of [7], wherein the amino acid residue from which the at least one disulfide bonds between the two antigen-binding domains is formed is present at position 126 according to Kabat numbering in the CL region.

[7.4] The method of [7], wherein at least one of the disulfide bonds linking the two antigen-binding domains is formed by linking an amino acid residue in the CL region of the first antigen-binding domain with an amino acid residue in the CL region of the second antigen-binding domain.

[7.5] The method of [7], wherein the amino acid residue from which the at least one disulfide bond between the two antigen-binding domains is formed is present at a position independently selected from the group consisting of positions 108, 109, 110, 111, and 112 according to Kabat numbering.

[7.6] The method of [7], wherein the amino acid residue from which the at least one disulfide bond between the two antigen-binding domains is formed is present at a position independently selected from the group consisting of positions 151, 152, 153, 154, 155, and 156 according to Kabat numbering.

[7.7] The method of [7], wherein the amino acid residue from which the at least one disulfide bond between the two antigen-binding domains is formed is present at a position independently selected from the group consisting of positions 184, 185, 186, 187, 188, 189, and 190 according to Kabat numbering.

[7.8] The method of [7], wherein the amino acid residue from which the at least one disulfide bond between the two antigen-binding domains is formed is present at a position independently selected from the group consisting of positions 200, 201, 202, and 203 according to Kabat numbering.

[7.9] The method of [7], wherein the amino acid residue from which the at least one disulfide bond between the two antigen-binding domains is formed is present at a position independently selected from the group consisting of positions 208, 209, 210, 211, 212, and 213 according to Kabat numbering.

[7.10] The method of [7] to [7.9], wherein the difference between the positions of the amino acid residues from which the at least one disulfide bond between the two antigen-binding domains is formed is three amino acids or less.

[7.11] The method of [7], wherein said at least one of the bonds linking the two antigen-binding domains is formed by linking amino acid residues at position 126 according to Kabat numbering in the CL region of the two antigen-binding domains with each other.

[8] The method of any one of [1] to [4], wherein said at least one of the disulfide bond is formed by linking an amino acid residue in a CH1 region of the first antigen-binding domain with an amino acid residue in a CL region of the second antigen-binding

domain.

[8.1] The method of [8], wherein the amino acid residue in the CH1 region is selected from the group consisting of positions 188, 189, 190, 191, 192, 193, 194, 195, 196, and 197 according to EU numbering, and the amino acid residue in the CL region is selected from the group consisting of positions 121, 122, 123, 124, 125, 126, 127, and 128 according to Kabat numbering.

[8.2] The method of [8], wherein at least one of the disulfide bond linking the two antigen-binding domains is formed by linking an amino acid residue at position 191 according to EU numbering in the CH1 region of the first antigen-binding domain with an amino acid residue at position 126 according to Kabat numbering in the CL region of the second antigen-binding domain.

[8A] The method of [7] to [8], wherein the subclass of the CL region is kappa or lambda.

[9] The method of any one of [1] to [4], wherein said at least one disulfide bond is formed between a variable region of the first antigen-binding domain and the second antigen-binding domain.

[9.1] The method of [9], wherein the amino acid residue from which the at least one disulfide bond between the antigen-binding domains is formed is present within a VH region.

[9.2] The method of [9], wherein the amino acid residue from which the at least one disulfide bond between the antigen-binding domains is formed is present at a position selected from the group consisting of positions 6, 8, 16, 20, 25, 26, 28, 74, and 82b according to Kabat numbering in the VH region.

[9.3] The method of [9], wherein the amino acid residue from which the at least one disulfide bond between the antigen-binding domains is formed is present within a VL region.

[9.4] The method of [9], wherein the amino acid residue from which the at least one disulfide bond between the antigen-binding domains is formed is present at a position selected from the group consisting of positions 21, 27, 58, 77, 100, 105, and 107 according to Kabat numbering in the VL region (subclass kappa).

[9.5] The method of [9], wherein the amino acid residue from which the at least one disulfide bond between the antigen-binding domains is formed is present at a position selected from the group consisting of positions 6, 19, 33, and 34 according to Kabat numbering in the VL region (subclass lambda).

[9A] The method of [4], wherein the amino acid residue from which the at least one disulfide bond between the two antigen-binding domains is formed is present within a VHH region.

[9B] The method of [9A], wherein the amino acid residue from which the at least one

disulfide bond between the antigen-binding domains is formed is present at a position selected from the group consisting of positions 4, 6, 7, 8, 9, 10, 11, 12, 14, 15, 17, 20, 24, 27, 29, 38, 39, 40, 41, 43, 44, 45, 46, 47, 48, 49, 67, 69, 71, 78, 80, 82, 82c, 85, 88, 91, 93, 94, and 107 according to Kabat numbering in the VHH region.

[10] The method of any one of [1] to [9B], characterized by one or more of the following:

(a) wherein said at least one disulfide bond restricts the antigen binding orientation of the two antigen-binding domains to cis antigen-binding (i.e. binding to two antigens on the same cell), or restrict binding of the two antigen binding domains to two antigens which are spatially close to each other;

(b) wherein said at least one disulfide bond holds the first antigen-binding domain and the second antigen-binding domain spatially closer to each other, as compared to a same corresponding antibody which does not have said at least one disulfide bond;

(c) wherein said at least one disulfide bond reduce the flexibility and/or mobility of first antigen-binding domain and the second antigen-binding domain, as compared to a corresponding same antibody which does not have said at least one disulfide bond;

(d) wherein said at least one disulfide bond increases resistance of the antibody to protease cleavage, as compared to a corresponding same antibody which does not have said at least one disulfide bond;

(e) wherein said at least one disulfide bond enhances or reduces interaction between two antigen molecules bound by the antigen-binding molecule, as compared to a corresponding same antibody which does not have said at least one disulfide bond;

(f) wherein said method produces an antibody preparation which is more homogeneous than the same antibody preparation that has not been treated by said method;

(g) wherein said method produces an antibody preparation having increase in its biological activity compared to the same antibody that has not been treated by said method;

(h) wherein said method produces an antibody having enhanced activity of holding two antigen molecules at spatially close positions compared to the same antibody that has not been treated by said method;

(i) wherein said method produces an antibody having enhanced stability compared to the same antibody that has not been treated by said method; and

(j) wherein said method preferentially enriches an antibody having at least one disulfide bond formed outside of hinge regions and said preferentially enriched form has a pharmaceutically desirable property selected from any of (a) to (i) above, as compared to a preparation that has not been treated by said method.

[11] The method of any one of [1] to [10], wherein each of the first and second antigen-binding domains has a Fab, Fab', scFab, Fv, scFv, or VHH structure.

[11A] The method of [11], wherein the first and second antigen-binding domains each comprises a Fab and a hinge region, forming a F(ab')2 structure.
[12] The method of any one of [1] to [11A], wherein the antigen-binding molecule further comprises an Fc region.
[12A] The method of [12], wherein the Fc region is a Fc region having reduced binding activity against Fc gamma R as compared with that of the Fc region of a wild-type human IgG1 antibody.
[13] The method of any one of [1] to [12A], wherein said antibody is an IgG antibody, preferably an IgG1, IgG2, IgG3 or IgG4 antibody.
[14] The method of any one of [1] to [13], wherein both the first and second antigen-binding domains bind to the same antigen.
[14A] The method of any one of [1] to [13], wherein both the first and second antigen-binding domains bind to the same epitope on said antigen.
[14B] The method of any one of [1] to [13], wherein each of the first and second antigen-binding domains binds to a different epitope on said antigen.
[14C] The method of any one of [1] to [13], wherein each of the first and second antigen-binding domains binds to a different antigen.
[14D] The method of any one of [1] to [13], wherein both the first and second antigen-binding domains have the same amino acid sequence.
[14E] The method of any one of [1] to [13], wherein each of the first and second antigen-binding domains has a different amino acid sequence.
[14F] The method of any one of [1] to [14E], wherein at least one of two antigens to which the first and second antigen-binding domains bind is a soluble protein.
[14G] The method of any one of [1] to [14E], wherein at least one of two antigens to which the first and second antigen-binding domains bind is a membrane protein.
[14H] The method of any one of [1] to [14G], which has activity of regulating interaction between two antigen molecules.
[14I] The method of [14H], which is capable of enhancing or diminishing interaction between two antigen molecules as compared to a same corresponding antibody which does not have said at least one disulfide bond.
[14J] The method of any one of [14H] to [14I], wherein the two antigen molecules are a ligand and a receptor thereof, respectively, and wherein the antibody has activity of promoting activation of the receptor by the ligand.
[14K] The method of any one of [14H] to [14I], wherein the two antigen molecules are an enzyme and a substrate thereof, respectively, and wherein the antigen-binding molecule has activity of promoting catalytic reaction of the enzyme with the substrate.
[14L] The method of any one of [14H] to [14I], wherein both of the two antigen molecules are proteins present on cellular surfaces, and wherein the antibody has

activity of promoting interaction between a cell expressing the first antigen and a cell expressing the second antigen.

[14M] The method of any of [14L], wherein the cell expressing the first antigen is a cell with cytotoxic activity, and the cell expressing the second antigen is a target cell thereof, and wherein the antibody promotes damage of said target cell by said cell with cytotoxic activity.

[14N] The method of [14M], wherein the cell with cytotoxic activity is a T cell, NK cell, monocyte, or macrophage.

[14O] The method of [14N], wherein the antibody having said at least one disulfide bond enhances or diminishes activation of two antigen molecules as compared to a same corresponding antibody which does not have said at least one disulfide bond.

[14P] The method of any one of [14] to [14O], wherein the antigen molecules are selected from the group consisting of receptors belonging to cytokine receptor super-families, G protein-coupled receptors, ion channel receptors, tyrosine kinase receptors, immune checkpoint receptors, antigen receptors, CD antigens, costimulatory molecules, and cell adhesion molecules.

[15] The method of any one of [14] to [14P], wherein the first antigen-binding domain and the second antigen-binding domain are each capable of binding to CD3.

[16] The method of any one of [1] to [15], wherein the pH of said reducing reagent contacting with the antibody is from about 3 to about 10.

[16A] The method of [16], wherein the pH of said reducing reagent contacting with the antibody is about 6, 7 or 8.

[16B] The method of [16], wherein the pH of said reducing reagent contacting with the antibody is about 7.

[16C] The method of [16], wherein the pH of said reducing reagent contacting with the antibody is about 3.

[17] The method of any one of [1] to [16B], wherein the reducing agent is selected from the group consisting of TCEP, 2-MEA, DTT, Cysteine, GSH and Na_2SO_3.

[17A] The method of [17], wherein the reducing agent is TCEP.

[18] The method of any one of [17] to [17A], wherein the concentration of the reducing agent is from about 0.01 mM to about 100 mM.

[19] The method of [18], wherein the concentration of the reducing agent is about 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100 mM, preferably about 0.01 mM to 25 mM.

[20] The method of any one of [1] to [19], wherein the contacting step is performed for at least 30 minutes.

[20A] The method of any one of [1] to [19], wherein the contacting step is performed for about 2 to about 48 hours.

[20B] The method of any one of [1] to [19], wherein the contacting step is performed for about 2 hours or about 16 hours.

[21] The method of any one of [1] to [20B], wherein the contacting step is performed at a temperature of about 20 degrees C to 37 degrees C, preferably at 23 degrees C, 25 degrees C or 37 degrees C, more preferably at 23 degrees C.

[22] The method of any one of [1] to [21], wherein said antibody is at least partially purified prior to said contacting step with reducing agent.

[22A] The method of [22], wherein said antibody is partially purified by affinity chromatography (preferably Protein A chromatography) prior to said contacting.

[23] The method of any one of [1] to [22], wherein the concentration of the antibody is from about 1 mg/ml and about 50 mg/ml.

[23A] The method of [23], wherein the concentration of the antibody is about 1 mg/ml or about 20 mg/ml.

[24] The method of any one of [1] to [23], further comprising isolating a fraction of the contacted antibody having a desired conformation.

[24A] The method of [24], wherein the procedure for said isolating is selected from the group consisting of: reversed-phase chromatography HPLC, size-exclusion chromatography, ion-exchange chromatography, hydrophobic interaction chromatography, affinity chromatography, dialysis and electrophoresis.

[24B] The method of [24], wherein the procedure for said isolating is ion exchange chromatography (IEC) and/or hydrophobic interaction chromatography (HIC).

[24C] The method of any one of [1] to [24B], further comprising a step of removing the reducing agent, preferably by dialysis, more preferably by a chromatography method.

[25] A preparation of an IgG antibody prepared according to the method of any one of [1] to [24B], said preparation having a homogeneous population of said IgG antibody having at least one disulfide bond outside of the hinge regions.

[26] A preparation of an IgG antibody prepared according to the method of any one of [1] to [25], said preparation having at least 50%, 60%, 70%, 80%, 90%, preferably at least 95% molar ratio of said IgG antibody having at least one disulfide bond outside of the hinge regions.

[27] The preparation of [25] or [26], further comprising a pharmaceutically acceptable carrier, excipient or diluent.

[28] A pharmaceutical composition comprising a homogeneous population of antibody as defined in [25] and a pharmaceutically acceptable carrier, excipient or diluent.

[0017] In another aspect, the present invention also provides the following:

[1] An antigen-binding molecule comprising a first antigen-binding domain and a second antigen-binding domain, wherein the two antigen-binding domains are linked

with each other via one or more bonds.

[2] The antigen-binding molecule of [1], wherein at least one of the bonds linking the two antigen-binding domains is a covalent bond.

[3] The antigen-binding molecule of [2], wherein the covalent bond is formed by direct crosslinking of an amino acid residue in the first antigen-binding domain with an amino acid residue in the second antigen-binding domain.

[4] The antigen-binding molecule of [3], wherein the crosslinked amino acid residues are cysteine.

[5] The antigen-binding molecule of [4], wherein the formed covalent bond is a disulfide bond.

[6] The antigen-binding molecule of [2], wherein the covalent bond is formed by crosslinking of an amino acid residue in the first antigen-binding domain with an amino acid residue in the second antigen-binding domain via a crosslinking agent.

[7] The antigen-binding molecule of [6], wherein the crosslinking agent is an amine-reactive crosslinking agent.

[8] The antigen-binding molecule of [7], wherein the crosslinked amino acid residues are lysine.

[9] The antigen-binding molecule of [1], wherein at least one of the bonds linking the two antigen-binding domains is a noncovalent bond.

[10] The antigen-binding molecule of [9], wherein the noncovalent bond is an ionic bond, hydrogen bond, or hydrophobic bond.

[11] The antigen-binding molecule of [10], wherein the ionic bond is formed between an acidic amino acid and a basic amino acid.

[12] The antigen-binding molecule of [11], wherein the acidic amino acid is aspartic acid (Asp) or glutamic acid (Glu), and the basic amino acid is histidine (His), lysine (Lys), or arginine (Arg).

[13] The antigen-binding molecule of any one of [1] to [12], wherein at least one of amino acid residues from which the bonds between the antigen-binding domains originate is an artificially-introduced mutated amino acid residue.

[14] The antigen-binding molecule of [13], wherein the mutated amino acid residue is a cysteine residue.

[15] The antigen-binding molecule of any one of [1] to [14], wherein at least one of the first and second antigen-binding domains has, by itself, activity of binding to an antigen.

[16] The antigen-binding molecule of any one of [1] to [15], wherein the first and second antigen-binding domains are both antigen-binding domains of the same type.

[17] The antigen-binding molecule of any one of [1] to [16], wherein at least one of the bonds linking the two antigen-binding domains is formed by linking amino acid

residues present at the same position on the first antigen-binding domain and the second antigen-binding domain with each other.

[18] The antigen-binding molecule of any one of [1] to [16], wherein at least one of the bonds linking the two antigen-binding domains is formed by linking amino acid residues present at different positions on the first antigen-binding domain and the second antigen-binding domain with each other.

[19] The antigen-binding molecule of any one of [1] to [18], wherein at least one of the first and second antigen-binding domains comprises an antibody fragment which binds to a particular antigen.

[20] The antigen-binding molecule of [19], wherein the antibody fragment is a Fab, Fab', scFab, Fv, scFv, or single domain antibody.

[21] The antigen-binding molecule of [19] or [20], wherein at least one of amino acid residues from which the bonds between the antigen-binding domains originate is present within the antibody fragment.

[22] The antigen-binding molecule of [21], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present within a constant region.

[23] The antigen-binding molecule of [22], wherein the constant region is derived from human.

[24] The antigen-binding molecule of [22] or [23], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present within a CH1 region.

[25] The antigen-binding molecule of [24], wherein the subclass of the CH1 region is gamma 1, gamma 2, gamma 3, gamma 4, alpha 1, alpha 2, mu, delta, or epsilon.

[26] The antigen-binding molecule of [24] or [25], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present at any one of positions 119 to 123, 131 to 140, 148 to 150, 155 to 167, 174 to 178, 188 to 197, 201 to 214, and 218 to 219, according to EU numbering, in the CH1 region.

[27] The antigen-binding molecule of [26], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present at a position selected from the group consisting of positions 119, 122, 123, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 148, 150, 155, 156, 157, 159, 160, 161, 162, 163, 164, 165, 167, 174, 176, 177, 178, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 201, 203, 205, 206, 207, 208, 211, 212, 213, 214, 218, and 219, according to EU numbering, in the CH1 region.

[28] The antigen binding molecule of [27], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present at position 134, 135, 136, 137, 191, 192, 193, 194, 195, or 196, according to EU numbering, in the

CH1 region.

[29] The antigen binding molecule of [28], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present at position 135, 136, or 191, according to EU numbering, in the CH1 region.

[30] The antigen binding molecule of any one of [24] to [29], wherein at least one of the bonds linking the two antigen-binding domains is formed by linking an amino acid residue in the CH1 region of the first antigen-binding domain with an amino acid residue in the CH1 region of the second antigen-binding domain.

[31] The antigen binding molecule of [30], wherein the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 119, 120, 121, 122, and 123 according to EU numbering.

[32] The antigen binding molecule of [30], wherein the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 131, 132, 133, 134, 135, 136, 137, 138, 139, and 140 according to EU numbering.

[33] The antigen binding molecule of [30], wherein the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 148, 149, and 150 according to EU numbering.

[34] The antigen binding molecule of [30], wherein the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, and 167 according to EU numbering.

[35] The antigen binding molecule of [30], wherein the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 174, 175, 176, 177, and 178 according to EU numbering.

[36] The antigen binding molecule of [30], wherein the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 188, 189, 190, 191, 192, 193, 194, 195, 196, and 197 according to EU numbering.

[37] The antigen binding molecule of [30], wherein the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, and 214 according to EU numbering.

[38] The antigen binding molecule of [30], wherein the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each inde-

pendently selected from the group consisting of positions 218 and 219 according to EU numbering.

[39] The antigen binding molecule of any one of [30] to [38], wherein the difference between the positions of the amino acid residues in the first antigen-binding domain and the second antigen-binding domain is three amino acids or less.

[40] The antigen-binding molecule of [39], wherein at least one of the bonds linking the two antigen-binding domains is formed by linking an amino acid residue at position 135 according to EU numbering in the CH1 region of the first antigen-binding domain with an amino acid residue at any one of positions 132 to 138 according to EU numbering in the CH1 region of the second antigen-binding domain.

[41] The antigen-binding molecule of [39], wherein at least one of the bonds linking the two antigen-binding domains is formed by linking an amino acid residue at position 136 according to EU numbering in the CH1 region of the first antigen-binding domain with an amino acid residue at any one of positions 133 to 139 according to EU numbering in the CH1 region of the second antigen-binding domain.

[42] The antigen-binding molecule of [39], wherein at least one of the bonds linking the two antigen-binding domains is formed by linking an amino acid residue at position 191 according to EU numbering in the CH1 region of the first antigen-binding domain with an amino acid residue at any one of positions 188 to 194 according to EU numbering in the CH1 region of the second antigen-binding domain.

[43] The antigen-binding molecule of [40], wherein at least one of the bonds linking the two antigen-binding domains is formed by linking amino acid residues at position 135 according to EU numbering in the CH1 region of the two antigen-binding domains with each other.

[44] The antigen-binding molecule of [41], wherein at least one of the bonds linking the two antigen-binding domains is formed by linking amino acid residues at position 136 according to EU numbering in the CH1 region of the two antigen-binding domains with each other.

[45] The antigen-binding molecule of [42], wherein at least one of the bonds linking the two antigen-binding domains is formed by linking amino acid residues at position 191 according to EU numbering in the CH1 region of the two antigen-binding domains with each other.

[45A] The antigen-binding molecule of [42], wherein one disulfide bond is formed between the amino acid residues at position 191 according to EU numbering in the respective CH1 regions of the first antigen-binding domain and the second antigen-binding domain.

[45B] The antigen-binding molecule of [45A], wherein additional one, two or more disulfide bond(s) is/are formed between the first antigen-binding domain and the

second antigen-binding domain via the amino acid residues at the following positions according to EU numbering in each of the respective CH1 regions of the first antigen-binding domain and the second antigen-binding domain:

(a) between amino acid residues at any position of 131 to 138, 194 and 195 in each of the two antigen-binding domains;

(b) between the amino acid residues at position 131 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;

(c) between the amino acid residues at position 132 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;

(d) between the amino acid residues at position 133 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;

(e) between the amino acid residues at position 134 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;

(f) between the amino acid residues at position 135 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;

(g) between the amino acid residues at position 136 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;

(h) between the amino acid residues at position 137 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;

(i) between the amino acid residues at position 138 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;

(j) between the amino acid residues at position 131 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;

(k) between the amino acid residues at position 132 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;

(l) between the amino acid residues at position 133 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;

(m) between the amino acid residues at position 134 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;
(n) between the amino acid residues at position 135 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;
(o) between the amino acid residues at position 136 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;
(p) between the amino acid residues at position 137 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains; and
(q) between the amino acid residues at position 138 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains.

[45C] The antigen-binding molecule of [45A] or [45B], wherein any one of the first and second antigen-binding domains comprises one, two or more charged amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more oppositely charged amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

[45D] The antigen-binding molecule of [45A] or [45B], wherein any one of the first and second antigen-binding domains comprises one, two or more positively charged amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more negatively charged amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

[45E] The antigen-binding molecule of [45A] or [45B], wherein any one of the first and second antigen-binding domains comprises one, two or more negatively charged amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more positively charged amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

[45F] The antigen-binding molecule of [45A] or [45B], wherein any one of the first and second antigen-binding domains comprises one, two or more of the following amino acid residues in the respective CH1 region (according to EU numbering):
(a) the amino acid residue at position 136 is glutamic acid (E) or aspartic acid (D);
(b) the amino acid residue at position 137 is glutamic acid (E) or aspartic acid (D);

(c) the amino acid residue at position 138 is glutamic acid (E) or aspartic acid (D); and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more of the following amino acid residues in the respective CH1 region (according to EU numbering):
(d) the amino acid residue at position 193 is lysine (K), arginine (R), or histidine (H);
(e) the amino acid residue at position 194 is lysine (K), arginine (R), or histidine (H); and
(f) the amino acid residue at position 195 is lysine (K), arginine (R), or histidine (H).

[45G-1] The antigen-binding molecule of [45A] or [45B], wherein any one of the first and second antigen-binding domains comprises one or more of the following amino acid residues in the respective CH1 region (according to EU numbering):
(a) the amino acid residue at position 136 is lysine (K), arginine (R), or histidine (H);
(b) the amino acid residue at position 137 is lysine (K), arginine (R), or histidine (H);
(c) the amino acid residue at position 138 is lysine (K), arginine (R), or histidine (H); and
the other antigen-binding domain out of the first and second antigen-binding domains comprises one or more of the following amino acid residues in the respective CH1 region (according to EU numbering):
(d) the amino acid residue at position 193 is glutamic acid (E) or aspartic acid (D);
(e) the amino acid residue at position 194 is glutamic acid (E) or aspartic acid (D); and
(f) the amino acid residue at position 195 is glutamic acid (E) or aspartic acid (D).

[45G-2] The antigen-binding molecule of [45A] or [45B], wherein each of the first and second antigen-binding domains comprises any of the specific charged mutation combination in the respective CH1 region (according to EU numbering) as listed in Tables 7, Table 82 or Table 85.

[45H] The antigen-binding molecule of [45A] or [45B], wherein any one of the first and second antigen-binding domains comprises one, two or more hydrophobic amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more hydrophobic amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

[45I-1] The antigen-binding molecule of [45H], wherein said hydrophobic amino acid residue(s) is/are alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), and/or tryptophan (Trp).

[45I-2] The method of [45A] or [45B], wherein each of the first and second antigen-binding domains comprises any of the specific hydrophobic amino acid mutation combination in the respective CH1 region (according to EU numbering) as listed in Table 10.

[45J] The antigen-binding molecule of [45A] or [45B], wherein any one of the first and second antigen-binding domains comprises one "knob" amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more "hole" amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

[45K] The antigen-binding molecule of [45A] or [45B], wherein any one of the first and second antigen-binding domains comprises one, two or more "hole" amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one "knob" amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

[45L] The antigen-binding molecule of [45J] or [45K], wherein said "knob" amino acid residue(s) is/are selected from the group consisting of tryptophan (Trp) and phenylalanine (Phe); and said "hole" amino acid residue(s) is/are selected from the group consisting of alanine (Ala), valine (Val), threonine (T) or serine (S).

[45M] The antigen-binding molecule of [45A] or [45B], wherein any one of the first and second antigen-binding domains comprises one, two or more aromatic amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more positively charged amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

[45N] The antigen-binding molecule of [45A] or [45B], wherein any one of the first and second antigen-binding domains comprises one, two or more positively charged amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more aromatic amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

[45O] The antigen-binding molecule of [45M] or [45N], wherein said aromatic amino acid residue(s) is/are selected from the group consisting of tryptophan (Trp), tyrosine (Tyr), histidine (His), and phenylalanine (Phe); and said positively charged amino acid residue(s) is/are selected from a group consisting of lysine (K), arginine (R), or histidine (H).

[46] The antigen-binding molecule of [22] or [23], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present within a CL region.

[47] The antigen-binding molecule of [46], wherein the subclass of the CL region is kappa or lambda.

[48] The antigen-binding molecule of [46] or [47], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present at any one of positions 108 to 112, 121 to 128, 151 to 156, 184 to 190, 195 to 196, 200 to 203, and 208 to 213, according to Kabat numbering, in the CL region.

[49] The antigen-binding molecule of [48], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present at a position selected from the group consisting of positions 108, 109, 112, 121, 123, 126, 128, 151, 152, 153, 156, 184, 186, 188, 189, 190, 195, 196, 200, 201, 202, 203, 208, 210, 211, 212, and 213 according to Kabat numbering in the CL region.

[50] The antigen-binding molecule of [49], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present at position 126 according to Kabat numbering in the CL region.

[51] The antigen-binding molecule of any one of [46] to [50], wherein at least one of the bonds linking the two antigen-binding domains is formed by linking an amino acid residue in the CL region of the first antigen-binding domain with an amino acid residue in the CL region of the second antigen-binding domain.

[52] The antigen-binding molecule of [51], wherein the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 108, 109, 110, 111, and 112 according to Kabat numbering.

[53] The antigen-binding molecule of [51], wherein the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 121, 122, 123, 124, 125, 126, 127, and 128 according to Kabat numbering.

[54] The antigen-binding molecule of [51], wherein the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 151, 152, 153, 154, 155, and 156 according to Kabat numbering.

[55] The antigen-binding molecule of [51], wherein the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 184, 185, 186, 187, 188, 189, and 190 according to Kabat numbering.

[56] The antigen-binding molecule of [51], wherein the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 195 and 196 according to Kabat numbering.

[57] The antigen-binding molecule of [51], wherein the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each inde-

pendently selected from the group consisting of positions 200, 201, 202, and 203 according to Kabat numbering.

[58] The antigen-binding molecule of [51], wherein the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 208, 209, 210, 211, 212, and 213 according to Kabat numbering.

[59] The antigen-binding molecule of any one of [51] to [58], wherein the difference between the positions of the amino acid residues in the first antigen-binding domain and the second antigen-binding domain is three amino acids or less

[60] The antigen-binding molecule of [59], wherein at least one of the bonds linking the two antigen-binding domains is formed by linking amino acid residues at position 126 according to Kabat numbering in the CL region of the two antigen-binding domains with each other.

[61] The antigen-binding molecule of any one of [24] to [29] and [46] to [50], wherein at least one of the bonds linking the two antigen-binding domains is formed by linking an amino acid residue in the CH1 region of the first antigen-binding domain with an amino acid residue in the CL region of the second antigen-binding domain.

[62] The antigen-binding molecule of [61], wherein the amino acid residue in the CH1 region is selected from the group consisting of positions 188, 189, 190, 191, 192, 193, 194, 195, 196, and 197 according to EU numbering, and the amino acid residue in the CL region is selected from the group consisting of positions 121, 122, 123, 124, 125, 126, 127, and 128 according to Kabat numbering.

[63] The antigen-binding molecule of [62], wherein at least one of the bonds linking the two antigen-binding domains is formed by linking an amino acid residue at position 191 according to EU numbering in the CH1 region of the first antigen-binding domain with an amino acid residue at position 126 according to Kabat numbering in the CL region of the second antigen-binding domain.

[64] The antigen-binding molecule of [21], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present within a variable region.

[65] The antigen-binding molecule of [64], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present within a VH region.

[66] The antigen-binding molecule of [65], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present at a position selected from the group consisting of positions 6, 8, 16, 20, 25, 26, 28, 74, and 82b according to Kabat numbering in the VH region.

[67] The antigen-binding molecule of [64], wherein the amino acid residue from which

the bonds between the antigen-binding domains originate is present within a VL region.

[68] The antigen-binding molecule of [67], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present at a position selected from the group consisting of positions 21, 27, 58, 77, 100, 105, and 107 according to Kabat numbering in the VL region (subclass kappa).

[69] The antigen-binding molecule of [67], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present at a position selected from the group consisting of positions 6, 19, 33, and 34 according to Kabat numbering in the VL region (subclass lambda).

[70] The antigen-binding molecule of [64], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present within a VHH region.

[71] The antigen-binding molecule of [70], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present at a position selected from the group consisting of positions 4, 6, 7, 8, 9, 10, 11, 12, 14, 15, 17, 20, 24, 27, 29, 38, 39, 40, 41, 43, 44, 45, 46, 47, 48, 49, 67, 69, 71, 78, 80, 82, 82c, 85, 88, 91, 93, 94, and 107 according to Kabat numbering in the VHH region.

[72] The antigen-binding molecule of any one of [1] to [18], wherein at least one of the first and second antigen-binding domains comprises a non-antibody protein binding to a particular antigen, or a fragment thereof.

[73] The antigen-binding molecule of [72], wherein the non-antibody protein is either of a pair of a ligand and a receptor which specifically bind to each other.

[74] The antigen-binding molecule of any one of [1] to [73], wherein the antigen-binding domains comprise a hinge region.

[75] The antigen-binding molecule of [74], wherein at least one of cysteine residues present within the wild-type hinge region is substituted with another amino acid residue.

[76] The antigen-binding molecule of [75], wherein the cysteine residue is present at positions 226 and/or 229 according to EU numbering in the hinge region.

[77] The antigen-binding molecule of [74] or [76], wherein at least one of amino acid residues from which the bonds between the antigen-binding domains originate is present within the hinge region.

[78] The antigen-binding molecule of [77], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present at a position selected from the group consisting of positions 216, 218, and 219 according to EU numbering in the hinge region.

[79] The antigen-binding molecule of any one of [1] to [78], wherein the first antigen-

binding domain and the second antigen-binding domain are linked with each other via two or more bonds.

[80] The antigen-binding molecule of [79], wherein at least one of amino acid residues from which the bonds between the antigen-binding domains originate is an amino acid residue present in a wild-type sequence.

[81] The antigen-binding molecule of [80], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is present within a hinge region.

[82] The antigen-binding molecule of [81], wherein the amino acid residue from which the bonds between the antigen-binding domains originate is a cysteine residue in the hinge region.

[83] The antigen-binding molecule of any one of [80] to [82], wherein at least one of the bonds linking the two antigen-binding domains is a disulfide bond formed by crosslinking of cysteine residues present within the hinge region with each other.

[84] The antigen-binding molecule of [83], wherein the cysteine residues are present at positions 226 and/or 229 according to EU numbering in the hinge region.

[85] The antigen-binding molecule of any one of [79] to [84], wherein at least one of amino acid residues from which the bonds between the antigen-binding domains originate is present within the antibody fragment, and at least one of the amino acid residues is present within the hinge region.

[86] The antigen-binding molecule of [85], wherein the first and second antigen-binding domains each comprise a Fab and a hinge region, and wherein the antigen-binding molecule comprising the two antigen-binding domains is F(ab')2.

[87] The antigen-binding molecule of any one of [1] to [86], wherein the antigen-binding domains comprise an Fc region.

[88] The antigen-binding molecule of [87], wherein one or more amino acid mutations promoting multimerization of Fc regions are introduced into the Fc region.

[89] The antigen-binding molecule of [88], wherein the amino acid mutations promoting the multimerization comprise an amino acid mutation at at least one position selected from the group consisting of positions 247, 248, 253, 254, 310, 311, 338, 345, 356, 359, 382, 385, 386, 430, 433, 434, 436, 437, 438, 439, 440, and 447 according to EU numbering.

[90] The antigen-binding molecule of [88] or [89], wherein the multimerization is hexamerization.

[91] The antigen-binding molecule of any one of [87] to [90], which is a full-length antibody.

[0018] In another aspect, the present invention also provides the following:

[92] The antigen-binding molecule of any one of [1] to [91], wherein both the first

and second antigen-binding domains bind to the same antigen.
[93] The antigen-binding molecule of [92], wherein both the first and second antigen-binding domains bind to the same epitope on said antigen.
[94] The antigen-binding molecule of [92], wherein each of the first and second antigen-binding domains binds to a different epitope on said antigen.
[95] The antigen-binding molecule of any one of [1] to [91], wherein each of the first and second antigen-binding domains binds to a different antigen.
[96] The antigen-binding molecule of [93], wherein both the first and second antigen-binding domains have the same amino acid sequence.
[97] The antigen-binding molecule of any one of [93] to [95], wherein each of the first and second antigen-binding domains has a different amino acid sequence.
[98] The antigen-binding molecule of any one of [1] to [91], wherein at least one of two antigens to which the first and second antigen-binding domains bind is a soluble protein.
[99] The antigen-binding molecule of any one of [1] to [91], wherein at least one of two antigens to which the first and second antigen-binding domains bind is a membrane protein.
In another aspect, the present invention also provides the following:
[100] The antigen-binding molecule of any one of [1] to [99], which has activity of regulating interaction between two antigen molecules.
[101] The antigen-binding molecule of [100], which is capable of enhancing or diminishing interaction between two antigen molecules as compared to a control antigen-binding molecule, wherein the control antigen-binding molecule differs from the antigen-binding molecule of [100] only in that the control antigen-binding molecule has one less bond between the two antigen-binding domains.
[102] The antigen-binding molecule of [100] or [101], wherein the two antigen molecules are a ligand and a receptor thereof, respectively, and wherein the antigen-binding molecule has activity of promoting activation of the receptor by the ligand.
[103] The antigen-binding molecule of [100] or [101], wherein the two antigen molecules are an enzyme and a substrate thereof, respectively, and wherein the antigen-binding molecule has activity of promoting catalytic reaction of the enzyme with the substrate.
[104] The antigen-binding molecule of [100] or [101], wherein both of the two antigen molecules are proteins present on cellular surfaces, and wherein the antigen-binding molecule has activity of promoting interaction between a cell expressing the first antigen and a cell expressing the second antigen.
[105] The antigen-binding molecule of [104], wherein the cell expressing the first antigen is a cell with cytotoxic activity, and the cell expressing the second antigen is a

target cell thereof, and wherein the antigen-binding molecule promotes damage of said target cell by said cell with cytotoxic activity.

[106] The antigen-binding molecule of [105], wherein the cell with cytotoxic activity is a T cell, NK cell, monocyte, or macrophage.

[107] The antigen-binding molecule of any one of [1] to [99], which has activity of regulating activation of two antigen molecules which are activated by association with each other.

[108] The antigen-binding molecule of [107], which enhances or diminishes activation of two antigen molecules as compared to a control antigen-binding molecule, wherein the control antigen-binding molecule differs from the antigen-binding molecule of [107] only in that the control antigen-binding molecule has one less bond between the two antigen-binding domains.

[109] The antigen-binding molecule of [107] or [108], wherein the antigen molecules are selected from the group consisting of receptors belonging to cytokine receptor superfamilies, G protein-coupled receptors, ion channel receptors, tyrosine kinase receptors, immune checkpoint receptors, antigen receptors, CD antigens, costimulatory molecules, and cell adhesion molecules.

[110] The antigen-binding molecule of any one of [1] to [99], which has activity of holding two antigen molecules at spatially close positions.

[111] The antigen-binding molecule of [110], which is capable of holding two antigen molecules at closer positions than a control antigen-binding molecule, wherein the control antigen-binding molecule differs from the antigen-binding molecule of [110] only in that the control antigen-binding molecule has one less bond between the two antigen-binding domains.

[112] The antigen-binding molecule of any one of [1] to [99], wherein the two antigen-binding domains are at spatially close positions and/or the mobility of the two antigen-binding domains is reduced.

[113] The antigen-binding molecule of [112], wherein the two antigen-binding domains are at closer positions and/or the two antigen-binding domains have less mobility than a control antigen-binding molecule, wherein the control antigen-binding molecule differs from the antigen-binding molecule of [112] only in that the control antigen-binding molecule has one less bond between the two antigen-binding domains.

[114] The antigen-binding molecule of any one of [1] to [99], which has resistance to protease cleavage.

[115] The antigen-binding molecule of [114], which has increased resistance to protease cleavage as compared to a control antigen-binding molecule, wherein the control antigen-binding molecule differs from the antigen-binding molecule of [114] only in that the control antigen-binding molecule has one less bond between the two

antigen-binding domains.

[116] The antigen-binding molecule of [115], wherein the proportion of the full-length molecule remaining after protease treatment is increased as compared to the control antigen-binding molecule.

[117] The antigen-binding molecule of [115] or [116], wherein the proportion of a particular fragment produced after protease treatment is reduced as compared to the control antigen-binding molecule.

[118] The antigen-binding molecule of any one of [1] to [99], wherein when the molecule is treated with a protease, a dimer of the antigen-binding domains or fragments thereof is excised.

[119] The antigen-binding molecule of [118], wherein when the control antigen-binding molecule is treated with said protease, monomers of the antigen-binding domains or fragments thereof are excised, and wherein the control antigen-binding molecule differs from the antigen-binding molecule of [118] only in that the control antigen-binding molecule has one less bond between the two antigen-binding domains.

[120] The antigen-binding molecule of [118] or [119], wherein the protease cleaves the hinge region.

[121] The antigen binding molecule of any one of [101] to [106], [108] to [109], [111], [113], [115] to [117], and [119] to [120], wherein the one less bond is a bond formed originating from a mutated amino acid residue.

[122] The antigen-binding molecule of [121], wherein the mutated amino acid residue is a cysteine residue.

[0019] In another aspect, the present invention also provides the following:

[123] A pharmaceutical composition comprising the antigen-binding molecule of any one of [1] to [122] and a pharmaceutically acceptable carrier.

[0020] In another aspect, the present invention also provides the following:

[124] A method for regulating interaction between two antigen molecules, comprising:

(a) providing an antigen-binding molecule comprising two antigen-binding domains,

(b) adding to the antigen-binding molecule at least one bond which links the two antigen-binding domains with each other, and

(c) contacting the antigen-binding molecule produced in (b) with the two antigen molecules.

[125] A method for regulating activity of two antigen molecules which are activated by association with each other, comprising:

(a) providing an antigen-binding molecule comprising two antigen-binding domains,

(b) adding to the antigen-binding molecule at least one bond which links the two antigen-binding domains with each other, and

(c) contacting the antigen-binding molecule produced in (b) with the two antigen molecules.

[126] A method for holding two antigen molecules at spatially close positions, comprising:

(a) providing an antigen-binding molecule comprising two antigen-binding domains,

(b) adding to the antigen-binding molecule at least one bond which links the two antigen-binding domains with each other, and

(c) contacting the antigen-binding molecule produced in (b) with the two antigen molecules.

[127] A method for placing two antigen-binding domains at spatially close positions and/or reducing the mobility of the two antigen-binding domains, comprising:

(a) providing an antigen-binding molecule comprising two antigen-binding domains, and

(b) adding to the antigen-binding molecule at least one bond which links the two antigen-binding domains with each other.

[128] A method for increasing resistance of an antigen-binding molecule to protease cleavage, comprising:

(a) providing an antigen-binding molecule comprising two antigen-binding domains, and

(b) adding to the antigen-binding molecule at least one bond which links the two antigen-binding domains with each other.

[0021] In another aspect, the present invention also provides the following:

[129] A method for producing an antigen-binding molecule which has activity of regulating interaction between two antigen molecules, comprising:

(a) providing a nucleic acid encoding a polypeptide comprising a first antigen-binding domain and a nucleic acid encoding a polypeptide comprising a second antigen-binding domain,

(b) introducing a mutation into the nucleic acids encoding the two antigen-binding domains such that at least one bond linking the two antigen-binding domains is added,

(c) introducing the nucleic acids produced in (b) into a host cell,

(d) culturing the host cell such that the two polypeptides are expressed, and

(e) obtaining an antigen-binding molecule which is a polypeptide comprising the first and second antigen-binding domains, wherein the two antigen-binding domains are linked with each other via one or more bonds.

[130] A method for producing an antigen-binding molecule which has activity of regulating activation of two antigen molecules which are activated by association with each other, comprising:

(a) providing a nucleic acid encoding a polypeptide comprising a first antigen-

binding domain and a nucleic acid encoding a polypeptide comprising a second antigen-binding domain,
(b) introducing a mutation into the nucleic acids encoding the two antigen-binding domains such that at least one bond linking the two antigen-binding domains is added,
(c) introducing the nucleic acids produced in (b) into a host cell,
(d) culturing the host cell such that the two polypeptides are expressed, and
(e) obtaining an antigen-binding molecule which is a polypeptide comprising the first and second antigen-binding domains, wherein the two antigen-binding domains are linked with each other via one or more bonds.

[131] A method for producing an antigen-binding molecule which has activity of holding two antigen molecules at spatially close positions, comprising:
(a) providing a nucleic acid encoding a polypeptide comprising a first antigen-binding domain and a nucleic acid encoding a polypeptide comprising a second antigen-binding domain,
(b) introducing a mutation into the nucleic acids encoding the two antigen-binding domains such that at least one bond linking the two antigen-binding domains is added,
(c) introducing the nucleic acids produced in (b) into a host cell,
(d) culturing the host cell such that the two polypeptides are expressed, and
(e) obtaining an antigen-binding molecule which is a polypeptide comprising the first and second antigen-binding domains, wherein the two antigen-binding domains are linked with each other via one or more bonds.

[132] A method for producing an antigen-binding molecule in which two antigen-binding domains are present at spatially close positions and/or the mobility of the two antigen binding domains is reduced, comprising:
(a) providing a nucleic acid encoding a polypeptide comprising a first antigen-binding domain and a nucleic acid encoding a polypeptide comprising a second antigen-binding domain,
(b) introducing a mutation into the nucleic acids encoding the two antigen-binding domains such that at least one bond linking the two antigen-binding domains is added,
(c) introducing the nucleic acids produced in (b) into a host cell,
(d) culturing the host cell such that the two polypeptides are expressed, and
(e) obtaining an antigen-binding molecule which is a polypeptide comprising the first and second antigen-binding domains, wherein the two antigen-binding domains are linked with each other via one or more bonds.

[133] A method for producing an antigen-binding molecule which has increased resistance to protease cleavage, comprising:
(a) providing a nucleic acid encoding a polypeptide comprising a first antigen-binding domain and a nucleic acid encoding a polypeptide comprising a second antigen-

binding domain,

(b) introducing a mutation into the nucleic acids encoding the two antigen-binding domains such that at least one bond linking the two antigen-binding domains is added,

(c) introducing the nucleic acids produced in (b) into a host cell,

(d) culturing the host cell such that the two polypeptides are expressed, and

(e) obtaining an antigen-binding molecule which is a polypeptide comprising the first and second antigen-binding domains, wherein the two antigen-binding domains are linked with each other via one or more bonds.

[0022] In another aspect, the present invention also provides the following:

[134] A method for identifying a novel pair of protein molecules which are activated by association with each other, comprising:

(a) providing two arbitrary protein molecules,

(b) producing, by the method of any one of [129] to [133], an antigen-binding molecule comprising two antigen-binding domains which respectively bind to the two protein molecules,

(c) contacting the antigen-binding molecule produced in (b) with the two protein molecules, and

(d) assessing whether or not the two protein molecules are activated.

[135] The method of [134], wherein at least one of the protein molecules is selected from the group consisting of receptors belonging to cytokine receptor superfamilies, G protein-coupled receptors, ion channel receptors, tyrosine kinase receptors, immune checkpoint receptors, antigen receptors, CD antigens, costimulatory molecules, and cell adhesion molecules.

Brief Description of Drawings

[0023] [fig.1]Fig. 1 shows a non-reducing SDS-PAGE gel image for analyzing OKT3 and its variants with the cysteine substitution (see Example 1). Two broken lines indicate upper and lower bands. The lower band can be considered to correspond to the antibody having one or more engineered disulfide bond(s) formed between the CH1 regions.

[fig.2]Fig. 2 shows a non-reducing SDS-PAGE gel image for analyzing OKT3 variants with the cysteine substitution and OKT3-KiH (see Example 1). Two broken lines indicate upper and lower bands.

[fig.3]Fig. 3 shows a non-reducing SDS-PAGE gel image for analyzing OKT3-KiH variants with the cysteine substitution (see Example 1). Two broken lines indicate upper and lower bands.

[fig.4]Fig. 4 shows a non-reducing SDS-PAGE gel image for analyzing OKT3-KiH variants with the cysteine substitution (see Example 1). Two broken lines indicate

upper and lower bands.

[fig.5]Fig. 5 shows an image of non-reducing SDS-PAGE gel in which 2-MEA concentrations of each sample are described (left panel); and a graph showing the lower band to upper band ratio (crosslinking ratio or crosslinking %) of each sample (right panel) (see Example 4). 20 mg/mL of the antibody was reacted by mixing with 2-MEA of different concentrations. The leftmost bar and dotted line represent the lower band to upper band ratio (crosslinking ratio or crosslinking %) of the control (0 mM 2-MEA). Numbers in the bars are the values of the lower band to upper band ratios (crosslinking ratio or crosslinking %).

[fig.6]Fig. 6 shows an image of non-reducing SDS-PAGE gel in which 2-MEA concentrations of each sample are described (upper panel); and a graph showing the lower band to upper band ratio (crosslinking ratio or crosslinking %) of each sample (lower panel) (see Example 4). 20 mg/mL of the antibody was reacted by mixing with 2-MEA of different concentrations. The leftmost bar and dotted line represent the lower band to upper band ratio (crosslinking ratio or crosslinking %) of the control (0 mM 2-MEA). Numbers in the bars are the values of the lower band to upper band ratio (crosslinking ratio or crosslinking %).

[fig.7]Fig. 7 shows an image of non-reducing SDS-PAGE gel in which 2-MEA concentrations of each sample are described (left panel); and a graph showing the lower band to upper band ratio (crosslinking ratio or crosslinking %) of each sample (right panel) (see Example 4). 1 mg/mL of the antibody was reacted by mixing with 2-MEA of different concentrations. The leftmost bar and dotted line represent the lower band to upper band ratio (crosslinking ratio or crosslinking %) of the control (0 mM 2-MEA). Numbers in the bars are the value of the lower band to upper band ratio (crosslinking ratio or crosslinking %).

[fig.8]Fig. 8 shows an image of non-reducing SDS-PAGE gel in which 2-MEA concentrations of each sample are described (upper panel); and a graph showing the lower band to upper band ratio (crosslinking ratio or crosslinking %) of each sample (lower panel) (see Example 4). 1 mg/mL of the antibody was reacted by mixing with 2-MEA of different concentrations. The leftmost bar and dotted line represent the lower band to upper band ratio (crosslinking ratio or crosslinking %) of the control (0 mM 2-MEA). Numbers in the bars are the values of the lower band to upper band ratio (crosslinking ratio or crosslinking %).

[fig.9]Fig. 9 shows an image of non-reducing SDS-PAGE gel in which TCEP concentrations of each sample are described (left panel); and a graph showing the lower band to upper band ratio (crosslinking ratio or crosslinking %) of each sample (right panel) (see Example 5). 20 mg/mL of the antibody was reacted by mixing with TCEP of different concentrations. The leftmost bar and dotted line represent the lower band to

upper band ratio (crosslinking ratio or crosslinking %) of the control (0 mM TCEP). Numbers in the bars are the values of the lower band to upper band ratio (crosslinking ratio or crosslinking %).

[fig.10]Fig. 10 shows an image of non-reducing SDS-PAGE gel in which TCEP concentrations of each sample are described (upper panel); and a graph showing the lower band to upper band ratio (crosslinking ratio or crosslinking %) of each sample (lower panel) (see Example 5). 20 mg/mL of the antibody was reacted by mixing with TCEP of each concentration. N.D. means that no band was detected. The leftmost bar and dotted line represent the lower band to upper band ratio (crosslinking ratio or crosslinking %) of the control (0 mM TCEP). Numbers in the bars are the values of the lower band to upper band ratio (crosslinking ratio or crosslinking %).

[fig.11]Fig. 11 shows an image of non-reducing SDS-PAGE gel in which TCEP concentrations of each sample are described (upper panel); and a graph showing the lower band to upper band ratio (crosslinking ratio or crosslinking %) of each sample (lower panel) (see Example 5). 1 mg/mL of the antibody was reacted by mixing with TCEP of each concentration. N.D. means that no band was detected. The leftmost bar and dotted line represent the lower band to upper band ratio (crosslinking ratio or crosslinking %) of the control (0 mM TCEP). Numbers in the bars are the values of the lower band to upper band ratio (crosslinking ratio or crosslinking %).

[fig.12]Fig. 12 shows an image of non-reducing SDS-PAGE gel in which reagent concentrations of each sample are described (upper panel); and a graph showing the lower band to upper band ratio (crosslinking ratio or crosslinking %) of samples reacted with DTT (left) or Cysteine (right) (see Example 6). 20 mg/mL of the antibody was reacted by mixing with DTT or Cysteine of each concentration. The leftmost bar and dotted line represent the lower band to upper band ratio (crosslinking ratio or crosslinking %) of the control (without reducing agent). Numbers in the bars are the values of the lower band to upper band ratio (crosslinking ratio or crosslinking %).

[fig.13]Fig. 13 shows an image of non-reducing SDS-PAGE gel in which reagent concentrations of each sample are described (upper panel); and a graph showing the lower band to upper band ratio (crosslinking ratio or crosslinking %) of samples reacted with GSH (left) or Na_2SO_3 (right) (lower panel) (see Example 6). 20 mg/mL of the antibody was reacted by mixing with GSH or Na_2SO_3 of each concentration. The leftmost bar and dotted line represent the lower band to upper band ratio (crosslinking ratio or crosslinking %) of the control (without reducing agent). Numbers in the bars are the values of the lower band to upper band ratio (crosslinking ratio or crosslinking %).

[fig.14]Fig. 14 shows an image of non-reducing SDS-PAGE gel (see Example 7). 20 mg/mL of the antibody was reacted by mixing with 2-MEA or TCEP in pH 3, 4, and 5 conditions. Buffer pH of each sample is described in the figure. Lanes 3, 6 and 9:

without reducing agent. Lanes 4, 7 and 10: mixed with 1 mM 2-MEA. Lanes 5, 8, and 11: mixed with 0.25 mM TCEP.

[fig.15]Fig. 15 shows an image of non-reducing SDS-PAGE gel (see Example 7). 20 mg/mL of the antibody was reacted by mixing with 2-MEA or TCEP in pH 6, 7 and 8 conditions. Buffer pH of each sample is described in the figure. Lanes 3, 6 and 9: without reducing agent. Lanes 4, 7 and 10: mixed with 1 mM 2-MEA. Lanes 5, 8, and 11: mixed with 0.25 mM TCEP.

[fig.16]Fig. 16 shows a graph showing the lower band to upper band ratio (crosslinking ratio) of the antibody samples in Figures 14 and 15 (see Example 7). For each pH, the leftmost (white) bar represents the lower band to upper band ratio (crosslinking ratio) of the control (without reducing agent treatment). The middle (shaded) bars represent the lower band to upper band ratio (crosslinking ratio) of samples mixed with 1 mM 2-MEA. The rightmost (black) bars represent the lower band to upper band ratio (crosslinking ratio) of samples mixed with 0.25 mM TCEP. Numbers in the bars are the values of the lower band to upper band ratio (crosslinking ratio).

[fig.17]Fig. 17 shows a chromatogram of cation exchange chromatography performed on the OKT3.S191C antibody sample as described in Example 8-1.

[fig.18]Fig. 18 shows a gel image of the non-reducing SDS-PAGE analysis of the OKT3.S191C antibody sample separated by cation exchange chromatography as described in Example 8-1. Lanes 5 and 10: OKT3.S191C (non-fractionated). Lane 6: mixture of RA3 and RA4. Lane 7: mixture of RA5 and RA6. Lane 8: mixture of RA7 and RA8. Lane 9: mixture of RA9 and RA10.

[fig.19]Fig. 19 shows a chromatogram of cation exchange chromatography performed on the OKT3.S191C0110 antibody sample as described in Example 8-2.

[fig.20]Fig. 20 shows a gel image of the non-reducing SDS-PAGE analysis of the OKT3.S191C0110 antibody sample separated by cation exchange chromatography as described in Example 8-2. Lane 3: OKT3.S191C0110 (non-fractionated). Lane 4: mixture of RA4 and RA5. Lane 5: mixture of RA6 and RA7. Lane 6: mixture of RA8 and RA9. Lane 7: mixture of RA10 and RA11. Lane 8: mixture of RB11 and RB10. Lane 9: mixture of RB8 and RB7. Lane 10: mixture of RB6 and RB5. Lane 11: mixture of RB4 and RB3.

[fig.21]Fig. 21 depicts examples of modified antibodies in which the Fabs are crosslinked with each other as described in Reference Example 1. The figure schematically shows structural differences between a wild-type antibody (WT) and a modified antibody in which the CH1 regions of antibody H chain are crosslinked with each other (HH type), a modified antibody in which the CL regions of antibody L chain are crosslinked with each other (LL type), and a modified antibody in which the CH1 region of antibody H chain is crosslinked with the CL region of antibody L chain

(HL or LH type).

[fig.22]Fig. 22 shows the results of assaying the CD3-mediated agonist activity of a wild-type anti-CD3 epsilon antibody molecule (CD3-G4s) and modified antibody molecules produced by linking the Fab-Fab of the wild-type molecule via an additional disulfide bond (CD3-G4sLL, CD3-G4sHH), as described in Reference Example 4-3.

[fig.23]Fig. 23 shows the results of assaying the CD3-mediated agonist activity of a wild-type anti-CD3 epsilon antibody molecule (OKT3-G1s) and modified antibody molecules produced by linking the Fab-Fab of the wild-type molecule via an additional disulfide bond (OKT3-G1sLL, OKT3-G1sHH), as described in Reference Example 4-3.

[fig.24]Fig. 24 shows the results of assaying the CD3- and/or CD28-mediated agonist activity of a wild-type anti-CD3 epsilon antibody molecule (CD3-G1s), an anti-CD28 antibody molecule (CD28-G1s), and an anti-CD3 epsilon x anti-CD28 bispecific antibody (CD3//CD28-G1s), and modified antibody molecules produced by linking the Fab-Fab of the bispecific antibody via an additional disulfide bond (CD3//CD28-G1sLL, CD3//CD28-G1sHH, CD3//CD28-G1sLH, CD3//CD28-G1sHL), as described in Reference Example 4-3.

[fig.25]Fig. 25 shows the results of assaying the CD3- and/or CD28-mediated agonist activity of a wild-type anti-CD3 epsilon antibody molecule (OKT3-G1s), an anti-CD28 antibody molecule (CD28-G1s), and an anti-CD3 epsilon x anti-CD28 bispecific antibody (OKT3//CD28-G1s), and modified antibody molecules produced by linking the Fab-Fab of the bispecific antibody via an additional disulfide bond (OKT3//CD28-G1sHH, OKT3//CD28-G1sHL), as described in Reference Example 4-3.

[fig.26]Fig. 26 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the heavy chain variable region of the anti-IL6R antibody (MRAH.xxx-G1T4), and modified antibodies produced by introducing a cysteine substitution into the heavy chain constant region of the anti-IL6R antibody (MRAH-G1T4.xxx), as described in Reference Example 5-2 (1/8). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.27]Fig. 27 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the heavy chain variable region of the anti-IL6R antibody (MRAH.xxx-G1T4), and modified antibodies produced by introducing a cysteine substitution into the heavy chain constant region of the anti-IL6R antibody (MRAH-G1T4.xxx), as described in Reference Example 5-2 (2/8). Each protease-treated antibody was applied to non-

reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.28]Fig. 28 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the heavy chain variable region of the anti-IL6R antibody (MRAH.xxx-G1T4), and modified antibodies produced by introducing a cysteine substitution into the heavy chain constant region of the anti-IL6R antibody (MRAH-G1T4.xxx), as described in Reference Example 5-2 (3/8). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.29]Fig. 29 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the heavy chain variable region of the anti-IL6R antibody (MRAH.xxx-G1T4), and modified antibodies produced by introducing a cysteine substitution into the heavy chain constant region of the anti-IL6R antibody (MRAH-G1T4.xxx), as described in Reference Example 5-2 (4/8). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.30]Fig. 30 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the heavy chain variable region of the anti-IL6R antibody (MRAH.xxx-G1T4), and modified antibodies produced by introducing a cysteine substitution into the heavy chain constant region of the anti-IL6R antibody (MRAH-G1T4.xxx), as described in Reference Example 5-2 (5/8). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.31]Fig. 31 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the heavy chain variable region of the anti-IL6R antibody (MRAH.xxx-G1T4), and modified antibodies produced by introducing a cysteine substitution into the heavy chain constant region of the anti-IL6R antibody (MRAH-G1T4.xxx), as described in Reference Example 5-2 (6/8). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.32]Fig. 32 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the heavy chain variable region of the anti-IL6R antibody (MRAH.xxx-G1T4), and modified antibodies produced by introducing a cysteine substitution into the heavy

chain constant region of the anti-IL6R antibody (MRAH-G1T4.xxx), as described in Reference Example 5-2 (7/8). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.33]Fig. 33 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the heavy chain variable region of the anti-IL6R antibody (MRAH.xxx-G1T4), and modified antibodies produced by introducing a cysteine substitution into the heavy chain constant region of the anti-IL6R antibody (MRAH-G1T4.xxx), as described in Reference Example 5-2 (8/8). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.34]Fig. 34 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the light chain variable region of the anti-IL6R antibody (MRAL.xxx-k0), and modified antibodies produced by introducing a cysteine substitution into the light chain constant region of the anti-IL6R antibody (MRAL-k0.xxx), as described in Reference Example 6-2 (1/10). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.35]Fig. 35 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the light chain variable region of the anti-IL6R antibody (MRAL.xxx-k0), and modified antibodies produced by introducing a cysteine substitution into the light chain constant region of the anti-IL6R antibody (MRAL-k0.xxx), as described in Reference Example 6-2 (2/10). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.36]Fig. 36 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the light chain variable region of the anti-IL6R antibody (MRAL.xxx-k0), and modified antibodies produced by introducing a cysteine substitution into the light chain constant region of the anti-IL6R antibody (MRAL-k0.xxx), as described in Reference Example 6-2 (3/10). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.37]Fig. 37 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the light chain variable region of the anti-IL6R antibody (MRAL.xxx-k0), and modified antibodies produced by introducing a cysteine substitution into the light chain constant region of the anti-IL6R antibody (MRAL-k0.xxx), as described in Reference Example

6-2 (4/10). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.38]Fig. 38 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the light chain variable region of the anti-IL6R antibody (MRAL.xxx-k0), and modified antibodies produced by introducing a cysteine substitution into the light chain constant region of the anti-IL6R antibody (MRAL-k0.xxx), as described in Reference Example 6-2 (5/10). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.39]Fig. 39 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the light chain variable region of the anti-IL6R antibody (MRAL.xxx-k0), and modified antibodies produced by introducing a cysteine substitution into the light chain constant region of the anti-IL6R antibody (MRAL-k0.xxx), as described in Reference Example 6-2 (6/10). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.40]Fig. 40 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the light chain variable region of the anti-IL6R antibody (MRAL.xxx-k0), and modified antibodies produced by introducing a cysteine substitution into the light chain constant region of the anti-IL6R antibody (MRAL-k0.xxx), as described in Reference Example 6-2 (7/10). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.41]Fig. 41 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the light chain variable region of the anti-IL6R antibody (MRAL.xxx-k0), and modified antibodies produced by introducing a cysteine substitution into the light chain constant region of the anti-IL6R antibody (MRAL-k0.xxx), as described in Reference Example 6-2 (8/10). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.42]Fig. 42 shows the results of protease treatment of an anti-IL6R antibody (MRA), modified antibodies produced by introducing a cysteine substitution into the light chain variable region of the anti-IL6R antibody (MRAL.xxx-k0), and modified antibodies produced by introducing a cysteine substitution into the light chain constant region of the anti-IL6R antibody (MRAL-k0.xxx), as described in Reference Example 6-2 (9/10). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.43]Fig. 43 shows the results of protease treatment of an anti-IL6R antibody

(MRA), modified antibodies produced by introducing a cysteine substitution into the light chain variable region of the anti-IL6R antibody (MRAL.xxx-k0), and modified antibodies produced by introducing a cysteine substitution into the light chain constant region of the anti-IL6R antibody (MRAL-k0.xxx), as described in Reference Example 6-2 (10/10). Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody.

[fig.44]Fig. 44 shows the results of protease treatment of an anti-IL6R antibody (MRA) and a modified antibody produced by introducing a cysteine substitution into the light chain constant region of the anti-IL6R antibody (MRAL-k0.K126C), as described in Reference Example 7-2. Each protease-treated antibody was applied to non-reducing capillary electrophoresis, followed by band detection with an anti-kappa chain antibody or an anti-human Fc antibody.

[fig.45]Fig. 45 shows the correspondence between the molecular weight of each band obtained by protease treatment of the antibody sample and its putative structure, as described in Reference Example 7-2. It is also noted below the structure of each molecule whether the molecule may react with an anti-kappa chain antibody or an anti-Fc antibody (whether a band is detected in the electrophoresis of Fig. 44).

[fig.46]Fig. 46 shows the results of assaying the CD3-mediated agonist activity of an anti-CD3 antibody molecule (OKT3), modified antibody molecules produced by linking the Fab-Fab of that antibody molecule via an additional disulfide bond (H_T135C, H_S136C, H_S191C, and L_K126C), and an anti-KLH antibody molecule (IC17) (negative control), as described in Reference Example 13-4.

[fig.47]Fig. 47 shows the results of assaying the CD3-mediated agonist activity of an anti-CD3 antibody molecule (OKT3), a modified antibody molecule produced by introducing Knobs-into-Holes (KiH) modifications, which facilitate heterodimerization, into the heavy chain constant region of OKT3 (OKT3_KiH), modified antibody molecules produced by linking the Fab-Fab of that antibody molecule via an additional disulfide bond (H_S191C_KiH, H_S191C/V188C_KiH, H_S191C/P189C_KiH, H_S191C/S190C_KiH, H_S191C/S192C_KiH, H_S191C/L193C_KiH, H_S191C/G194C_KiH), and an anti-KLH antibody (IC17) (negative control), as described in Reference Example 14-4.

[fig.48]Fig. 48 shows the results of assaying the CD3-mediated agonist activity of an anti-CD3 antibody molecule (OKT3), a modified antibody molecule produced by linking the Fab-Fab of that antibody molecule via an additional disulfide bond (H_S191C), a modified antibody molecule produced by introducing Knobs-into-Holes (KiH) modifications, which facilitate heterodimerization, into the heavy chain constant region of OKT3 (OKT3_KiH), a modified antibody molecule produced by linking the Fab-Fab of that antibody molecule via an additional disulfide bond (H_S191C_KiH),

modified antibody molecules produced by introducing a positively-charged amino acid substitution into one of the heavy chain constant regions of OKT3_KiH and introducing a negatively-charged amino acid substitution into the other heavy chain constant region (0004//0004, 0004//0006), modified antibody molecules produced by introducing a positively- or negatively-charged amino acid substitution into one of the heavy chain constant regions of OKT3_KiH (0004//OKT3, OKT3//0004, OKT3//0006), and an anti-KLH antibody molecule (IC17) (negative control), as described in Reference Example 15-4.

[fig.49]Fig. 49 shows the results of assaying the CD3-mediated agonist activity of an anti-CD3 antibody molecule (OKT3), modified antibody molecules produced by removing a disulfide bond in the hinge region of that antibody molecule (dh1, dh2, dh3), modified antibody molecules produced by linking the Fab-Fab of those molecules via an additional disulfide bond (H_S191C_dh1, H_S191C_dh2, H_S191C_dh3), and an anti-KLH antibody molecule (IC17) (negative control) as described in Reference Example 16-4.

[fig.50]Fig. 50 shows the results of assaying the CD3-mediated agonist activity of an anti-CD3 monospecific antibody molecule (OKT3-G1s), a modified antibody molecule produced by linking the Fab-Fab of that antibody molecule via an additional disulfide bond (OKT3-G1sHH), a modified antibody molecule produced by linking the Fab-Fab of an anti-CD3 monospecific antibody (CD3-G1s) via an additional disulfide bond (CD3-G1sLL), an anti-CD3 biparatopic antibody molecule (CD3//OKT3-G1s), modified antibody molecules produced by linking the Fab-Fab of that antibody molecule via an additional disulfide bond (CD3//OKT3-G1sHH, CD3//OKT3-G1sLH), and a combination of CD3-G1sLL and OKT3-G1s (CD3-G1sLL+OKT3-G1s), as described in Reference Example 20.

[fig.51A]Fig. 51A shows the results of assaying the CD3- and/or PD1-mediated agonist activity of anti-CD3 x anti-PD1 bispecific antibodies and modified antibody molecules produced by linking the Fab-Fab of those antibodies via an additional disulfide bond, as described in Reference Example 22-1. Fig. 51A shows the agonist activity of an anti-CD3 x anti-PD1 bispecific antibody molecule (OKT3//117-G1silent) which is composed of an anti-CD3 antibody (OKT3) and an anti-PD1 antibody (117), and modified antibody molecules produced by linking the Fab-Fab of that antibody molecule via an additional disulfide bond (OKT3//117-G1silentHH, OKT3//117-G1silentHL, OKT3//117-G1silentLL).

[fig.51B]Fig. 51B shows the results of assaying the CD3- and/or PD1-mediated agonist activity of anti-CD3 x anti-PD1 bispecific antibodies and modified antibody molecules produced by linking the Fab-Fab of those antibodies via an additional disulfide bond, as described in Reference Example 22-1. Fig. 51B shows the agonist activity of an

anti-CD3 x anti-PD1 bispecific antibody molecule (OKT3//10-G1silent) which is composed of an anti-CD3 antibody (OKT3) and an anti-PD1 antibody (10), and modified antibody molecules produced by linking the Fab-Fab of that antibody molecule via an additional disulfide bond (OKT3//10-G1silentHH, OKT3//10-G1silentHL).

[fig.51C]Fig. 51C shows the results of assaying the CD3- and/or PD1-mediated agonist activity of anti-CD3 x anti-PD1 bispecific antibodies and modified antibody molecules produced by linking the Fab-Fab of those antibodies via an additional disulfide bond, as described in Reference Example 22-1. Fig. 51C shows the agonist activity of an anti-CD3 x anti-PD1 bispecific antibody molecule (CD3//949-G1silent) which is composed of an anti-CD3 antibody (CD3) and an anti-PD1 antibody (949), and modified antibody molecules produced by linking the Fab-Fab of that antibody molecule via an additional disulfide bond (CD3//949-G1silentLH, CD3//949-G1silentHH, CD3//949-G1silentLL, CD3//949-G1silentHL).

[fig.51D]Fig. 51D shows the results of assaying the CD3- and/or PD1-mediated agonist activity of anti-CD3 x anti-PD1 bispecific antibodies and modified antibody molecules produced by linking the Fab-Fab of those antibodies via an additional disulfide bond, as described in Reference Example 22-1. Fig. 51D shows the agonist activity of an anti-CD3 x anti-PD1 bispecific antibody molecule (OKT3//949-G1silent) which is composed of an anti-CD3 antibody (OKT3) and an anti-PD1 antibody (949), and modified antibody molecules produced by linking the Fab-Fab of that antibody molecule via an additional disulfide bond (OKT3//949-G1silentHL, OKT3//949-G1silentHH, OKT3//949-G1silentLL).

[fig.52]Fig. 52 shows the results of assaying the CD3- and/or PD1-mediated agonist activity of an anti-CD3 x anti-PD1 bispecific antibody molecule (OKT3//949-G1silent) which is composed of an anti-CD3 antibody (OKT3) and an anti-PD1 antibody (949), and modified antibody molecules produced by linking the Fab-Fab of that antibody molecule via an additional disulfide bond (OKT3//949-G1silentHH, OKT3//949-G1silentHL, OKT3//949-G1silentLH, OKT3//949-G1silentLL), as described in Reference Example 22-2.

[fig.53A]Fig. 53A shows the results of evaluating the T cell-dependent inhibitory effect on cancer cell growth when using a CD28/CD3 clamping bispecific antibody and a GPC3/binding-attenuated CD3 bispecific antibody in combination, as described in Reference Example 23-1. When the above-mentioned CD28/CD3 clamping bispecific antibody and GPC3/binding-attenuated CD3 bispecific antibody are combined and allowed to act in the presence of target cells (GPC3-expressing cancer cells) and effector cells (T cells), the GPC3/binding-attenuated CD3 bispecific antibody brings the target cell and the effector cell close together, and the CD28/CD3

clamping bispecific antibody activates the effector cell. Fig. 53A shows the inhibitory effect on cancer cell growth when a GPC3/binding-attenuated CD3 bispecific antibody molecule (GPC3/attCE115) was used as an antibody to target T cells to cancer cells, and a GPC3/ CD3 clamping bispecific antibody molecule (GPC3/clamp CD3), a KLH/ CD3 clamping bispecific antibody molecule (KLH/clamp CD3), a CD28/CD3 clamping bispecific antibody molecule (CD28/clamp CD3), or a modified antibody molecule produced by linking the Fab-Fab of that antibody molecule via an additional disulfide bond (CD28/clamp CD3_HH) was used as an antibody for activating T cells.
[fig.53B]Fig. 53B shows, as with Fig. 53A, the results of evaluating the T cell-dependent inhibitory effect on cancer cell growth when using a CD28/CD3 clamping bispecific antibody and a GPC3/binding-attenuated CD3 bispecific antibody in combination, as described in Reference Example 23-1. Fig. 53B shows the inhibitory effect on cancer cell growth when a modified antibody molecule produced by linking the Fab-Fab of the GPC3/binding-attenuated CD3 bispecific antibody via an additional disulfide bond (GPC3/attCE115_LL) was used as an antibody to target T cells to cancer cells, and a GPC3/CD3 clamping bispecific antibody molecule (GPC3/clamp CD3), a KLH/CD3 clamping bispecific antibody molecule (KLH/clamp CD3), a CD28/CD3 clamping bispecific antibody molecule (CD28/clamp CD3), or a modified antibody molecule produced by linking the Fab-Fab of that antibody molecule via an additional disulfide bond (CD28/clamp CD3_HH) was used as an antibody for activating T cells.
[fig.54A]Fig. 54A shows the results of evaluating cytokine production from T cells when a CD28/CD3 clamping bispecific antibody and a GPC3/binding-attenuated CD3 bispecific antibody were used in combination as described in Reference Example 23-2. When the above-mentioned CD28/CD3 clamping bispecific antibody and GPC3/binding-attenuated CD3 bispecific antibody are used in combination in the presence of target cells (GPC3-expressing cancer cells) and effector cells (T cells), the GPC3/binding-attenuated CD3 bispecific antibody brings the target cell and the effector cell close together, and the CD28/CD3 clamping bispecific antibody activates the effector cell. Fig. 54A shows the level of IL-6 production when a GPC3/binding-attenuated CD3 bispecific antibody molecule (GPC3/attCE115) and a modified antibody molecule produced by linking the Fab-Fab of the CD28/CD3 clamping bispecific antibody via an additional disulfide bond (CD28/clamp CD3_HH) were used each alone or in combination in the presence of target cells (GPC3-expressing cancer cells) and effector cells (T cells).
[fig.54B]Fig. 54B shows, as with Fig. 54A, the results of evaluating cytokine production from T cells when a CD28/CD3 clamping bispecific antibody and a GPC3/binding-attenuated CD3 bispecific antibody were used in combination as

described in Reference Example 23-2. Fig. 54B shows the level of IL-6 production when a GPC3/binding-attenuated CD3 bispecific antibody molecule (GPC3/attCE115) and a modified antibody molecule produced by linking the Fab-Fab of the CD28/CD3 clamping bispecific antibody via an additional disulfide bond (CD28/clamp CD3_HH) were used each alone or in combination in the presence of effector cells (T cells) only.

[fig.54C]Fig. 54C shows, as with Fig. 54A, the results of evaluating cytokine production from T cells when a CD28/CD3 clamping bispecific antibody and a GPC3/binding-attenuated CD3 bispecific antibody were used in combination as described in Reference Example 23-2. Fig. 54C shows the cancer cell growth inhibitory effect when a GPC3/binding-attenuated CD3 bispecific antibody molecule (GPC3/attCE115) and a modified antibody molecule produced by linking the Fab-Fab of the CD28/CD3 clamping bispecific antibody via an additional disulfide bond (CD28/clamp CD3_HH) were used each alone or in combination in the presence of target cells (GPC3-expressing cancer cells) and effector cells (T cells).

[fig.55A]Fig. 55A is a schematic diagram showing the mechanism of action of the T cell-dependent cancer cell growth inhibition when a CD28/CD3 clamping bispecific antibody and a GPC3/binding-attenuated CD3 bispecific antibody are used in combination, as described in Reference Examples 23-1 ("epsilon" in the diagrams indicates CD3 epsilon). Fig. 55A shows the mechanism of action of the cancer cell growth inhibition when a CD28/CD3 clamping bispecific antibody and a GPC3/binding-attenuated CD3 bispecific antibody are used in combination in the presence of target cells (GPC3-expressing cancer cells) and effector cells (T cells).

[fig.55B]Fig. 55B is a schematic diagram showing the mechanism of action of the T cell-dependent cancer cell growth inhibition when a CD28/CD3 clamping bispecific antibody and a GPC3/binding-attenuated CD3 bispecific antibody are used in combination, as described in Reference Examples 23-1 (epsilon in the diagrams indicates CD3 epsilon). Fig. 55B shows the mechanism of action of the cancer cell growth inhibition when a modified antibody molecule which has been modified to introduce an additional disulfide bond into the Fab-Fab of a CD28/CD3 clamping bispecific antibody, and a GPC3/binding-attenuated CD3 bispecific antibody, are used in combination in the presence of target cells (GPC3-expressing cancer cells) and effector cells (T cells).

[fig.56A]Fig. 56A is a schematic diagram showing the mechanism of action of the cytokine production from T cells when a CD28/CD3 clamping bispecific antibody and a GPC3/binding-attenuated CD3 bispecific antibody are used in combination, as described in Reference Examples 23-2 (epsilon in the diagrams indicates CD3 epsilon). Fig. 56A shows the mechanism of action of the cytokine production when a modified antibody molecule which has been modified to introduce an additional disulfide bond

into the Fab-Fab of a CD28/CD3 clamping bispecific antibody, and a GPC3/binding-attenuated CD3 bispecific antibody, are used in combination in the presence of target cells (GPC3-expressing cancer cells) and effector cells (T cells).

[fig.56B]Fig. 56B is a schematic diagram showing the mechanism of action of the cytokine production from T cells when a CD28/CD3 clamping bispecific antibody and a GPC3/binding-attenuated CD3 bispecific antibody are used in combination, as described in Reference Examples 23-2 (epsilon in the diagrams indicates CD3 epsilon). Fig. 56B shows the mechanism of action of the cytokine production when a modified antibody molecule which has been modified to introduce an additional disulfide bond into the Fab-Fab of a CD28/CD3 clamping bispecific antibody, and a GPC3/binding-attenuated CD3 bispecific antibody, are used in combination in the presence of effector cells (T cells) only.

[fig.57A]Fig. 57A shows the results of assaying the agonist activity of a CD8/CD28 bispecific antibody molecule (CD8/CD28-P587), and modified antibody molecules produced by linking the Fab-Fab of that antibody via an additional disulfide bond (CD8/CD28-P587(HH), CD8/CD28-P587(LL), CD8/CD28-P587(HL), CD8/CD28-P587(LH)) as described in Reference Example 24. An anti-KLH antibody molecule (KLH-P587) was used as a negative control. The results obtained by using peripheral blood mononuclear cells (PBMC) from two different donors are shown (upper panel: donor A, lower panel: donor B). Fig. 57A shows the proportion of divided regulatory T cells (Treg) in PBMCs.

[fig.57B]Fig. 57B shows the results of assaying the agonist activity of a CD8/CD28 bispecific antibody molecule (CD8/CD28-P587), and modified antibody molecules produced by linking the Fab-Fab of that antibody via an additional disulfide bond (CD8/CD28-P587(HH), CD8/CD28-P587(LL), CD8/CD28-P587(HL), CD8/CD28-P587(LH)) as described in Reference Example 24. Fig. 57B shows the proportion of divided CD8 alpha-positive T cells in PBMCs.

[fig.58]Fig. 58 shows chromatograms of cation exchange chromatography (CIEX) performed on the antibody sample of OKT3 variants with charged amino acid substitution as described in Example 9-3.

[fig.59]Fig. 59 shows chromatograms of cation exchange chromatography (CIEX) performed on the antibody sample of OKT3 variants with charged amino acid substitution as described in Examples 2-2 and Examples 9-3.

[fig.60]Fig. 60 shows a scatter diagram of lower band-to-upper band ratio (non-reducing SDS-PAGE gel image) of OKT3 and MRA antibody variants produced in Example 10-1. Y-axis represents the ratio of the lower band to upper band of MRA variants sample as shown in Table 87, whereas X-axis represents the ratio of the lower band to upper band of OKT3 variants sample as shown in Table 87.

[fig.61A]Fig. 61A shows chromatograms of cation exchange chromatography (CIEX) performed on the antibody sample of OKT3 variants with charged amino acid substitution as described in Example 10-3.

[fig.61B]Fig. 61B shows chromatograms of cation exchange chromatography (CIEX) performed on the antibody sample of MRA variants with charged amino acid substitution as described in Example 10-3.

[fig.62A]Fig. 62A is a schematic diagram showing the effect of additional amino acid mutation for enhancement of Fab crosslinking of the engineered disulfide bond. (Left) G1T4.S191C variant with cysteine substitution e.g. at the S191C of CH1 (EU numbering) contain mixtures of cross-linked and non-cross-linked antibodies. (Middle) G1T4.S191C variants which comprise additional amino acid mutation X (X can be either charged amino acid, hydrophobic amino acid or Knob-hole amino acids) shows higher proportion of cross-linked antibodies. (Right) Amino acid position at CH1-CH1 interface (EU numbering) in which additional amino acid mutation X (X can be either charged amino acid, hydrophobic amino acid or Knob-hole amino acids) can facilitate the crosslinking of the engineered disulfide bond.

[fig.62B]Fig. 62B is a schematic diagram showing the effect of additional mutation for separation between crosslinked and non-crosslinked Fabs by chromatography methods such as CIEX.

Description of Embodiments

[0024] I. Definitions

Herein, the term "antigen-binding molecule" refers, in its broadest sense, to a molecule that specifically binds to an antigenic determinant (epitope). In one embodiment, the antigen-binding molecule is an antibody, antibody fragment, or antibody derivative. In one embodiment, the antigen-binding molecule is a non-antibody protein, or a fragment thereof, or a derivative thereof.

[0025] Herein, "antigen-binding domain" refers to a region that specifically binds and is complementary to the whole or a portion of an antigen. Herein, an antigen-binding molecule comprises an antigen-binding domain. When the molecular weight of an antigen is large, an antigen-binding domain can only bind to a particular portion of the antigen. The particular portion is called "epitope". In one embodiment, an antigen-binding domain comprises an antibody fragment which binds to a particular antigen. An antigen-binding domain can be provided from one or more antibody variable domains. In a non-limiting embodiment, the antigen-binding domains comprise both the antibody light chain variable region (VL) and antibody heavy chain variable region (VH). Examples of such antigen-binding domains include "single-chain Fv (scFv)", "single-chain antibody", "Fv", "single-chain Fv2 (scFv2)", "Fab", and "Fab'". In other

embodiments, an antigen-binding domain comprises a non-antibody protein which binds to a particular antigen, or a fragment thereof. In a specific embodiment, an antigen-binding domain comprises a hinge region.

[0026] In the present invention, "specifically binds" means binding in a state where one of the molecules involved in specific binding does not show any significant binding to molecules other than a single or a number of binding partner molecules. Furthermore, it is also used when an antigen-binding domain is specific to a particular epitope among multiple epitopes contained in an antigen. When an epitope bound by an antigen-binding domain is contained in multiple different antigens, antigen-binding molecules comprising the antigen-binding domain can bind to various antigens that have the epitope.

[0027] In the present disclosure, the recitation "binds to the same epitope" means that the epitopes to which two antigen-binding domains bind at least partially overlap each other. The degree of the overlap is, but not limited to, at least 10% or more, preferably 20% or more, 30% or more, 40% or more, 50% or more, 60% or more, 70% or more, and 80% or more, particularly preferably 90% or more, and most preferably 100%.

The term "antibody" herein is used in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired antigen-binding activity.

[0028] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies composing the population are identical and/or bind the same epitope, except for possible variant antibodies, e.g., containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic animals containing all or part of the human immunoglobulin loci, such methods and other exemplary methods for making monoclonal antibodies being described herein.

[0029] "Native antibodies" refer to naturally occurring immunoglobulin molecules with

varying structures. For example, native IgG antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light chains and two identical heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant domains (CH1, CH2, and CH3). Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain. The light chain of an antibody may be assigned to one of two types, called kappa and lambda, based on the amino acid sequence of its constant domain.

[0030] The term "chimeric" antibody refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

[0031] The "class" of an antibody refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively.

[0032] In one embodiment of the present invention, constant regions are preferably antibody constant regions, more preferably IgG1, IgG2, IgG3, and IgG4-type antibody constant regions, and even more preferably human IgG1, IgG2, IgG3, and IgG4-type antibody constant regions. Furthermore, in another embodiment of the present invention, constant regions are preferably heavy chain constant regions, more preferably IgG1, IgG2, IgG3, and IgG4-type heavy chain constant regions, and even more preferably human IgG1, IgG2, IgG3, and IgG4-type heavy chain constant regions. The amino acid sequences of the human IgG1 constant region, the human IgG2 constant region, the human IgG3 constant region, and the human IgG4 constant region are known. For the constant regions of human IgG1, human IgG2, human IgG3, and human IgG4, a plurality of allotype sequences with genetic polymorphism are described in Sequences of proteins of immunological interest, NIH Publication No.91-3242, and any of them can be used in the present invention. Amino acid-modified constant regions of the present invention may contain other amino acid mutations or modifications, as long as they include an amino acid mutation of the present invention.

[0033] The term "hinge region" denotes an antibody heavy chain polypeptide portion in a wild-type antibody heavy chain that joins the CH1 domain and the CH2 domain, e.g., from about position 216 to about position 230 according to the EU numbering system, or from about position 226 to about position 243 according to the Kabat numbering system. It is known that in a native IgG antibody, cysteine residue at position 220

according to EU numbering in the hinge region forms a disulfide bond with cysteine residue at position 214 in the antibody light chain. It is also known that between the two antibody heavy chains, disulfide bonds are formed between cysteine residues at position 226 and between cysteine residues at position 229 according to EU numbering in the hinge region. In general, a "hinge region" is defined as extending from human IgG1 from 216 to 238 (EU numbering) or from 226 to 251 (Kabat numbering). This hinge can be further divided into three different regions, an upper hinge, a central hinge and a lower hinge. In human IgG1 antibodies, these regions are generally defined as follows:
Upper hinge: 216-225 (EU numbering) or 226-238 (Kabat numbering),
Central hinge: 226-230 (EU numbering) or 239-243 (Kabat numbering),
Lower hinge: 231-238 (EU numbering) or 244-251 (Kabat numbering).
The hinge region of other IgG isotypes can be aligned with the IgG1 sequence by placing the first and last cysteine residues that form an interheavy chain SS bond in the same position (e.g., Brekke et al., 1995, Immunol (See Table 1 of Today 16: 85-90). A hinge region herein includes wild-type hinge regions as well as variants in which amino acid residue(s) in a wild-type hinge region is altered by substitution, addition, or deletion.
The term "disulfide bond formed between amino acids which are not in a hinge region" (or "disulfide bond formed between amino acids outside of a hinge region") means disulfide bond formed, connected or linked through amino acids located in any antibody region which is outside of the "hinge region" defined above. For example, such disulfide bond is formed, connected or linked through amino acids in any position in an antibody other than in a hinge region (e.g., from about position 216 to about position 230 according to the EU numbering system, or from about position 226 to about position 243 according to the Kabat numbering system). In some embodiments, such disulfide bond is formed, connected or linked through amino acids located in a CH1 region, a CL region, a VL region, a VH region and/or a VHH region. In some embodiments, such disulfide bond is formed, connected or linked through amino acids located in positions 119 to 123, 131 to 140, 148 to 150, 155 to 167, 174 to 178, 188 to 197, 201 to 214, according to EU numbering, in the CH1 region. In some embodiments, such disulfide bond is formed, connected or linked through amino acids located in positions 119, 122, 123, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 148, 150, 155, 156, 157, 159, 160, 161, 162, 163, 164, 165, 167, 174, 176, 177, 178, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 201, 203, 205, 206, 207, 208, 211, 212, 213, 214 according to EU numbering, in the CH1 region. In some embodiments, such disulfide bond is formed, connected or linked through amino acids located in positions 188, 189, 190, 191, 192, 193, 194, 195, 196, and 197, according to EU

numbering, in the CH1 region. In one preferred embodiment, such disulfide bond is formed, connected or linked through amino acids located in position 191, according to EU numbering, in the CH1 region.

[0034] The term "Fc region" herein is used to define a C-terminal region of an immunoglobulin heavy chain that contains at least a portion of the constant region. The term includes native sequence Fc regions and variant Fc regions. In one embodiment, a human IgG heavy chain Fc region extends from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, the C-terminal lysine (Lys447) or glycine-lysine (residues 446-447) of the Fc region may or may not be present. Unless otherwise specified herein, numbering of amino acid residues in the Fc region or constant region is according to the EU numbering system, also called the EU index, as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991.

[0035] "Effector functions" refer to those biological activities attributable to the Fc region of an antibody, which vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor); and B cell activation.

[0036] The term "Fc receptor" or "FcR" refers to a receptor that binds to the Fc region of an antibody. In some embodiments, an FcR is a native human FcR. In some embodiments, an FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the Fc gamma RI, Fc gamma RII, and Fc gamma RIII subclasses, including allelic variants and alternatively spliced forms of those receptors. Fc gamma RII receptors include Fc gamma RIIA (an "activating receptor") and Fc gamma RIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor Fc gamma RIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor Fc gamma RIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see, e.g., Daeron, Annu. Rev. Immunol. 15:203-234 (1997)). FcRs are reviewed, for example, in Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991); Capel et al., Immunomethods 4:25-34 (1994); and de Haas et al., J. Lab. Clin. Med. 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein.

[0037] The term "Fc receptor" or "FcR" also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., J. Immunol. 117:587 (1976) and Kim et al., J. Immunol. 24:249 (1994)) and regulation of homeostasis of immunoglobulins. Methods of measuring binding to FcRn are known

(see, e.g., Ghetie and Ward., Immunol. Today 18(12):592-598 (1997); Ghetie et al., Nature Biotechnology, 15(7):637-640 (1997); Hinton et al., J. Biol. Chem. 279(8):6213-6216 (2004); WO 2004/92219 (Hinton et al.).

[0038] The term "variable region" or "variable domain" refers to the domain of an antibody heavy or light chain that is involved in binding the antibody to antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable regions (HVRs). (See, e.g., Kindt et al. Kuby Immunology, 6th ed., W.H. Freeman and Co., page 91 (2007).) A single VH or VL domain may be sufficient to confer antigen-binding specificity. Furthermore, antibodies that bind a particular antigen may be isolated using a VH or VL domain from an antibody that binds the antigen to screen a library of complementary VL or VH domains, respectively. See, e.g., Portolano et al., J. Immunol. 150:880-887 (1993); Clarkson et al., Nature 352:624-628 (1991).

[0039] The term "hypervariable region" or "HVR" as used herein refers to each of the regions of an antibody variable domain which are hypervariable in sequence ("complementarity determining regions" or "CDRs") and/or form structurally defined loops ("hypervariable loops") and/or contain the antigen-contacting residues ("antigen contacts"). Generally, antibodies comprise six HVRs: three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). Exemplary HVRs herein include:

(a) hypervariable loops occurring at amino acid residues 26-32 (L1), 50-52 (L2), 91-96 (L3), 26-32 (H1), 53-55 (H2), and 96-101 (H3) (Chothia and Lesk, J. Mol. Biol. 196:901-917 (1987));

(b) CDRs occurring at amino acid residues 24-34 (L1), 50-56 (L2), 89-97 (L3), 31-35b (H1), 50-65 (H2), and 95-102 (H3) (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991));

(c) antigen contacts occurring at amino acid residues 27c-36 (L1), 46-55 (L2), 89-96 (L3), 30-35b (H1), 47-58 (H2), and 93-101 (H3) (MacCallum et al. J. Mol. Biol. 262: 732-745 (1996)); and

(d) combinations of (a), (b), and/or (c), including HVR amino acid residues 46-56 (L2), 47-56 (L2), 48-56 (L2), 49-56 (L2), 26-35 (H1), 26-35b (H1), 49-65 (H2), 93-102 (H3), and 94-102 (H3).

Unless otherwise indicated, HVR residues and other residues in the variable domain (e.g., FR residues) are numbered herein according to Kabat et al., supra.

[0040] "Framework" or "FR" refers to variable domain residues other than hypervariable region (HVR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences

generally appear in the following sequence in VH (or VL): FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4.

[0041] The terms "full length antibody," "intact antibody," and "whole antibody" are used herein interchangeably to refer to an antibody having a structure substantially similar to a native antibody structure or having heavy chains that contain an Fc region as defined herein.

[0042] The terms "host cell," "host cell line," and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

[0043] The term "vector," as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression vectors."

[0044] A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

[0045] A "humanized" antibody refers to a chimeric antibody comprising amino acid residues from non-human HVRs and amino acid residues from human FRs. In certain embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the HVRs (e.g., CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region derived from a human antibody. A "humanized form" of an antibody, e.g., a non-human antibody, refers to an antibody that has undergone humanization.

[0046] An "antibody fragment" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, $F(ab')_2$; diabodies; linear antibodies; single-chain antibody molecules

(e.g. scFv); single chain Fabs (scFabs); single domain antibodies; and multispecific antibodies formed from antibody fragments.

[0047] By "contacting" is meant subjecting to, exposing to, in solution. The antibody, protein or polypeptide can be contacted with the reducing reagents while also bound to a solid support (e.g., an affinity column or a chromatography matrix). Preferably, the solution is buffered. In order to maximize the yield of antibody/protein with a desired conformation, the pH of the solution is chosen to protect the stability of the antibody/protein and to be optimal for disulfide exchange. In the practice of the invention, the pH of the solution is preferably not strongly acidic. Thus, some pH ranges are greater than pH 5, preferably about pH 6 to about pH 11, more preferably from about pH 7 to about pH 10, and still more preferably from about pH 6 to about pH 8. In one non-limiting embodiment of the invention, the optimal pH was found to be about pH 7. However, the optimal pH for a particular embodiment of the invention can be easily determined experimentally by those skilled in the art.

[0048] The term "reduction reagent" and "reducing agent" is used interchangeably. In some embodiments, said reducing agents are free thiols. The reducing reagent is preferably comprised of a compound from the group consisting of glutathione (GSH), dithiothreitol (DTT), 2-mercaptoethanol, 2-aminoethanethiol (2-MEA), TCEP (tris(2-carboxyethyl)phosphine), dithionitrobenzoate, cysteine and Na_2SO_3. In some embodiments, TCEP, 2-MEA, DTT, cysteine, GSH or Na_2SO_3 can be used. In some preferred embodiments, 2-MEA can be used. In some preferred embodiments, TCEP can be used.

[0049] The reducing agent may be added to the fermentation media in which the cells producing the recombinant protein are grown. In additional embodiments, the reducing agent also may be added to the LC mobile phase during the LC separation step for separating the recombinant protein. In certain embodiments, the protein is immobilized to a stationary phase of the LC column and the reducing agents are part of the mobile phase. In specific embodiments, the untreated IgG antibody may elute as a heterogeneous mixture as indicated by the number of peaks. The use of the reduction/oxidation coupling reagent produces a simpler and more uniform peak pattern. It is contemplated that this more uniform peak of interest may be isolated as a more homogeneous preparation of the IgG.

[0050] The reducing agent is present at a concentration that is sufficient to increase the relative proportion of the desired conformation (e.g., the "paired cysteines" form of an antibody which has one or more engineered disulfide bond(s) formed between the two Fabs of the antibody, e.g., between amino acid residues which are not in the hinge region). The optimal absolute concentration and molar ratio of the reducing agent depends upon the concentration of total IgG and in some circumstances the specific

IgG subclass. When used for preparing IgG1 molecules it also will depend on the number and accessibility of the unpaired cysteines in the protein. Generally, the concentration of free thiols from the reducing agent can be from about 0.05 mM to about 100 mM, more preferably about 0.1 mM to about 50 mM, and still more preferably about 0.2 mM to about 20 mM. In some preferred embodiments, the concentration of the reducing agent is 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100 mM. In some preferred embodiments, 0.05 mM to 1 mM of 2-MEA can be used. In some preferred embodiments, 0.01 mM to 25 mM TCEP can be used.

[0051] Contacting the preparation of recombinant protein with a reducing agent is performed for a time sufficient to increase the relative proportion of the desired conformation. Any relative increase in proportion is desirable, including for, example, at least 10%, 20%, 30%, 40%, 50%, 60%, 70% and even 80% or 90% of the protein with an undesired conformation is converted to protein with the desired conformation. The contacting may be performed by providing the reducing agent to the fermentation medium in which the protein is being generated. Alternatively, the contacting takes place upon partial purification of the protein from the cell culture in which it is generated. In still other embodiments, the contacting is performed after the protein has been eluted from the chromatography column but before any further processing. Essentially, the contacting may be performed at any stage during preparation, purification, storage or formulation of the antibody. In some embodiments, partial purification by affinity chromatography (e.g., Protein A chromatography) may be conducted prior to the contacting.

[0052] The contacting may be also performed with antibodies attached to a stationary phase of a chromatographic columns, while the reducing agent are a part of the mobile phase; In this case the contacting may be performed as a part of chromatographic purification procedure. Examples of representative chromatographic refolding processes may include size exclusion (SEC); solvent exchange during reversible adsorption on protein A column; hydrophobic interaction chromatography (HIC); immobilized metal affinity chromatography (IMAC); reversed-phase chromatography (RPC); use of immobilized folding catalyst, such as GroE1, GroES or other proteins with folding properties. The on-column refolding is attractive because it is easily automated using commercially available preparative chromatographic systems. The refolding on column of recombinant proteins produced in microbial cell was recently reviewed in (Li et al., 2004).

[0053] If the contacting step is performed on a partially or highly purified preparation of recombinant protein, the contacting step can be performed for as short as about 1 hour to about 4 hours, and as long as about 6 hours to about 4 days. It has been found that a contacting step of about 2 to about 48 hours, or about 16 hours works well. The

contacting step can also take place during another step, such as on a solid phase or during filtering or any other step in purification.

[0054] The methods of the invention can be performed over a wide temperature range. For example, the methods of the invention have been successfully carried out at temperatures from about 4 degrees Celsius ("degrees C") to about 37 degrees C, however the best results were achieved at lower temperatures. A typical temperature for contacting a partially or fully purified preparation of the recombinant protein is about 4 degrees C to about 25 degrees C (ambient), or preferably at 23 degrees C, but can also be performed at lower temperatures and at higher temperature.

[0055] In addition, it is contemplated that the method may be performed at high pressure. Previously, high hydrostatic pressures (1000-2000 bar), combined with low, nondenaturing concentrations of guanidine hydrochloride below 1M has been used to disaggregate (solubilize) and refold several denatured proteins produced by E-coli as inclusion bodies that included human growth hormone and lysozyme, and b-lactamase (St John et al., Proc Natl Acad Sci USA, 96:13029-13033 (1999)). B-lactamase was refolded at high yields of active protein, even without added GdmHCl. In another study (Seefeldt et al., Protein Sci, 13:2639-2650 (2004)), the refolding yield of mammalian cell produced protein bikunin obtained with high pressure modulated refolding at 2000 bas was 70% by RP HPLC, significantly higher than the value of 55% (by RP-HPLC) obtained with traditional guanidine hydrochloride "dilution-refolding". These findings indicate that high hydrostatic pressure facilitates disruption of inter- and intra-molecular interactions, leading to protein unfolding and disaggregation. The interaction of the high pressure on protein is similar to the interaction of proteins with chaotropic agents. Thus, it is contemplated that in the methods of the invention, instead of using chaotropic agents, high pressure is used for protein unfolding. Of course, a combination of high pressure and chaotropic agents also may be used in some instances.

[0056] The preparation of recombinant antibody/protein can be contacted with the reducing agent in various volumes as appropriate. For example, the methods of the invention have been carried out successfully at the analytical laboratory-scale (1-50 mL), preparative-scale (50 mL-10 L) and manufacturing-scale (10 L or more). The methods of the invention can be carried out on both small and large scale with reproducibility. As such, the concentration of antibody may be an industrial quantity (in terms of weight in grams) (e.g., an industrial amount of a specific IgG) or alternatively may be in milligram quantities. In specific embodiments, the concentration of the recombinant antibody in the reaction mixture is from about 1 mg/ml and about 50 mg/ml, more specifically, 10 mg/ml, 15 mg/ml or 20 mg/ml. The recombinant IgG1 molecules in these concentrations are particularly contemplated.

[0057] In certain embodiments, the proteins produced using media contain reducing agent are further processed in a separate processing step which employs chaotropic denaturants such as, for example, sodium dodecyl sulfate (SDS), urea or guanidium hydrochloride (GuHCl). Significant amounts of chaotropic agents are needed to observe perceptible unfolding. In some embodiments the processing step uses between 0.1M and 2 M chaotrope that produces an effect equivalent to the use of 0.1 M to 2M guanidine hydrochloride. In a specific embodiment, the oxidative refolding is achieved in the presence of approximately 1.0 M guanidine hydrochloride or an amount of other chaotropic agent that produces the same or similar amount of refolding as 1M guanidine hydrochloride. In some embodiments, the methods use between about 1.5 M and 0.5 M chaotrope. The amount of chaotropic agent used is based on the structural stability of the protein in the presence of the said chaotrope. One needs to have enough chaotrope present to perturb the local tertiary structure and/or quaternary structure of domain interactions of the protein, but less than that required to fully unfold secondary structure of the molecule and/or individual domains. To determine the point at which a protein will start to unfold by equilibrium denaturation, one practiced in the art would titrate a chaotrope into a solution containing the protein and monitor structure by a technique such as circular dichroism or fluorescence. There are other parameters that could be used to unfold or slightly perturb the structure of a protein that may be used instead of a chaotrope. Temperature and pressure are two fundamental parameters that have been previously used to alter the structure of a protein and may be used in place of a chaotropic agent while contacting with a redox agent. The inventors contemplate that any parameter that has been shown to denature or perturb a protein structure may be used by a person practiced in the art in place of a chaotropic agent.

[0058] Disulfide exchange can be quenched in any way known to those of skill in the art. For example, the reducing agent can be removed or its concentration can be reduced through a purification step, and/or it can be chemically inactivated by, e.g., acidifying the solution. Typically, when the reaction is quenched by acidification, the pH of the solution containing the reducing agent will be brought down below pH 7. In some embodiment, the pH is brought to below pH 6. Generally, the pH is reduced to between about pH 2 and about pH 6.

In some embodiments, removing the reducing agent may be conducted by dialysis, buffer exchange or any chromatography method described herein.

[0059] The term by "preferentially enriched (or increased)" means an increase in relative abundance of a desired form, or increase in relative proportion of a desired form, or increase the population of a desired form (structural isoform). In some embodiments, the methods described herein increase relative abundance of an antibody structural isoform such as an antibody having at least one disulfide bond formed between amino

acid residues outside of the hinge region. In one embodiment, said at least one disulfide bond is formed between the amino acid residues at position 191 according to EU numbering in the respective CH1 regions of the first antigen-binding domain and the second antigen-binding domain. In certain embodiment, said methods produce a homogenous antibody preparation having at least 50%, 60%, 70%, 80%, 90%, preferably at least 95% molar ratio of said antibody having at least one disulfide bond formed outside of the hinge region.

[0060] A "homogeneous" population of an antibody means an antibody population that comprises largely a single form of the antibody, for example, at least 50%, 60%, 70%, 80% or more, preferably at least 90%, 95%, 96%, 97%, 99% or 100% of the antibody in the solution or composition is in the properly folded form. Similarly, a "homogeneous" population of an antibody having at least one disulfide bond formed outside of the hinge region means a population of said antibody which comprises largely a single, properly folded form, for example, at least 50%, 60%, 70%, 80% or more, preferably at least 90%, 95%, 96%, 97%, 99% or 100% molar ratio of said antibody having at least one disulfide bond formed outside of the hinge region. In one preferred embodiment, said "homogeneous" population of an antibody comprises at least one disulfide bond which is formed between the amino acid residues at position 191 according to EU numbering in the respective CH1 regions of the first antigen-binding domain and the second antigen-binding domain (i.e. "paired cysteines" at the position 191 according to EU number in the CH1 region).

In preferred embodiments, the methods of the present invention produce a homogeneous antibody population or a homogeneous antibody preparation by the steps described herein.

[0061] Determining whether an antibody population is homogenous, and the relative abundance or proportions of a conformation of a protein/antibody in a mixture, can be done using any of a variety of analytical and/or qualitative techniques. If the two conformations resolve differently during separation techniques such as chromatography, electrophoresis, filtering or other purification technique, then the relative proportion of a conformation in the mixture can be determined using such purification techniques. For example, at least two different conformations of the recombinant IgG could be resolved by way of hydrophobic interaction chromatography. Further, since far UV Circular Dichroism has been used to estimate secondary structure composition of proteins (Perczel et al., 1 991, Protein Engrg. 4:669-679), such a technique can determine whether alternative conformations of a protein are present. Still another technique used to determine conformation is fluorescence spectroscopy which can be employed to ascertain complementary differences in tertiary structure assignable to tryptophan and tyrosine fluorescence. Other techniques that can be used to determine

differences in conformation and, hence, the relative proportions of a conformation, are on-line SEC to measure aggregation status, differential scanning calorimetry to measure melting transitions (Tm's) and component enthalpies, and chaotrope unfolding. Yet another technique that can be used to determine differences in conformation and, hence, the relative proportions of a conformation is LC/MS detection to determine the heterogeneity of the protein.

[0062] Alternatively, if there is a difference in activity between the conformations of the antibody/protein, determining the relative proportion of a conformation in the mixture can be done by way of an activity assay (e.g., binding to a ligand, enzymatic activity, biological activity, etc.). Biological activity of the protein also could be used. Alternatively, the binding assays can be used in which the activity is expressed as activity units/mg of protein.

[0063] In some embodiments described in detail herein below, the invention uses IEC chromatography, to determine the heterogeneity of the antibody/protein. In such a case, the antibody is purified or considered to be "homogenous", which means that no polypeptide peaks or fractions corresponding to other polypeptides are detectable upon analysis by IEC chromatography. In certain embodiments, the antibody is purified or considered to be "homogenous" such that no polypeptide bands corresponding to other polypeptides are detectable upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It will be recognized by one skilled in the pertinent field that multiple bands corresponding to the polypeptide can be visualized by SDS-PAGE, due to differential glycosylation, differential post-translational processing, and the like. Most preferably, the polypeptide of the invention is purified to substantial homogeneity, as indicated by a single polypeptide band upon analysis by SDS-PAGE. The polypeptide band can be visualized by silver staining, Coomassie blue staining, and/or (if the polypeptide is radiolabeled) by auto radiography.

[0064] Herein, examples of conditions of SDS-PAGE analysis are as follows. Sample Buffer Solution without 2-mercaptoethanol (x4) may be used for preparation of electrophoresis samples. The samples may be treated for 10 minutes under the condition of specimen concentration 50 or 100 microgram/mL and 70 degrees C, and then subjected to non-reducing SDS-PAGE. In non-reducing SDS-PAGE, electrophoresis may be carried out for 90 minutes at 125 V, using a 4% SDS-PAGE gel. Then, the gel may be stained with CBB, and the gel image may be captured, and the bands may be quantified using an imaging device. In the gel image, several, for example, two bands, i.e., "upper band" and "lower band", may be observed for an antibody variant sample. In this case, the molecular weight of the upper band may correspond to that of the parent antibody (before modification). Structural changes such as crosslinking via disulfide bonds of Fabs may be caused by cysteine substitution, which may result in

the change in electrophoretic mobility. In this case, the lower band may be considered to correspond to the antibody having one or more engineered disulfide bond(s) formed between the CH1 regions. Antibody variant samples with additional cysteine substitutions may show a higher lower band to upper band ratio, compared to control samples. Additional cysteine substitutions may enhance/promote disulfide bond crosslinking of Fabs; and may increase the percentage or structural homogeneity of an antibody preparation having an engineered disulfide bond formed at a mutated position; and may decrease the percentage of an antibody preparation having no engineered disulfide bond formed at the mutated position. Herein, the term "lower band to upper band ratio" refers to a ratio between the quantities/intensities of the lower and upper bands that may be quantified during the above-mentioned SDS-PAGE experiments.

[0065] Variable fragment (Fv)

Herein, the term "variable fragment (Fv)" refers to the minimum unit of an antibody-derived antigen-binding domain that is composed of a pair of the antibody light chain variable region (VL) and antibody heavy chain variable region (VH). In 1988, Skerra and Pluckthun found that homogeneous and active antibodies can be prepared from the E. coli periplasm fraction by inserting an antibody gene downstream of a bacterial signal sequence and inducing expression of the gene in E. coli (Science (1988) 240(4855), 1038-1041). In the Fv prepared from the periplasm fraction, VH associates with VL in a manner so as to bind to an antigen.

[0066] scFv, single-chain antibody, and sc(Fv)2

Herein, the terms "scFv", "single-chain antibody", and "sc(Fv)2" all refer to an antibody fragment of a single polypeptide chain that contains variable regions derived from the heavy and light chains, but not the constant region. In general, a single-chain antibody also contains a polypeptide linker between the VH and VL domains, which enables formation of a desired structure that is thought to allow antigen binding. The single-chain antibody is discussed in detail by Pluckthun in "The Pharmacology of Monoclonal Antibodies, Vol. 113, Rosenburg and Moore, eds., Springer-Verlag, New York, 269-315 (1994)". See also International Patent Publication WO 1988/001649; US Patent Nos. 4,946,778 and 5,260,203. In a particular embodiment, the single-chain antibody can be bispecific and/or humanized.

[0067] scFv is an antigen-binding domain in which VH and VL forming Fv are linked together by a peptide linker (Proc. Natl. Acad. Sci. U.S.A. (1988) 85(16), 5879-5883). VH and VL can be retained in close proximity by the peptide linker.

[0068] sc(Fv)2 is a single-chain antibody in which four variable regions of two VL and two VH are linked by linkers such as peptide linkers to form a single chain (J Immunol. Methods (1999) 231(1-2), 177-189). The two VH and two VL may be derived from

different monoclonal antibodies. Such sc(Fv)2 preferably includes, for example, a bispecific sc(Fv)2 that recognizes two epitopes present in a single antigen as disclosed in the Journal of Immunology (1994) 152(11), 5368-5374. sc(Fv)2 can be produced by methods known to those skilled in the art. For example, sc(Fv)2 can be produced by linking scFv by a linker such as a peptide linker.

[0069] Herein, the forms of an antigen-binding domain forming an sc(Fv)2 include an antibody in which the two VH units and two VL units are arranged in the order of VH, VL, VH, and VL ([VH]-linker-[VL]-linker-[VH]-linker-[VL]) beginning from the N terminus of a single-chain polypeptide. The order of the two VH units and two VL units is not limited to the above form, and they may be arranged in any order. Example order of the form is listed below.

[VL]-linker-[VH]-linker-[VH]-linker-[VL]
[VH]-linker-[VL]-linker-[VL]-linker-[VH]
[VH]-linker-[VH]-linker-[VL]-linker-[VL]
[VL]-linker-[VL]-linker-[VH]-linker-[VH]
[VL]-linker-[VH]-linker-[VL]-linker-[VH]

[0070] Fab, F(ab')2, and Fab'

"Fab" consists of a single light chain, and a CH1 region and variable region from a single heavy chain. The heavy chain of a wild-type Fab molecule cannot form disulfide bonds with another heavy chain molecule. Herein, in addition to wild-type Fab molecules, Fab variants in which amino acid residue(s) in a wild-type Fab molecule is altered by substitution, addition, or deletion are also included. In a specific embodiment, mutated amino acid residue(s) comprised in Fab variants (e.g., cysteine residue(s) or lysine residue(s) after substitution, addition, or insertion) can form disulfide bond(s) with another heavy chain molecule or a portion thereof (e.g., Fab molecule).

[0071] scFab is an antigen-binding domain in which a single light chain, and a CH1 region and variable region from a single heavy chain which form Fab are linked together by a peptide linker. The light chain, and the CH1 region and variable region from the heavy chain can be retained in close proximity by the peptide linker.

[0072] "F(ab')2" or "Fab" is produced by treating an immunoglobulin (monoclonal antibody) with a protease such as pepsin and papain, and refers to an antibody fragment generated by digesting an immunoglobulin (monoclonal antibody) at near the disulfide bonds present between the hinge regions in each of the two H chains. For example, papain cleaves IgG upstream of the disulfide bonds present between the hinge regions in each of the two H chains to generate two homologous antibody fragments, in which an L chain comprising VL (L-chain variable region) and CL (L-chain constant region) is linked to an H-chain fragment comprising VH (H-chain variable region) and CH

gamma 1 (gamma 1 region in an H-chain constant region) via a disulfide bond at their C-terminal regions. Each of these two homologous antibody fragments is called Fab'.

[0073] "F(ab')2" consists of two light chains and two heavy chains comprising the constant region of a CH1 domain and a portion of CH2 domains so that disulfide bonds are formed between the two heavy chains. The F(ab')2 disclosed herein can be preferably produced as follows. A whole monoclonal antibody or such comprising a desired antigen-binding domain is partially digested with a protease such as pepsin; and Fc fragments are removed by adsorption onto a Protein A column. The protease is not particularly limited, as long as it can cleave the whole antibody in a selective manner to produce F(ab')2 under an appropriate setup enzyme reaction condition such as pH. Such proteases include, for example, pepsin and ficin.

[0074] Single domain antibodies

Herein, those referred to by the term "single domain antibodies" are not particularly limited in their structure, as long as the domain can exert antigen-binding activity by itself. Ordinary antibodies exemplified by IgG antibodies exert antigen-binding activity in a state where a variable region is formed by the pairing of VH and VL. In contrast, a single domain antibody is known to be able to exert antigen-binding activity by its own domain structure alone without pairing with another domain. Single domain antibodies usually have a relatively low molecular weight and exist in the form of a monomer.

Examples of a single domain antibody include, but are not limited to, antigen binding molecules which naturally lack light chains, such as VHH of Camelidae animals and V_{NAR} of sharks, and antibody fragments comprising the whole or a portion of an antibody VH domain or the whole or a portion of an antibody VL domain. Examples of a single domain antibody which is an antibody fragment comprising the whole or a portion of an antibody VH/VL domain include, but are not limited to, artificially prepared single domain antibodies originating from a human antibody VH or a human antibody VL as described, e.g., in US Patent No. 6,248,516 B1. In some embodiments of the present invention, one single domain antibody has three CDRs (CDR1, CDR2, and CDR3).

Single domain antibodies can be obtained from animals capable of producing single domain antibodies or by immunizing animals capable of producing single domain antibodies. Examples of animals capable of producing single domain antibodies include, but are not limited to, camelids and transgenic animals into which gene(s) for the capability of producing a single domain antibody has been introduced. Camelids include camel, llama, alpaca, dromedary, guanaco, and such. Examples of a transgenic animal into which gene(s) for the capability of producing a single domain antibody has been introduced include, but are not limited to, the transgenic animals described in International Publication No. WO2015/143414 or US Patent Publication No. US2011/0123527 A1. Humanized single chain antibodies can also be obtained, by

replacing framework sequences of a single domain antibody obtained from an animal with human germline sequences or sequences similar thereto. A humanized single domain antibody (e.g., humanized VHH) is one embodiment of the single domain antibody of the present invention.

Alternatively, single domain antibodies can be obtained from polypeptide libraries containing single domain antibodies by ELISA, panning, and such. Examples of polypeptide libraries containing single domain antibodies include, but are not limited to, naive antibody libraries obtained from various animals or humans (e.g., Methods in Molecular Biology 2012 911 (65-78) and Biochimica et Biophysica Acta - Proteins and Proteomics 2006 1764:8 (1307-1319)), antibody libraries obtained by immunizing various animals (e.g., Journal of Applied Microbiology 2014 117:2 (528-536)), and synthetic antibody libraries prepared from antibody genes of various animals or humans (e.g., Journal of Biomolecular Screening 2016 21:1 (35-43), Journal of Biological Chemistry 2016 291:24 (12641-12657), and AIDS 2016 30:11 (1691-1701)).

[0075] "Binding activity" refers to the strength of the sum total of noncovalent interactions between one or more binding sites of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Herein, binding activity is not strictly limited to a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). For example, when the members of a binding pair reflect a monovalent 1:1 interaction, the binding activity refers to the intrinsic binding affinity (affinity). When a member of a binding pair is capable of both monovalent binding and multivalent binding, the binding activity is the sum of each binding strength. The binding activity of a molecule X for its partner Y can generally be represented by the dissociation constant (KD) or "amount of bound analyte per unit amount of ligand". Binding activity can be measured by common methods known in the art, including those described herein.

[0076] An "agonist" antigen-binding molecule or "agonist" antibody, as used herein, is an antigen-binding molecule or antibody which significantly potentiates a biological activity of the antigen it binds.

[0077] A "blocking" antigen-binding molecule or "blocking" antibody, or an "antagonist" antigen-binding molecule or "antagonist" antibody, as used herein, is an antigen-binding molecule or antibody which significantly inhibits (either partially or completely) a biological activity of the antigen it binds.

[0078] The phrase "substantially reduced" or "substantially different," as used herein, refers to a sufficiently high degree of difference between two numeric values (generally one associated with a molecule and the other associated with a reference/comparator molecule) such that one of skill in the art would consider the difference between the two values to be of statistical significance within the context of the biological characteristic measured by said values (e.g., KD values).

[0079] The term "substantially similar" or "substantially the same," as used herein, refers to a sufficiently high degree of similarity between two numeric values (for example, one associated with an antibody of the invention and the other associated with a reference/ comparator antibody), such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (e.g., KD values).

[0080] The terms "pharmaceutical formulation" and "pharmaceutical composition" refer to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

[0081] A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

[0082] An "individual" or "subject" is a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). In certain embodiments, the individual or subject is a human.

[0083] II. Antigen-binding molecule

In an aspect, the present disclosure is partly based on the discovery that various activities of an antigen-binding molecule that contains a first antigen-binding domain and a second antigen-binding domain in which the antigen-binding domains are linked with each other via one or more bonds, are enhanced or diminished compared to a control antigen-binding molecule containing antigen-binding domains without the linkage or linked via less bonds. In certain embodiments, an antigen-binding molecule that has activity of holding two or more antigen molecules at spatially close positions is provided. The antigen-binding molecule of the present disclosure is useful, for example, in that it can regulate the activation of two antigen molecules which are activated by association with each other. In certain other embodiments, an antigen-binding molecule that has acquired resistance to protease digestion by the linkage between the antigen-binding domains is provided.

[0084] A. Exemplary antigen-binding molecules

< Structures of antigen-binding molecules >

In an aspect, the present disclosure provides an antigen-binding molecule comprising a first antigen-binding domain and a second antigen-binding domain, and the antigen-binding domains are linked with each other via one or more bonds.

[0085] In an embodiment of the above aspects, at least one of the one or more bonds linking the two antigen-binding domains is a covalent bond. In certain embodiments, the covalent bond is formed by direct crosslinking of an amino acid residue in the first antigen-binding domain and an amino acid residue in the second antigen-binding domain. The crosslinked amino acid residues are, for example, cysteine, and the formed covalent bond is, for example, a disulfide bond.

In certain other embodiments, the covalent bond is formed by crosslinking of an amino acid residue in the first antigen-binding domain and an amino acid residue in the second antigen-binding domain via a crosslinking agent. The crosslinking agent is, for example, an amine-reactive crosslinking agent, and the crosslinked amino acid residues are, for example, lysine.

[0086] In an embodiment of the above aspects, at least one of the one or more bonds linking the antigen-binding domains is a noncovalent bond. In certain embodiments, the non-covalent bond is an ionic bond, hydrogen bond, or hydrophobic bond. The ionic bond is formed, for example, between an acidic amino acid and a basic amino acid. The acidic amino acid is, for example, aspartic acid (Asp) or glutamic acid (Glu). The basic amino acid is, for example, histidine (His), lysine (Lys), or arginine (Arg).

[0087] Amino acid residues from which the bonds between the antigen-binding domains (the bonds which link two antigen-binding domains) originate are respectively present in the first and second antigen-binding domains, and the bonds between the antigen-binding domains are formed by linking these amino acid residues. In an embodiment of the above aspects, at least one of the amino acid residues from which the bond between the antigen-binding domains originates is an artificially introduced mutated amino acid residue and, for example, it is an artificially introduced cysteine residue. Such a mutated amino acid residue can be introduced into a wild-type antigen-binding domain by, for example, a method of amino acid substitution. The present specification discloses the sites of amino acid residues from which the bond between the antigen-binding domains can originate for each of the CH1, CL, and hinge regions as constant regions and the VH, VL, and VHH regions as variable regions when the antigen-binding domains comprise, for example, an antibody fragment, and for example, cysteine residues can be introduced into such sites.

[0088] In an embodiment of the above aspects, at least one of the first and second antigen-binding domains has, by itself, activity of binding to an antigen (i.e., a single antigen-binding domain independently has antigen-binding activity). In certain embodiments, each of the first and second antigen-binding domains has, by itself, activity of binding to an antigen.

[0089] In an embodiment of the above aspects, the first and second antigen-binding domains are both antigen-binding domains of the same type. As stated below, examples of

proteins that constitute the antigen-binding domains include polypeptides derived from an antibody or a non-antibody protein, and fragments thereof (for example, a Fab, Fab', scFab, Fv, scFv, and single domain antibody). From the viewpoint of such molecular forms, when the structures of the proteins constituting the first and second antigen-binding domains are identical, the antigen-binding domains are determined to be of the same type.

[0090] In an embodiment of the above aspects, the at least one bond which links the first antigen-binding domain and the second antigen-binding domain may be formed by linking amino acid residues present at the same position in the first antigen-binding domain and in the second antigen-binding domain with each other, or it may be formed by linking amino acid residues present at a respectively different position with each other.

[0091] Positions of amino acid residues in the antigen-binding domain can be shown according to the Kabat numbering or EU numbering system (also called the EU index) described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991. For example, if the amino acid residues from which the bonds between the first and second antigen-binding domains originate are present at an identical position corresponding in the antigen-binding domains, the position of these amino acid residues can be indicated as the same number according to the Kabat numbering or EU numbering system. Alternatively, if the amino acid residues from which the bonds between the first and second antigen-binding domains originate are present at different positions which are not corresponding in the antigen-binding domains, the positions of these amino acid residues can be indicated as different numbers according to the Kabat numbering or EU numbering system.

[0092] In an embodiment of the above aspects, at least one of the first and second antigen-binding domains comprises an antibody fragment which binds to a specific antigen. In certain embodiments, the antibody fragment is a Fab, Fab', scFab, Fv, scFv, or single domain antibody. In certain embodiments, at least one of the amino acid residues from which the bonds between the antigen-binding domains originate is present in an antibody fragment.

[0093] In an embodiment of the above aspects, at least one of amino acid residues from which the bonds between the antigen-binding domains originate is present within a constant region. In certain embodiments, the amino acid residue is present within a CH1 region, and for example, it is present at any of positions 119 to 123, 131 to 140, 148 to 150, 155 to 167, 174 to 178, 188 to 197, 201 to 214, and 218 to 219 according to EU numbering in the CH1 region. In certain embodiments, the amino acid residue is present at a position selected from the group consisting of positions 119, 122, 123,

131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 148, 150, 155, 156, 157, 159, 160, 161, 162, 163, 164, 165, 167, 174, 176, 177, 178, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 201, 203, 205, 206, 207, 208, 211, 212, 213, 214, 218, and 219 according to EU numbering in the CH1 region. In certain embodiments, the amino acid residue is present at position 134, 135, 136, 137, 191, 192, 193, 194, 195, or 196 according to EU numbering in the CH1 region. In certain embodiments, the amino acid residue is present at position 135, 136, or 191 according to EU numbering in the CH1 region.

In an embodiment of the above aspects, the constant region is derived from human. In certain embodiments, the subclass of the heavy chain constant region is any of IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgM, IgD, and IgE. In certain embodiments, the subclass of the CH1 region is any of gamma 1, gamma 2, gamma 3, gamma 4, alpha 1, alpha 2, mu, delta, and epsilon.

[0094] In an embodiment of the above aspects, the at least one bond which links the first antigen-binding domain and the second antigen-binding domain is formed by linking an amino acid residue in the CH1 region of the first antigen-binding domain and an amino acid residue in the CH1 region of the second antigen-binding domain. In certain embodiments, the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 119, 120, 121, 122, and 123 according to EU numbering. In certain embodiments, the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 131, 132, 133, 134, 135, 136, 137, 138, 139, and 140 according to EU numbering. In certain embodiments, the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 148, 149, and 150 according to EU numbering. In certain embodiments, the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, and 167 according to EU numbering. In certain embodiments, the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 174, 175, 176, 177, and 178 according to EU numbering. In certain embodiments, the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 188, 189, 190, 191, 192, 193, 194, 195, 196, and 197 according to EU numbering. In certain embodiments, the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group

consisting of positions 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, and 214 according to EU numbering. In certain embodiments, the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 218 and 219 according to EU numbering.

[0095] In an embodiment of the above aspects, the difference in the positions of the amino acid residues from which the bonds originate in each of the first antigen-binding domain and the second antigen-binding domain is three amino acids or less. This means that when the position of the amino acid residue from which a bond originates in the CH1 region of the first antigen-binding domain and the position of the amino acid residue from which the bond originates in the CH1 region of the second antigen-binding domain are respectively compared according to EU numbering, the difference (i.e., distance) is three amino acids or less. In certain embodiments, the at least one bond which links the first antigen-binding domain and the second antigen-binding domain is formed by linking the amino acid residue at position 135 according to EU numbering in the CH1 region of the first antigen-binding domain and an amino acid residue at any of positions 132 to 138 according to EU numbering in the CH1 region of the second antigen-binding domain. In certain embodiments, the at least one bond which links the first antigen-binding domain and the second antigen-binding domain is formed by linking the amino acid residue at position 136 according to EU numbering in the CH1 region of the first antigen-binding domain and an amino acid residue at any of positions 133 to 139 according to EU numbering in the CH1 region of the second antigen-binding domain.

In certain embodiments, the at least one bond which links the first antigen-binding domain and the second antigen-binding domain is formed by linking the amino acid residue at position 191 according to EU numbering in the CH1 region of the first antigen-binding domain and an amino acid residue at any of positions 188 to 194 according to EU numbering in the CH1 region of the second antigen-binding domain. In an exemplary embodiment, the at least one bond which links the first antigen-binding domain and the second antigen-binding domain is formed by linking the amino acid residues at position 135 according to EU numbering in the CH1 regions of the two antigen-binding domains with each other. In an exemplary embodiment, the at least one bond which links the first antigen-binding domain and the second antigen-binding domain is formed by linking the amino acid residues at position 136 according to EU numbering in the CH1 regions of the two antigen-binding domains with each other. In an exemplary embodiment, the at least one bond which links the first antigen-binding domain and the second antigen-binding domain is formed by linking the amino acid residues at position 191 according to EU numbering in the CH1 regions of the two

antigen-binding domains with each other.

[0096] In an embodiment of the above aspects, at least one of amino acid residues from which the bonds between the antigen-binding domains originate is present within a CL region, and for example, it is present at any of positions 108 to 112, 121 to 128, 151 to 156, 184 to 190, 195 to 196, 200 to 203, and 208 to 213 according to Kabat numbering in the CL region. In certain embodiments, the amino acid residue is present at a position selected from the group consisting of positions 108, 109, 112, 121, 123, 126, 128, 151, 152, 153, 156, 184, 186, 188, 189, 190, 195, 196, 200, 201, 202, 203, 208, 210, 211, 212, and 213 according to Kabat numbering in the CL region. In certain embodiments, the amino acid residue is present at position 126 according to Kabat numbering in the CL region.

In an embodiment of the above aspects, the constant region is derived from human. In certain embodiments, the subclass of the CL region is kappa or lambda.

[0097] In an embodiment of the above aspects, the at least one bond which links the first antigen-binding domain and the second antigen-binding domain is formed by linking an amino acid residue in the CL region of the first antigen-binding domain and an amino acid residue in the CL region of the second antigen-binding domain. In certain embodiments, the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 108, 109, 110, 111, and 112 according to Kabat numbering. In certain embodiments, the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 121, 122, 123, 124, 125, 126, 127, and 128 according to Kabat numbering. In certain embodiments, the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 151, 152, 153, 154, 155, and 156 according to Kabat numbering. In certain embodiments, the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 184, 185, 186, 187, 188, 189, and 190 according to Kabat numbering. In certain embodiments, the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 195 and 196 according to Kabat numbering. In certain embodiments, the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 200, 201, 202, and 203 according to Kabat numbering. In certain embodiments, the amino acid residues in the first antigen-binding domain and the second antigen-binding domain are each independently selected from the group consisting of positions 208, 209, 210, 211, 212, and

213 according to Kabat numbering.

[0098] In an embodiment of the above aspects, the difference in (i.e., distance between) the positions of the amino acid residues from which the bonds originate in each of the first antigen-binding domain and the second antigen-binding domain is three amino acids or less. This means that when the position of the amino acid residue from which a bond originates in the CL region of the first antigen-binding domain and the position of the amino acid residue from which the bond originates in the CL region of the second antigen-binding domain are respectively compared according to EU numbering, the difference (i.e., distance) is three amino acids or less. In an exemplary embodiment, the at least one bond which links the first antigen-binding domain and the second antigen-binding domain is formed by linking the amino acid residues at position 126 according to Kabat numbering in the CL regions of the two antigen-binding domains with each other.

[0099] In an embodiment of the above aspects, the at least one bond which links the first antigen-binding domain and the second antigen-binding domain is formed by linking an amino acid residue in the CH1 region of the first antigen-binding domain and an amino acid residue in the CL region of the second antigen-binding domain. In certain embodiments, the amino acid residues in the CH1 region of the first antigen-binding domain are selected from the group consisting of positions 188, 189, 190, 191, 192, 193, 194, 195, 196, and 197 according to EU numbering, and the amino acid residues in the CL region of the second antigen-binding domain are selected from the group consisting of positions 121, 122, 123, 124, 125, 126, 127, and 128 according to Kabat numbering. In an exemplary embodiment, the at least one bond which links the first antigen-binding domain and the second antigen-binding domain is formed by linking the amino acid residue at position 191 according to EU numbering in the CH1 region of the first antigen-binding domain and the amino acid residue at position 126 according to Kabat numbering in the CL region of the second antigen-binding domain.

[0100] In an embodiment of the above aspects, at least one of amino acid residues from which the bonds between the antigen-binding domains originate is present within a variable region. In certain embodiments, the amino acid residue is present within a VH region, and for example, it is present at a position selected from the group consisting of positions 6, 8, 16, 20, 25, 26, 28, 74, and 82b according to Kabat numbering in the VH region. In certain embodiments, the amino acid residue is present within a VL region, and for example, it is present at a position selected from the group consisting of positions 21, 27, 58, 77, 100, 105, and 107 according to Kabat numbering in the VL region (subclass kappa) and positions 6, 19, 33, and 34 according to Kabat numbering in the VL region (subclass lambda). In certain embodiments, the amino acid residue is present within a VHH region, and for example, it is present at a position selected from

the group consisting of positions 4, 6, 7, 8, 9, 10, 11, 12, 14, 15, 17, 20, 24, 27, 29, 38, 39, 40, 41, 43, 44, 45, 46, 47, 48, 49, 67, 69, 71, 78, 80, 82, 82c, 85, 88, 91, 93, 94, and 107 according to Kabat numbering in the VHH region.

[0101] In an embodiment of the above aspects, at least one of the first and second antigen-binding domains comprises a non-antibody protein binding to a particular antigen, or a fragment thereof. In certain embodiments, the non-antibody protein is either of a pair of a ligand and a receptor which specifically bind to each other. Such receptors include, for example, receptors belonging to cytokine receptor superfamilies, G protein-coupled receptors, ion channel receptors, tyrosine kinase receptors, immune checkpoint receptors, antigen receptors, CD antigens, costimulatory molecules, and cell adhesion molecules.

[0102] In an embodiment of the above aspects, the first and/or second antigen-binding domains comprise a hinge region. In certain embodiments, at least one of the cysteine residues present within a wild-type hinge region is substituted to another amino acid residue. Such cysteine residues are present, for example, at positions 226 and/or 229 according to EU numbering in the wild-type hinge region. In certain embodiments, at least one of amino acid residues from which the bonds between the antigen-binding domains originate is present within a hinge region and, for example, it is present at a position selected from the group consisting of positions 216, 218, and 219 according to EU numbering in the hinge region.

[0103] In an embodiment of the above aspects, the first antigen-binding domain and the second antigen-binding domain are linked with each other via two or more bonds.

[0104] In certain embodiments, at least one of amino acid residues from which the bonds between the antigen-binding domains originate is an amino acid residue present in a wild-type sequence and, for example, it is a cysteine residue in a wild-type hinge region. In certain embodiments, the at least one bond which links the first antigen-binding domain and the second antigen-binding domain is a disulfide bond formed by crosslinking of cysteine residues present within wild-type hinge regions with each other. Such cysteine residues are present, for example, at positions 226 and/or 229 according to EU numbering of a wild-type hinge region.

[0105] In certain embodiments, at least one of amino acid residues from which the bonds between the antigen-binding domains originate is present within an antibody fragment, and at least one is present within a hinge region. In an exemplary embodiment, the antigen-binding molecule of the present disclosure is F(ab')2 in which both the first and second antigen-binding domains comprise a Fab and a hinge region.

[0106] In an embodiment of the above aspects, the antigen-binding molecule of the present disclosure further comprises an Fc region, and for example, it is a full-length antibody. In certain embodiments, one or more amino acid mutations promoting multimerization

of Fc regions are introduced into the Fc region of the antigen-binding molecule of the present disclosure. Such amino acid mutations include, for example, the amino acid mutations at at least one position selected from the group consisting of positions 247, 248, 253, 254, 310, 311, 338, 345, 356, 359, 382, 385, 386, 430, 433, 434, 436, 437, 438, 439, 440, and 447 according to EU numbering (see, e.g., WO 2016/164480). In certain embodiments, the multimerization is hexamerization.

[0107] < Antigens bound by antigen-binding molecules >

In an embodiment of the above aspects, both the first and second antigen-binding domains bind to the same antigen. In certain embodiments, both the first and second antigen-binding domains bind to the same epitope on the same antigen. In certain other embodiments, each of the first and second antigen-binding domains binds to a different epitope on the same antigen. In certain embodiments, the antigen-binding molecule of the present disclosure is a biparatopic antigen-binding molecule (for example, a biparatopic antibody) that targets one specific antigen.

In another embodiment of the above aspects, each of the first and second antigen-binding domains binds to a different antigen.

In another embodiment of the above aspects, the antigen-binding molecule of the present disclosure is a clamping antigen-binding molecule (for example, a clamping antibody). A clamping antigen-binding molecule in the present specification means an antigen-binding molecule which specifically binds to an antigen/antigen-binding molecule complex formed between a given antigen A and an antigen-binding molecule which binds to antigen A, and which thereby increases the binding activity toward antigen A of the antigen-binding molecule that binds to antigen A (or alternatively, stabilizes the antigen/antigen-binding molecule complex formed by antigen A and the antigen-binding molecule that binds to antigen A). For example, a CD3 clamping antibody specifically binds to the antigen-antibody complex formed between CD3 and an antibody with reduced binding ability toward CD3 (binding-attenuated CD3 antibody) and can thereby increase the binding activity of the binding-attenuated CD3 antibody toward CD3 (or alternatively, stabilize the antigen-antibody complex formed by CD3 and the binding-attenuated CD3 antibody). In certain embodiments, the first and/or second antigen-binding domains in the antigen-binding molecule of the present disclosure can be antigen-binding domains (clamping antigen-binding domains) from clamping antigen-binding molecules.

In an embodiment of the above aspects, both the first and second antigen-binding domains have the same amino acid sequence. In another embodiment, each of the first and second antigen-binding domains has a different amino acid sequence.

[0108] In an embodiment of the above aspects, at least one of two antigens to which the first and second antigen-binding domains bind is a soluble protein or a membrane protein.

[0109] < Functions of antigen-binding molecules >

In an embodiment of the above aspects, the antigen-binding molecule of the present disclosure has activity of holding two antigen molecules at spatially close positions. In certain embodiments, the antigen-binding molecule of the present disclosure is capable of holding two antigen molecules at closer positions than a control antigen-binding molecule, and the control antigen-binding molecule differs from the antigen-binding molecule of the present disclosure only in that the control antigen-binding molecule has one less bond between the two antigen-binding domains. In a further embodiment, the one less bond can be selected from bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

In another embodiment of the above aspects, the antigen-binding molecule of the present disclosure has activity of regulating interaction between two antigen molecules. Without being bound by a particular theory, the activity of regulating interaction is thought to be resulted from holding two antigen molecules at spatially closer positions by the antigen-binding molecule of the present disclosure. In certain embodiments, the antigen-binding molecule of the present disclosure is capable of enhancing or diminishing interaction between two antigen molecules as compared to a control antigen-binding molecule, and the control antigen-binding molecule differs from the antigen-binding molecule of the present disclosure only in that the control antigen-binding molecule has one less bond between the two antigen-binding domains. In a further embodiment, the one less bond can be selected from bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

In certain embodiments, the two antigen molecules bound by the antigen-binding molecule of the present disclosure are a ligand and a receptor thereof, respectively, and the antigen-binding molecule of the present disclosure has activity of promoting activation of the receptor by the ligand. In certain other embodiment, the two antigen molecules bound by the antigen-binding molecule of the present disclosure are an enzyme and a substrate thereof, respectively, and the antigen-binding molecule of the present disclosure has activity of promoting catalytic reaction of the enzyme with the substrate.

Further, in certain other embodiments, both of the two antigen molecules bound by the antigen-binding molecule of the present disclosure are antigens (for example,

proteins) present on cellular surfaces, and the antigen-binding molecule of the present disclosure has activity of promoting interaction between a cell expressing the first antigen and a cell expressing the second antigen. For example, the cell expressing the first antigen and the cell expressing the second antigen are, respectively, a cell with cytotoxic activity and a target cell thereof, and the antigen-binding molecule of the present disclosure promotes damage of the target cell by the cell with cytotoxic activity. The cell with cytotoxic activity is, for example, a T cell, NK cell, monocyte, or macrophage.

[0110] In an embodiment of the above aspects, the antigen-binding molecule of the present disclosure has activity of regulating activation of two antigen molecules which are activated by association with each other. Without being bound by a particular theory, the activity of regulating activation is thought to be resulted from holding two antigen molecules at spatially closer positions by the antigen-binding molecule of the present disclosure. In certain embodiments, the antigen-binding molecule of the present disclosure can enhance or diminish activation of two antigen molecules as compared to a control antigen-binding molecule, and the control antigen-binding molecule differs from the antigen-binding molecule of the present disclosure only in that the control antigen-binding molecule has one less bond between the two antigen-binding domains. In a further embodiment, the one less bond can be selected from bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region). For example, such antigen molecules are selected from the group consisting of receptors belonging to cytokine receptor superfamilies, G protein-coupled receptors, ion channel receptors, tyrosine kinase receptors, immune checkpoint receptors, antigen receptors, CD antigens, costimulatory molecules, and cell adhesion molecules.

[0111] In an embodiment of the above aspects, in the antigen-binding molecule of the present disclosure, two antigen-binding domains are present at spatially close positions and/or the mobility of the two antigen-binding domains is reduced. In certain embodiments, as compared with a control antigen-binding molecule, the antigen-binding molecule of the present disclosure has two antigen-binding domains that are present at closer positions and/or the mobility of the two antigen-binding domains is more reduced, and the control antigen-binding molecule differs from the antigen-binding molecule of the present disclosure only in that it has one less bond between the two antigen-binding domains. In a further embodiment, the one less bond can be selected from bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from mutated amino acid residues

which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

[0112] In an embodiment of the above aspects, the antigen-binding molecule of the present disclosure has resistance to protease cleavage. In certain embodiments, the antigen-binding molecule of the present disclosure has increased resistance to protease cleavage as compared to a control antigen-binding molecule, and the control antigen-binding molecule differs from the antigen-binding molecule of the present disclosure only in that the control antigen-binding molecule has one less bond between the two antigen-binding domains. In a further embodiment, the one less bond can be selected from bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region). In certain embodiments, in the antigen-binding molecule of the present disclosure, the proportion of the full-length molecule (for example, full-length IgG molecule) remaining after protease treatment is increased as compared to the control antigen-binding molecule. In certain embodiments, in the antigen-binding molecule of the present disclosure, the proportion of a particular fragment (for example, Fab monomer) produced after protease treatment is reduced as compared to the control antigen-binding molecule.

[0113] In an embodiment of the above aspects, when the antigen-binding molecule of the present disclosure is treated with a protease, a dimer of the antigen-binding domains or fragments thereof (for example, crosslinked Fab dimer) is excised. In certain embodiments, when the control antigen-binding molecule, which differs from the antigen-binding molecule of the present disclosure only in that it has one less bond between the two antigen-binding domains, is treated with the protease, monomers of the antigen-binding domains or fragments thereof are excised. In a further embodiment, the one less bond can be selected from bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region). In these embodiments, the protease can cleave the hinge region of the antigen-binding molecule.

[0114] In a further embodiment, the control antigen-binding molecule differs from the antigen-binding molecule of the present disclosure only in that it has one less bond between the two antigen-binding domains, and the one less bond is a bond which is formed originating from mutated amino acid residues. The mutated amino acid residues are, for example, artificially introduced cysteine residues.

[0115] < Pharmaceutical compositions >

In an aspect, the present disclosure provides a pharmaceutical composition comprising the antigen-binding molecule of the present disclosure and a pharmaceutically acceptable carrier.

[0116] < Use of antigen-binding molecules >

In an aspect, the present disclosure provides a method for holding two antigen molecules at spatially close positions, comprising:

(a) providing an antigen-binding molecule comprising two antigen-binding domains,

(b) adding to the antigen-binding molecule at least one bond which links the two antigen-binding domains with each other, and

(c) contacting the antigen-binding molecule produced in (b) with the two antigen molecules. In certain embodiments, the two antigen-binding domains in the antigen-binding molecule recited in (a) above may be linked with each other via one or more bonds, and in this case, some or all of the one or more bonds are bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said at least one bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region). The present disclosure also provides a method for holding two antigen molecules at spatially close positions which comprises contacting two antigen molecules with the antigen-binding molecule or pharmaceutical composition of the present disclosure. The present disclosure further provides an antigen-binding molecule or pharmaceutical composition of the present disclosure for use in holding two antigen molecules at spatially close positions.

[0117] In another aspect, the present disclosure provides a method for regulating interaction between two antigen molecules, comprising:

(a) providing an antigen-binding molecule comprising two antigen-binding domains,

(b) adding to the antigen-binding molecule at least one bond which links the two antigen-binding domains with each other, and

(c) contacting the antigen-binding molecule produced in (b) with the two antigen molecules. In certain embodiments, the two antigen-binding domains in the antigen-binding molecule recited in (a) above may be linked with each other via one or more bonds, and in this case, some or all of the one or more bonds are bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further em-

bodiment, said at least one bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region). The present disclosure also provides a method for regulating interaction between two antigen molecules which comprises contacting two antigen molecules with the antigen-binding molecule or pharmaceutical composition of the present disclosure. The present disclosure further provides an antigen-binding molecule or pharmaceutical composition of the present disclosure for use in regulating interaction between two antigen molecules.

[0118] Further, in another aspect, the present disclosure provides a method for regulating activity of two antigen molecules which are activated by association with each other, comprising:

(a) providing an antigen-binding molecule comprising two antigen-binding domains,

(b) adding to the antigen-binding molecule at least one bond which links the two antigen-binding domains with each other, and

(c) contacting the antigen-binding molecule produced in (b) with the two antigen molecules. In certain embodiments, the two antigen-binding domains in the antigen-binding molecule recited in (a) above may be linked with each other via one or more bonds, and in this case, some or all of the one or more bonds are bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said at least one bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region). The present disclosure also provides a method for regulating activity of two antigen molecules which are activated by association with each other, which comprises contacting two antigen molecules with the antigen-binding molecule or pharmaceutical composition of the present disclosure. The present disclosure further provides an antigen-binding molecule or pharmaceutical composition of the present disclosure for use in regulating activity of two antigen molecules which are activated by association with each other.

[0119] Further, in another aspect, the present disclosure provides a method for placing two antigen-binding domains at spatially close positions and/or reducing the mobility of two antigen-binding domains, comprising:

(a) providing an antigen-binding molecule comprising two antigen-binding domains,

and

(b) adding to the antigen-binding molecule at least one bond which links the two antigen-binding domains with each other. In certain embodiments, the two antigen-binding domains in the antigen-binding molecule recited in (a) above may be linked with each other via one or more bonds, and in this case, some or all of the one or more bonds are bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said at least one bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

[0120] Furthermore, in another aspect, the present disclosure provides a method for increasing resistance of an antigen-binding molecule to protease cleavage, comprising:

(a) providing an antigen-binding molecule comprising two antigen-binding domains, and

(b) adding to the antigen-binding molecule at least one bond which links the two antigen-binding domains with each other. In certain embodiments, the two antigen-binding domains in the antigen-binding molecule recited in (a) above may be linked with each other via one or more bonds, and in this case, some or all of the one or more bonds are bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said at least one bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

[0121] The antigen-binding molecule used in these various methods may have the characteristics of the antigen-binding molecules described herein.

[0122] < Methods for producing antigen-binding molecules >

In an aspect, the present disclosure provides a method for producing an antigen-binding molecule which has activity of holding two antigen molecules at spatially close positions, comprising:

(a) providing a nucleic acid encoding a polypeptide comprising a first antigen-binding domain and a nucleic acid encoding a polypeptide comprising a second antigen-binding domain,

(b) introducing a mutation into the nucleic acids encoding the two antigen-binding domains such that at least one bond linking the two antigen-binding domains is added,
(c) introducing the nucleic acids produced in (b) into a host cell,
(d) culturing the host cell such that the two polypeptides are expressed, and
(e) obtaining an antigen-binding molecule which is a polypeptide comprising the first and second antigen-binding domains, wherein the two antigen-binding domains are linked with each other via one or more bonds; and preferably further comprising a step of contacting the antibody preparation with a reducing reagent.

[0123] In certain embodiments, said contacting with a reducing agent ("said contacting step") preferentially enriches or increases the population of an antibody structural isoform having at least one disulfide bond formed between amino acid residues which are not in a hinge region. In certain embodiments, said method produces a homogenous antibody preparation having at least 50%, 60%, 70%, 80%, 90%, preferably at least 95% molar ratio of said antibody having at least one disulfide bond formed between amino acid residues which are not in a hinge region.

[0124] In certain embodiments, the pH of said reducing reagent contacting with the antibody is from about 3 to about 10. In certain embodiments, the pH of said reducing reagent contacting with the antibody is about 6, 7 or 8. In some embodiments, the pH of said reducing reagent contacting with the antibody is about 7 or about 3.

[0125] In certain embodiments, the reducing agent is selected from the group consisting of TCEP, 2-MEA, DTT, Cysteine, GSH and Na_2SO_3. In some preferred embodiments, the reducing agent is TCEP. In certain embodiments, the concentration of the reducing agent is from about 0.01 mM to about 100 mM.

In some preferred embodiments, the concentration of the reducing agent is about 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100 mM, preferably about 0.01 mM to 25 mM. In one preferred embodiment, the reducing agent is 0.01 mM to 25 mM of TCEP.

[0126] In certain embodiments, the contacting step with a reducing agent is performed for at least 30 minutes. In certain embodiments, the contacting step is performed for about 2 to about 48 hours. In some preferred embodiments, the contacting step is performed for about 2 hours or about 16 hours.

[0127] In certain embodiments, the contacting step is performed at a temperature of about 20 degrees C to 37 degrees C, preferably at 23 degrees C, 25 degrees C or 37 degrees C, more preferably at 23 degrees C. In certain embodiments, said antibody is partially purified by affinity chromatography (preferably Protein A chromatography) prior to said contacting. In certain embodiments, the concentration of the antibody is from about 1 mg/ml and about 50 mg/ml. In some preferred embodiments, the concentration of the antibody is about 1 mg/ml or about 20 mg/ml.

[0128] In certain embodiments, said contacting step preferentially enriches or increases the population of an antibody structural isoform having at least one disulfide bond formed between amino acid residues which are not in a hinge region. In certain embodiments, said contacting step produces a homogenous antibody preparation having at least 50%, 60%, 70%, 80%, 90%, preferably at least 95% molar ratio of said antibody having at least one disulfide bond formed between amino acid residues which are not in a hinge region.

[0129] In certain embodiments, said contacting step produces an antibody preparation which is more homogeneous than the same antibody preparation that has not been treated by said method.

In certain embodiments, said contacting step produces an antibody preparation having increase in its biological activity compared to the same antibody that has not been treated by said method.

In certain embodiments, said contacting step produces an antibody having enhanced activity of holding two antigen molecules at spatially close positions compared to the same antibody that has not been treated by said method.

In certain embodiments, said contacting step produces an antibody having enhanced stability compared to the same antibody that has not been treated by said method.

[0130] In certain embodiments, said contacting step preferentially enriches antibody having at least one disulfide bond formed outside of hinge regions and said preferentially enriched form has a pharmaceutically desirable property selected from any of (a) to (e) below, as compared to a preparation that has not been treated by said contacting step:

(a) wherein said at least one disulfide bond restricts the antigen binding orientation of the two antigen-binding domains to cis antigen-binding (i.e. binding to two antigens on the same cell), or restrict binding of the two antigen binding domains to two antigens which are spatially close to each other;

(b) wherein said at least one disulfide bond holds the first antigen-binding domain and the second antigen-binding domain spatially closer to each other, as compared to a same corresponding antibody which does not have said at least one disulfide bond;

(c) wherein said at least one disulfide bond reduce the flexibility and/or mobility of first antigen-binding domain and the second antigen-binding domain, as compared to a corresponding same antibody which does not have said at least one disulfide bond;

(d) wherein said at least one disulfide bond increases resistance of the antibody to protease cleavage, as compared to a corresponding same antibody which does not have said at least one disulfide bond; or

(e) wherein said at least one disulfide bond enhances or reduces interaction between two antigen molecules bound by the antigen-binding molecule, as compared to a corresponding same antibody which does not have said at least one disulfide bond.

In certain embodiments, each of the two antigen-binding domains recited in (a) above may comprise one or more amino acid residues from which the bonds for linking the two antigen-binding domains originate, and in this case, some or all of the one or more amino acid residues from which the bond between the antigen-binding domains originates are amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said at least one bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

[0131] In another aspect, the present disclosure provides a method for producing an antigen-binding molecule which has activity of regulating interaction between two antigen molecules, comprising:

(a) providing a nucleic acid encoding a polypeptide comprising a first antigen-binding domain and a nucleic acid encoding a polypeptide comprising a second antigen-binding domain,

(b) introducing a mutation into the nucleic acids encoding the two antigen-binding domains such that at least one bond linking the two antigen-binding domains is added,

(c) introducing the nucleic acids produced in (b) into a host cell,

(d) culturing the host cell such that the two polypeptides are expressed, and

(e) obtaining an antigen-binding molecule which is a polypeptide comprising the first and second antigen-binding domains, wherein the two antigen-binding domains are linked with each other via one or more bonds.

In certain embodiments, each of the two antigen-binding domains recited in (a) above may comprise one or more amino acid residues from which the bonds for linking the two antigen-binding domains originate, and in this case, some or all of the one or more amino acid residues from which the bond between the antigen-binding domains originates are amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said at least one bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

[0132] Further, in another aspect, the present disclosure provides a method for producing an antigen-binding molecule which has activity of regulating activation of two antigen molecules which are activated by association with each other, comprising:

(a) providing a nucleic acid encoding a polypeptide comprising a first antigen-binding domain and a nucleic acid encoding a polypeptide comprising a second antigen-binding domain,
(b) introducing a mutation into the nucleic acids encoding the two antigen-binding domains such that at least one bond linking the two antigen-binding domains is added,
(c) introducing the nucleic acids produced in (b) into a host cell,
(d) culturing the host cell such that the two polypeptides are expressed, and
(e) obtaining an antigen-binding molecule which is a polypeptide comprising the first and second antigen-binding domains, wherein the two antigen-binding domains are linked with each other via one or more bonds.
In certain embodiments, each of the two antigen-binding domains recited in (a) above may comprise one or more amino acid residues from which the bonds for linking the two antigen-binding domains originate, and in this case, some or all of the one or more amino acid residues from which the bond between the antigen-binding domains originates are amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said at least one bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

[0133] Further, in another aspect, the present disclosure provides a method for producing an antigen-binding molecule in which two antigen-binding domains are present at spatially close positions and/or the mobility of two antigen-binding domains is reduced, comprising:

(a) providing a nucleic acid encoding a polypeptide comprising a first antigen-binding domain and a nucleic acid encoding a polypeptide comprising a second antigen-binding domain,

(b) introducing a mutation into the nucleic acids encoding the two antigen-binding domains such that at least one bond linking the two antigen-binding domains is added,

(c) introducing the nucleic acids produced in (b) into a host cell,

(d) culturing the host cell such that the two polypeptides are expressed, and

(e) obtaining an antigen-binding molecule which is a polypeptide comprising the first and second antigen-binding domains, wherein the two antigen-binding domains are linked with each other via one or more bonds.

In certain embodiments, each of the two antigen-binding domains recited in (a) above may comprise one or more amino acid residues from which the bonds for linking the two antigen-binding domains originate, and in this case, some or all of the

one or more amino acid residues from which the bond between the antigen-binding domains originates are amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said at least one bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

[0134] Furthermore, in another aspect, the present disclosure provides a method for producing an antigen-binding molecule which has increased resistance to protease cleavage, comprising:

(a) providing a nucleic acid encoding a polypeptide comprising a first antigen-binding domain and a nucleic acid encoding a polypeptide comprising a second antigen-binding domain,

(b) introducing a mutation into the nucleic acids encoding the two antigen-binding domains such that at least one bond linking the two antigen-binding domains is added,

(c) introducing the nucleic acids produced in (b) into a host cell,

(d) culturing the host cell such that the two polypeptides are expressed, and

(e) obtaining an antigen-binding molecule which is a polypeptide comprising the first and second antigen-binding domains, wherein the two antigen-binding domains are linked with each other via one or more bonds.

In certain embodiments, each of the two antigen-binding domains recited in (a) above may comprise one or more amino acid residues from which the bonds for linking the two antigen-binding domains originate, and in this case, some or all of the one or more amino acid residues from which the bond between the antigen-binding domains originates are amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said at least one bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

[0135] The antigen-binding molecule produced in these various aspects may have the characteristics of the antigen-binding molecules described herein.

[0136] < Methods of screening for antigen-binding molecules >

In another aspect, the present disclosure provides a method for identifying a novel pair of protein molecules which are activated by association with each other, comprising:

(a) providing two arbitrary protein molecules,
(b) producing, by the production method of the present disclosure, an antigen-binding molecule comprising two antigen-binding domains which respectively bind to the two protein molecules,
(c) contacting the antigen-binding molecule produced in (b) with the two protein molecules, and
(d) assessing whether or not the two protein molecules are activated.
In certain embodiments, at least one of the two protein molecules is selected from the group consisting of receptors belonging to cytokine receptor superfamilies, G protein-coupled receptors, ion channel receptors, tyrosine kinase receptors, immune checkpoint receptors, antigen receptors, CD antigens, costimulatory molecules, and cell adhesion molecules.

[0137] A. Exemplary antigen-binding molecules

< Structures of antigen-binding molecules >

In an aspect, the present disclosure provides an antigen-binding molecule comprising a first antigen-binding domain and a second antigen-binding domain, and the antigen-binding domains are linked with each other via two or more bonds. In an embodiment, at least one of the first and second antigen-binding domains has, by itself, activity of binding to an antigen (i.e., a single antigen-binding domain independently has antigen-binding activity). In certain embodiments, each of the first and second antigen-binding domains has, by itself, activity of binding to an antigen.

[0138] In an embodiment of the above aspects, at least one of the first and second antigen-binding domains comprises an antibody fragment which binds to a particular antigen. In certain embodiments, the first and/or second antigen-binding domains comprise a hinge region. Amino acid residues from which the bonds between the antigen-binding domains originate are respectively present in the first and second antigen-binding domains, and the bonds between the antigen-binding domains are formed by linking these amino acid residues. In certain embodiments, at least one of amino acid residues from which the bonds between the antigen-binding domains originate is present within the antibody fragment. In certain embodiments, at least one of amino acid residues from which the bonds between the antigen-binding domains originate is present within a hinge region. In certain embodiments, at least one of amino acid residues from which the bonds between the antigen-binding domains originate is present within the antibody fragment, and at least one of the amino acid residues is present within a hinge region.

[0139] In an embodiment of the above aspects, in at least one of the first and second antigen-binding domains, multiple amino acid residues from which the bonds between the antigen-binding domains originate are present at positions at a distance of seven

amino acids or more from each other in the primary structure. This means that, between any two amino acid residues of the above multiple amino acid residues, six or more amino acid residues which are not said amino acid residues are present. In certain embodiments, combinations of multiple amino acid residues from which the bonds between the antigen-binding domains originate include a pair of amino acid residues which are present at positions at a distance of less than seven amino acids in the primary structure. In certain embodiments, if the first and second antigen-binding domains are linked each other via three or more bonds, the bonds between the antigen-binding domains may originate from three or more amino acid residues including a pair of amino acid residues which are present at positions at a distance of seven amino acids or more in the primary structure.

In certain embodiments, amino acid residues present at the same position in the first antigen-binding domain and in the second antigen-binding domain are linked with each other to form a bond. In certain embodiments, amino acid residues present at a different position in the first antigen-binding domain and in the second antigen-binding domain are linked with each other to form a bond.

[0140] Positions of amino acid residues in the antigen-binding domain can be shown according to the Kabat numbering or EU numbering system (also called the EU index) described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991. For example, if the amino acid residues from which the bonds between the first and second antigen-binding domains originate are present at an identical position corresponding in the antigen-binding domains, the position of these amino acid residues can be indicated as the same number according to the Kabat numbering or EU numbering system. Alternatively, if the amino acid residues from which the bonds between the first and second antigen-binding domains originate are present at different positions which are not corresponding in the antigen-binding domains, the positions of these amino acid residues can be indicated as different numbers according to the Kabat numbering or EU numbering system.

[0141] In an embodiment of the above aspects, at least one of the two or more bonds linking the antigen-binding domains is a covalent bond. In certain embodiments, the covalent bond is formed by direct crosslinking of an amino acid residue in the first antigen-binding domain and an amino acid residue in the second antigen-binding domain. The crosslinked amino acid residues are, for example, cysteine, and the formed covalent bond is, for example, a disulfide bond. At least one of the crosslinked cysteine residues may be present within a hinge region.

In certain other embodiments, the covalent bond is formed by crosslinking of an amino acid residue in the first antigen-binding domain and an amino acid residue in the

second antigen-binding domain via a crosslinking agent. The crosslinking agent is, for example, an amine-reactive crosslinking agent, and the crosslinked amino acid residues are, for example, lysine.

[0142] In an embodiment of the above aspects, at least one of the two or more bonds linking the antigen-binding domains is a noncovalent bond. In certain embodiments, the non-covalent bond is an ionic bond, hydrogen bond, or hydrophobic bond.

[0143] In an embodiment of the above aspects, the antibody fragment is a Fab, Fab', scFab, Fv, scFv, or single domain antibody.

[0144] In an embodiment of the above aspects, at least one of amino acid residues from which the bonds between the antigen-binding domains originate is present within a constant region. In certain embodiments, the amino acid residue is present within a CH1 region, and for example, it is present at a position selected from the group consisting of positions 119, 122, 123, 131, 132, 133, 134, 135, 136, 137, 139, 140, 148, 150, 155, 156, 157, 159, 160, 161, 162, 163, 165, 167, 174, 176, 177, 178, 190, 191, 192, 194, 195, 197, 213, and 214 according to EU numbering in the CH1 region. In an exemplary embodiment, the amino acid residue is present at position 191 according to EU numbering in the CH1 region, and the amino acid residues at position 191 according to EU numbering in the CH1 region of the two antigen-binding domains are linked with each other to form a bond.

In some embodiments of the above aspects, one disulfide bond is formed between the amino acid residues at position 191 according to EU numbering in the respective CH1 regions of the first antigen-binding domain and the second antigen-binding domain.

In some embodiments of the above aspects, additional one, two or more disulfide bond(s) is/are formed between the first antigen-binding domain and the second antigen-binding domain via the amino acid residues at the following positions according to EU numbering in each of the respective CH1 regions of the first antigen-binding domain and the second antigen-binding domain:

(a) between amino acid residues at any position of 131 to 138, 194 and 195 in each of the two antigen-binding domains;

(b) between the amino acid residues at position 131 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;

(c) between the amino acid residues at position 132 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;

(d) between the amino acid residues at position 133 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;

(e) between the amino acid residues at position 134 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;
(f) between the amino acid residues at position 135 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;
(g) between the amino acid residues at position 136 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;
(h) between the amino acid residues at position 137 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;
(i) between the amino acid residues at position 138 in each of the two antigen-binding domains, and between the amino acid residues at position 194 in each of the two antigen-binding domains;
(j) between the amino acid residues at position 131 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;
(k) between the amino acid residues at position 132 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;
(l) between the amino acid residues at position 133 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;
(m) between the amino acid residues at position 134 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;
(n) between the amino acid residues at position 135 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;
(o) between the amino acid residues at position 136 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains;
(p) between the amino acid residues at position 137 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two antigen-binding domains; and
(q) between the amino acid residues at position 138 in each of the two antigen-binding domains, and between the amino acid residues at position 195 in each of the two

antigen-binding domains.

In some embodiments of the above aspects, any one of the first and second antigen-binding domains comprises one, two or more charged amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more oppositely charged amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

In some embodiments of the above aspects, any one of the first and second antigen-binding domains comprises one, two or more positively charged amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more negatively charged amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

In some embodiments of the above aspects, any one of the first and second antigen-binding domains comprises one, two or more negatively charged amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more positively charged amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

In some embodiments of the above aspects, any one of the first and second antigen-binding domains comprises one, two or more of the following amino acid residues in the respective CH1 region (according to EU numbering):

(a) the amino acid residue at position 136 which is glutamic acid (E) or aspartic acid (D);

(b) the amino acid residue at position 137 which is glutamic acid (E) or aspartic acid (D);

(c) the amino acid residue at position 138 which is glutamic acid (E) or aspartic acid (D); and

the other antigen-binding domain of the first and second antigen-binding domains comprises one, two or more of the following amino acid residues in the respective CH1 region (according to EU numbering):

(d) the amino acid residue at position 193 which is lysine (K), arginine (R), or histidine (H);

(e) the amino acid residue at position 194 which is lysine (K), arginine (R), or histidine (H); and

(f) the amino acid residue at position 195 which is lysine (K), arginine (R), or histidine (H).

In some embodiments of the above as aspects, any one of the first and second antigen-

binding domains comprises one or more of the following amino acid residues in the respective CH1 region (according to EU numbering):

(a) the amino acid residue at position 136 which is lysine (K), arginine (R), or histidine (H);

(b) the amino acid residue at position 137 which is lysine (K), arginine (R), or histidine (H);

(c) the amino acid residue at position 138 which is lysine (K), arginine (R), or histidine (H); and

the other antigen-binding domain of the first and second antigen-binding domains comprises one or more of the following amino acid residues in the respective CH1 region (according to EU numbering):

(d) the amino acid residue at position 193 which is glutamic acid (E) or aspartic acid (D);

(e) the amino acid residue at position 194 which is glutamic acid (E) or aspartic acid (D); and

(f) the amino acid residue at position 195 which is glutamic acid (E) or aspartic acid (D).

In some embodiments of the above aspects, any one of the first and second antigen-binding domains comprises one, two or more hydrophobic amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more hydrophobic amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region.

In some embodiments of the above aspects, the hydrophobic amino acid residue(s) is/are alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), and/or tryptophan (Trp).

[0145] In some embodiments of the above aspects, any one of the first and second antigen-binding domains comprises one "knob" amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more "hole" amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region. In some embodiments of the above aspects, any one of the first and second antigen-binding domains comprises one, two or more "hole" amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one "knob" amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region. In some embodiments, said "knob" amino acid residue(s) is/are selected from the group consisting

of tryptophan (Trp) and phenylalanine (Phe); and said "hole" amino acid residue(s) is/are selected from the group consisting of alanine (Ala), valine (Val), threonine (T) or serine (S).

[0146] In some embodiments of the above aspects, any one of the first and second antigen-binding domains comprises one, two or more aromatic amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more positively charged amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region. In some embodiments of the above aspects, any one of the first and second antigen-binding domains comprises one, two or more positively charged amino acid residues at position 136-138 (according to EU numbering) in the respective CH1 region; and the other antigen-binding domain out of the first and second antigen-binding domains comprises one, two or more aromatic amino acid residues at position 193-195 (according to EU numbering) in the respective CH1 region. In some embodiments, said aromatic amino acid residue(s) is/are selected from the group consisting of tryptophan (Trp), tyrosine (Tyr), histidine (His), and phenylalanine (Phe); and said positively charged amino acid residue(s) is/are selected from a group consisting of lysine (K), arginine (R), or histidine (H).

In certain embodiments, at least one of amino acid residues from which the bonds between the antigen-binding domains originate is present within a hinge region, and for example, it is present at a position selected from the group consisting of positions 216, 218, and 219 according to EU numbering in the hinge region.

In certain embodiments, at least one of amino acid residues from which the bonds between the antigen-binding domains originate is present within a CL region, and for example, it is present at a position selected from the group consisting of positions 109, 112, 121, 126, 128, 151, 152, 153, 156, 184, 186, 188, 190, 200, 201, 202, 203, 208, 210, 211, 212, and 213 according to EU numbering in the CL region. In an exemplary embodiment, the amino acid residue is present at position 126 according to EU numbering in the CL region, and the amino acid residues at position 126 according to EU numbering in the CL region of the two antigen-binding domains are linked with each other to form a bond.

In certain embodiments, an amino acid residue in the CH1 region of the first antigen-binding domain and an amino acid residue in the CL region of the second antigen-binding domain are linked to form a bond. In an exemplary embodiment, an amino acid residue at position 191 according to EU numbering in the CH1 region of the first antigen-binding domain and an amino acid residue at position 126 according to EU numbering in the CL region of the second antigen-binding domain are linked to form a

bond.

[0147] In an embodiment of the above aspects, the constant region is derived from human. In certain embodiments, the subclass of the heavy chain constant region is any of IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgM, IgD, and IgE. In certain embodiments, the subclass of the CH1 region is any of gamma 1, gamma 2, gamma 3, gamma 4, alpha 1, alpha 2, mu, delta, and epsilon. In certain embodiments, the subclass of the CL region is kappa or lambda.

[0148] In an embodiment of the above aspects, at least one of amino acid residues from which the bonds between the antigen-binding domains originate is present within a variable region. In certain embodiments, the amino acid residue is present within a VH region, and for example, it is present at a position selected from the group consisting of positions 8, 16, 28, 74, and 82b according to Kabat numbering in the VH region. In certain embodiments, the amino acid residue is present within a VL region, and for example, it is present at a position selected from the group consisting of positions 100, 105, and 107 according to Kabat numbering in the VL region.

[0149] In an embodiment of the above aspects, both the first and second antigen-binding domains comprise a Fab and a hinge region.

In certain embodiments, at least one of amino acid residues from which the bonds between the antigen-binding domains originate is an amino acid residue present in a wild-type Fab or hinge region, and for example, it is a cysteine residue in the hinge region. Examples of such cysteine residues include the cysteine residues at positions 226 and 229 according to EU numbering.

In certain other embodiments, at least one of amino acid residues from which the bonds between the antigen-binding domains originate is a mutated amino acid residue which is not present in a wild-type Fab or hinge region, and for example, it is a cysteine residue which is not present in a wild-type Fab or hinge region. Such a mutated amino acid residue can be introduced into a wild-type Fab or hinge region by, for example, a method of amino acid substitution. The present specification discloses the sites of amino acid residues from which the bonds between the antigen-binding domains can originate for each of the CH1, hinge, CL, VH, and VL regions, and for example, cysteine residues can be introduced into such sites.

Alternatively, in another embodiment, an amino acid residue that is present in a wild-type Fab or hinge region and which is involved in a bond between the antigen-binding domains (for example, a cysteine residue) can be substituted with another amino acid or deleted. Examples of such cysteine residues include the cysteine residues at positions 220, 226, and 229 according to EU numbering in the hinge region, and the cysteine residue at position 214 in the CL region.

In certain embodiments, the antigen-binding molecule of the present disclosure is

F(ab')2 in which both the first and second antigen-binding domains comprise a Fab and a hinge region.

[0150] In an embodiment of the above aspects, at least one of the first and second antigen-binding domains comprises a non-antibody protein binding to a particular antigen, or a fragment thereof. In certain embodiments, the non-antibody protein is either of a pair of a ligand and a receptor which specifically bind to each other. Such receptors include, for example, receptors belonging to cytokine receptor superfamilies, G protein-coupled receptors, ion channel receptors, tyrosine kinase receptors, immune checkpoint receptors, antigen receptors, CD antigens, costimulatory molecules, and cell adhesion molecules.

[0151] In an embodiment of the above aspects, the antigen-binding molecule of the present disclosure further comprises an Fc region, and for example, it is a full-length antibody. In certain embodiments, one or more amino acid mutations promoting multimerization of Fc regions are introduced into the Fc region of the antigen-binding molecule of the present disclosure. Such amino acid mutations include, for example, the amino acid mutations at at least one position selected from the group consisting of positions 247, 248, 253, 254, 310, 311, 338, 345, 356, 359, 382, 385, 386, 430, 433, 434, 436, 437, 438, 439, 440, and 447 according to EU numbering (see, e.g., WO 2016/164480). In certain embodiments, the multimerization is hexamerization.

[0152] < Antigens bound by antigen-binding molecules >

In an embodiment of the above aspects, both the first and second antigen-binding domains bind to the same antigen. In certain embodiments, both the first and second antigen-binding domains bind to the same epitope on the same antigen. In certain other embodiments, each of the first and second antigen-binding domains binds to a different epitope on the same antigen. In certain embodiments, the antigen-binding molecule of the present disclosure is a biparatopic antigen-binding molecule (for example, bi-paratopic antibody) that targets one specific antigen.

In an embodiment of the above aspects, each of the first and second antigen-binding domains binds to a different antigen.

In another embodiment of the above aspects, the antigen-binding molecule of the present disclosure is a clamping antigen-binding molecule (for example, clamping antibody). Herein, a clamping antigen-binding molecule refers to an antigen-binding molecule that specifically binds to an antigen/antigen-binding molecule complex formed by a certain antigen A and an antigen-binding molecule binding to the antigen A, and thereby increases the activity of the antigen-binding molecule binding to the antigen A to bind the antigen A (or stabilize the antigen/antigen-binding molecule complex formed by the antigen A and the antigen-binding molecule binding to the antigen A). For example, a CD3 clamping antibody is able to bind to an antigen-

antibody complex formed by CD3 and an antibody with attenuated binding ability to CD3 (binding-attenuated CD3 antibody), and thereby increase the CD3-binding activity of the binding-attenuated CD3 antibody (or stabilize the antigen-antibody complex formed by CD3 and the binding-attenuated CD3 antibody). In certain embodiments, the first and/or second antigen-binding domains in the antigen-binding molecule of the present disclosure may be antigen-binding domains derived from clamping antigen-binding molecules (clamping antigen-binding domains).
In an embodiment of the above aspects, both the first and second antigen-binding domains have the same amino acid sequence. In another embodiment, each of the first and second antigen-binding domains has a different amino acid sequence.

[0153] In an embodiment of the above aspects, at least one of two antigens to which the first and second antigen-binding domains bind is a soluble protein or a membrane protein.

[0154] < Functions of antigen-binding molecules >

In an embodiment of the above aspects, the antigen-binding molecule of the present disclosure has activity of holding two antigen molecules at spatially close positions. In certain embodiments, the antigen-binding molecule of the present disclosure is capable of holding two antigen molecules at closer positions than a control antigen-binding molecule, and the control antigen-binding molecule differs from the antigen-binding molecule of the present disclosure only in that the control antigen-binding molecule has one less bond between the two antigen-binding domains. In a further embodiment, the one less bond can be selected from bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

[0155] In an embodiment of the above aspects, the antigen-binding molecule of the present disclosure has activity of regulating interaction between two antigen molecules. Without being bound by a particular theory, the activity of regulating interaction is thought to be resulted from holding two antigen molecules at spatially closer positions by the antigen-binding molecule of the present disclosure. In certain embodiments, the antigen-binding molecule of the present disclosure is capable of enhancing or diminishing interaction between two antigen molecules as compared to a control antigen-binding molecule, and the control antigen-binding molecule differs from the antigen-binding molecule of the present disclosure only in that the control antigen-binding molecule has one less bond between the two antigen-binding domains. In a further embodiment, the one less bond can be selected from bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from mutated amino acid residues which are not present in a wild-type Fab or

hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

[0156] In certain embodiments, the two antigen molecules bound by the antigen-binding molecule of the present disclosure are a ligand and a receptor thereof, respectively, and the antigen-binding molecule of the present disclosure has activity of promoting activation of the receptor by the ligand. In certain other embodiment, the two antigen molecules bound by the antigen-binding molecule of the present disclosure are an enzyme and a substrate thereof, respectively, and the antigen-binding molecule of the present disclosure has activity of promoting catalytic reaction of the enzyme with the substrate.

[0157] Further, in certain other embodiments, both of the two antigen molecules bound by the antigen-binding molecule of the present disclosure are antigens (for example, proteins) present on cellular surfaces, and the antigen-binding molecule of the present disclosure has activity of promoting interaction between a cell expressing the first antigen and a cell expressing the second antigen. For example, the cell expressing the first antigen and the cell expressing the second antigen are, respectively, a cell with cytotoxic activity and a target cell thereof, and the antigen-binding molecule of the present disclosure promotes damage of the target cell by the cell with cytotoxic activity. The cell with cytotoxic activity is, for example, a T cell, NK cell, monocyte, or macrophage.

[0158] In an embodiment of the above aspects, the antigen-binding molecule of the present disclosure has activity of regulating activation of two antigen molecules which are activated by association with each other. Without being bound by a particular theory, the activity of regulating activation is thought to be resulted from holding two antigen molecules at spatially closer positions by the antigen-binding molecule of the present disclosure. In certain embodiments, the antigen-binding molecule of the present disclosure can enhance or diminish activation of two antigen molecules as compared to a control antigen-binding molecule, and the control antigen-binding molecule differs from the antigen-binding molecule of the present disclosure only in that the control antigen-binding molecule has one less bond between the two antigen-binding domains. In a further embodiment, the one less bond can be selected from bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region). For example, such antigen molecules are selected from the group consisting of receptors belonging to cytokine receptor superfamilies, G protein-coupled receptors, ion channel receptors, tyrosine kinase receptors, immune checkpoint receptors, antigen receptors, CD antigens, costimulatory molecules, and

cell adhesion molecules.

[0159] In an embodiment of the above aspects, the antigen-binding molecule of the present disclosure has resistance to protease cleavage. In certain embodiments, the antigen-binding molecule of the present disclosure has increased resistance to protease cleavage as compared to a control antigen-binding molecule, and the control antigen-binding molecule differs from the antigen-binding molecule of the present disclosure only in that the control antigen-binding molecule has one less bond between the two antigen-binding domains. In a further embodiment, the one less bond can be selected from bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region). In certain embodiments, in the antigen-binding molecule of the present disclosure, the proportion of the full-length molecule (for example, full-length IgG molecule) remaining after protease treatment is increased as compared to the control antigen-binding molecule. In certain embodiments, in the antigen-binding molecule of the present disclosure, the proportion of a particular fragment (for example, Fab monomer) produced after protease treatment is reduced as compared to the control antigen-binding molecule.

[0160] In an embodiment of the above aspects, when the antigen-binding molecule of the present disclosure is treated with a protease, a dimer of the antigen-binding domains or fragments thereof (for example, crosslinked Fab dimer) is excised. In certain embodiments, when the control antigen-binding molecule, which differs from the antigen-binding molecule of the present disclosure only in that it has one less bond between the two antigen-binding domains, is treated with the protease, monomers of the antigen-binding domains or fragments thereof are excised. In a further embodiment, the one less bond can be selected from bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region). In these embodiments, the protease can cleave the hinge region of the antigen-binding molecule.

[0161] < Pharmaceutical compositions >

In an aspect, the present disclosure provides a pharmaceutical composition comprising the antigen-binding molecule of the present disclosure and a pharmaceutically acceptable carrier.

[0162] < Use of antigen-binding molecules >

In an aspect, the present disclosure provides a method for holding two antigen molecules at spatially close positions, comprising:

(a) providing an antigen-binding molecule comprising two antigen-binding domains, wherein the two antigen-binding domains are linked with each other via one or more bonds,

(b) adding to the antigen-binding molecule another bond which links the two antigen-binding domains with each other, and

(c) contacting the antigen-binding molecule produced in (b) with the two antigen-binding molecules.

In certain embodiments, some or all of the one or more bonds recited in (a) above are bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said another bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region). The present disclosure also provides a method for holding two antigen molecules at spatially close positions which comprises contacting two antigen molecules with the antigen-binding molecule or pharmaceutical composition of the present disclosure. The present disclosure further provides an antigen-binding molecule or pharmaceutical composition of the present disclosure for use in holding two antigen molecules at spatially close positions.

[0163] In another aspect, the present disclosure provides a method for regulating interaction between two antigen molecules, comprising:

(a) providing an antigen-binding molecule comprising two antigen-binding domains, wherein the two antigen-binding domains are linked with each other via one or more bonds,

(b) adding to the antigen-binding molecule another bond which links the two antigen-binding domains with each other, and

(c) contacting the antigen-binding molecule produced in (b) with the two antigen-binding molecules.

In certain embodiments, some or all of the one or more bonds recited in (a) above are bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said another bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in

the wild-type Fab or hinge region). The present disclosure also provides a method for regulating interaction between two antigen molecules which comprises contacting two antigen molecules with the antigen-binding molecule or pharmaceutical composition of the present disclosure. The present disclosure further provides an antigen-binding molecule or pharmaceutical composition of the present disclosure for use in regulating interaction between two antigen molecules.

[0164] Further, in another aspect, the present disclosure provides a method for regulating activity of two antigen molecules which are activated by association with each other, comprising:

(a) providing an antigen-binding molecule comprising two antigen-binding domains, wherein the two antigen-binding domains are linked with each other via one or more bonds,

(b) adding to the antigen-binding molecule another bond which links the two antigen-binding domains with each other, and

(c) contacting the antigen-binding molecule produced in (b) with the two antigen-binding molecules.

In certain embodiments, some or all of the one or more bonds recited in (a) above are bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said another bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region). The present disclosure also provides a method for regulating activity of two antigen molecules which are activated by association with each other, which comprises contacting two antigen molecules with the antigen-binding molecule or pharmaceutical composition of the present disclosure. The present disclosure further provides an antigen-binding molecule or pharmaceutical composition of the present disclosure for use in regulating activity of two antigen molecules which are activated by association with each other.

[0165] Furthermore, in another aspect, the present disclosure provides a method for increasing resistance of an antigen-binding molecule to protease cleavage, comprising:

(a) providing an antigen-binding molecule comprising two antigen-binding domains, wherein the two antigen-binding domains are linked with each other via one or more bonds, and

(b) adding to the antigen-binding molecule another bond which links the two antigen-binding domains with each other.

In certain embodiments, some or all of the one or more bonds recited in (a) above are bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said another bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

[0166] The antigen-binding molecule used in these various methods may have the characteristics of the antigen-binding molecules described herein.

[0167] < Methods for producing antigen-binding molecules >

In an aspect, the present disclosure provides a method for producing an antigen-binding molecule which has activity of holding two antigen molecules at spatially close positions, comprising:

(a) providing a nucleic acid encoding a polypeptide comprising a first antigen-binding domain and a nucleic acid encoding a polypeptide comprising a second antigen-binding domain, wherein each of the two antigen-binding domains comprises one or more amino acid residues from which a bond for linking the two antigen-binding domains originates,

(b) introducing a mutation into the nucleic acids encoding the two antigen-binding domains such that another bond linking the two antigen-binding domains is added,

(c) introducing the nucleic acids produced in (b) into a host cell,

(d) culturing the host cell such that the two polypeptides are expressed, and

(e) obtaining an antigen-binding molecule which is a polypeptide comprising the first and second antigen-binding domains, wherein the two antigen-binding domains are linked with each other via two or more bonds.

In certain embodiments, some or all of the one or more amino acid residues recited in (a) above from which the bond between the antigen-binding domains originates are amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said another bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

[0168] In another aspect, the present disclosure provides a method for producing an antigen-binding molecule which has activity of regulating interaction between two antigen molecules, comprising:

(a) providing a nucleic acid encoding a polypeptide comprising a first antigen-binding domain and a nucleic acid encoding a polypeptide comprising a second antigen-binding domain, wherein each of the two antigen-binding domains comprises one or more amino acid residues from which a bond for linking the two antigen-binding domains originates,
(b) introducing a mutation into the nucleic acids encoding the two antigen-binding domains such that another bond linking the two antigen-binding domains is added,
(c) introducing the nucleic acids produced in (b) into a host cell,
(d) culturing the host cell such that the two polypeptides are expressed, and
(e) obtaining an antigen-binding molecule which is a polypeptide comprising the first and second antigen-binding domains, wherein the two antigen-binding domains are linked with each other via two or more bonds.
In certain embodiments, some or all of the one or more amino acid residues recited in (a) above from which the bond between the antigen-binding domains originates are amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said another bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

[0169] Further, in another aspect, the present disclosure provides a method for producing an antigen-binding molecule which has activity of regulating activation of two antigen molecules which are activated by association with each other, comprising:

(a) providing a nucleic acid encoding a polypeptide comprising a first antigen-binding domain and a nucleic acid encoding a polypeptide comprising a second antigen-binding domain, wherein each of the two antigen-binding domains comprises one or more amino acid residues from which a bond for linking the two antigen-binding domains originates,

(b) introducing a mutation into the nucleic acids encoding the two antigen-binding domains such that another bond linking the two antigen-binding domains is added,

(c) introducing the nucleic acids produced in (b) into a host cell,

(d) culturing the host cell such that the two polypeptides are expressed, and

(e) obtaining an antigen-binding molecule which is a polypeptide comprising the first and second antigen-binding domains, wherein the two antigen-binding domains are linked with each other via two or more bonds.

In certain embodiments, some or all of the one or more amino acid residues recited in (a) above from which the bond between the antigen-binding domains originates are amino acid residues which are present in a wild-type Fab or hinge region (for example,

cysteine residues in the hinge region). In a further embodiment, said another bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

[0170] Furthermore, in another aspect, the present disclosure provides a method for producing an antigen-binding molecule which has increased resistance to protease cleavage, comprising:

(a) providing a nucleic acid encoding a polypeptide comprising a first antigen-binding domain and a nucleic acid encoding a polypeptide comprising a second antigen-binding domain, wherein each of the two antigen-binding domains comprises one or more amino acid residues from which a bond for linking the two antigen-binding domains originates,

(b) introducing a mutation into the nucleic acids encoding the two antigen-binding domains such that another bond linking the two antigen-binding domains is added,

(c) introducing the nucleic acids produced in (b) into a host cell,

(d) culturing the host cell such that the two polypeptides are expressed, and

(e) obtaining an antigen-binding molecule which is a polypeptide comprising the first and second antigen-binding domains, wherein the two antigen-binding domains are linked with each other via two or more bonds.

In certain embodiments, some or all of the one or more amino acid residues recited in (a) above from which the bond between the antigen-binding domains originates are amino acid residues which are present in a wild-type Fab or hinge region (for example, cysteine residues in the hinge region). In a further embodiment, said another bond recited in (b) above is a bond in which the amino acid residues from which the bond between the antigen-binding domains originates are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region).

The antigen-binding molecule produced in these various aspects may have the characteristics of the antigen-binding molecules described herein.

[0171] < Methods of screening for antigen-binding molecules >

In another aspect, the present disclosure provides a method for identifying a novel pair of protein molecules which are activated by association with each other, comprising:

(a) providing two arbitrary protein molecules,

(b) producing, by the production method of the present disclosure, an antigen-binding molecule comprising two antigen-binding domains which respectively bind to the two protein molecules, wherein the antigen-binding molecule has activity of

holding the two protein molecules at close positions,
(c) contacting the antigen-binding molecule produced in (b) with the two protein molecules, and
(d) assessing whether or not the two protein molecules are activated.
In certain embodiments, at least one of the protein molecules is selected from the group consisting of receptors belonging to cytokine receptor superfamilies, G protein-coupled receptors, ion channel receptors, tyrosine kinase receptors, immune checkpoint receptors, antigen receptors, CD antigens, costimulatory molecules, and cell adhesion molecules.

[0172] < Linkage of antigen-binding domains >
In a non-limiting embodiment, two or more antigen-binding domains contained in an antigen-binding molecule of the present disclosure are linked with each other via one or more bonds. In a preferred embodiment, an antigen-binding domain contained in an antigen-binding molecule of the present disclosure has, by itself, activity to bind to an antigen. In such an embodiment, the antigen-binding molecule of the present disclosure containing two antigen-binding domains can bind to two or more antigen molecules; the antigen-binding molecule of the present disclosure containing three antigen-binding domains can bind to three or more antigen molecules; the antigen-binding molecule of the present disclosure containing four antigen-binding domains can bind to four or more antigen molecules; and the antigen-binding molecule of the present disclosure containing N antigen-binding domains can bind to N or more antigen molecules.

[0173] In certain embodiments, at least one of the bonds between the antigen-binding domains contained in an antigen-binding molecule of the present disclosure is different from a bond found in a naturally-occurring antibody (for example, in a wild-type Fab or hinge region). Examples of the bonds found between the antigen-binding domains of a naturally-occurring antibody (for example, naturally-occurring IgG antibody) include disulfide bonds in the hinge region. Bonds between amino acid residues positioned in a region other than the hinge region may be bonds between amino acid residues within an antibody fragment (for example, Fab), and they include bonds between the heavy chains (HH), bonds between the light chains (LL), and bonds between the heavy and light chains (HL or LH) (see Figure 21). Examples of the amino acid residues in the heavy or light chain from which the bonds between the antigen-binding domains originate include amino acid residues at the above-mentioned positions within the variable region (VH region or VL region) or within the constant region (CH1 region, hinge region, or CL region).

[0174] In a non-limiting embodiment, the bonds between the antigen-binding domains may originate from multiple amino acid residues present at positions separate from each

other in the primary structure in at least one of two or more antigen-binding domains contained in an antigen-binding molecule of the present disclosure. The distance between the multiple amino acid residues is a distance that allows the achievement of the structures of two or more, sufficiently close antigen-binding domains as a result of linkage between the antigen-binding domains by the bonds which originate from the amino acid residues. The distance between the multiple amino acid residues may be, for example, 4 amino acids or more, 5 amino acids or more, 6 amino acids or more, 7 amino acids or more, 8 amino acids or more, 9 amino acids or more, 10 amino acids or more, 11 amino acids or more, 12 amino acids or more, 13 amino acids or more, 14 amino acids or more, 15 amino acids or more, 20 amino acids or more, 25 amino acids or more, 30 amino acids or more, 35 amino acids or more, 40 amino acids or more, 45 amino acids or more, 50 amino acids or more, 60 amino acids or more, 70 amino acids or more, 80 amino acids or more, 90 amino acids or more, 100 amino acids or more, 110 amino acids or more, 120 amino acids or more, 130 amino acids or more, 140 amino acids or more, 150 amino acids or more, 160 amino acids or more, 170 amino acids or more, 180 amino acids or more, 190 amino acids or more, 200 amino acids or more, 210 amino acids or more, or 220 amino acids or more.

Further, the number of the bonds between the antigen-binding domains and the number of the amino acid residues from which the bonds originate are a number that allows the achievement of the structures of two or more, sufficiently close antigen-binding domains as a result of linkage between the antigen-binding domains by the bonds. The number may be, for example, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten or more.

In certain embodiments, as long as the structures of two or more, sufficiently close antigen-binding domains are achieved as a result of linkage between the antigen-binding domains by three or more bonds which respectively originate from three or more amino acid residues in the antigen-binding domains, the distance in the primary structure between any two amino acid residues selected from the three amino acid residues may be seven amino acids or more in at least one amino acid residue pair, and may be less than seven amino acids in the remainder of amino acid residue pairs.

[0175] In connection with antigen-binding domains contained in antigen-binding molecules of the present disclosure, "sufficiently close" means that two or more antigen-binding domains are close to the extent that this is sufficient for achieving the desired functions (activities) of the antigen-binding molecule of the present disclosure. Examples of the desired functions (activities) include activity of holding two antigen molecules at spatially close positions; activity of regulating interaction between two antigen molecules; activity of promoting activation of a receptor by a ligand; activity of promoting catalytic reaction of an enzyme with a substrate; activity of promoting in-

teraction between a cell expressing a first antigen and a cell expressing a second antigen; activity of promoting damage of a target cell by a cell with cytotoxic activity (such as a T cell, NK cell, monocyte, macrophage); activity of regulating activation of two antigen molecules which are activated by association with each other; and resistance to protease cleavage of the antigen-binding molecules.

[0176] In a non-limiting embodiment, the bond between the antigen-binding domains contained in an antigen-binding molecule of the present disclosure may be a covalent bond or a non-covalent bond. The covalent bond may be a covalent bond formed by directly crosslinking an amino acid residue in a first antigen-binding domain and an amino acid residue of a second antigen-binding domain, for example, a disulfide bond between cysteine residues. The directly crosslinked amino acid residue may be present in an antibody fragment such as Fab, or within a hinge region.

In another embodiment, a covalent bond is formed by crosslinking an amino acid residue in a first antigen-binding domain and an amino acid residue of a second antigen-binding domain via a crosslinking agent. For example, when an amine-reactive crosslinking agent is used for crosslinking, the crosslinkage can be made via a free amino group of the N-terminal amino acid of the antigen-binding domain, or a primary amine of the side chain of a lysine residue in the antigen-binding domain. Amine-reactive crosslinking agents include a functional group that forms a chemical bond with a primary amine, such as isothiocyanate, isocyanate, acyl azide, NHS ester, sulfonyl chloride, aldehyde, glyoxal, epoxide, oxirane, carbonate, aryl halide, imide ester, carbodiimide, anhydride, and fluoroester. Representative examples include DSG (disuccinimidyl glutarate), DSS (disuccinimidyl suberate), BS3 (bis(sulfosuccinimidyl) suberate), DSP (dithiobis(succinimidyl propionate)), DTSSP (3,3'-dithiobis (sulfosuccinimidyl propionate)), DST (disuccinimidyl tartrate), BSOCOES (bis(2-(succinimidooxycarbonyloxy) ethyl)sulfone) , EGS (ethylene glycol bis(succinimidyl succinate)), Sulfo-EGS (ethylene glycol bis(sulfosuccinimidyl succinate)), DMA (dimethyl adipimidate), DMP (dimethyl pimelimidate) , DMS (dimethyl suberimidate), and DFDNB (1,5-difluoro-2,4-dinitrobenzene). Examples of other crosslinking agents include carboxyl/amine-reactive, sulfhydryl-reactive, aldehyde-reactive, and light-reactive crosslinking agents.

The non-covalent bond for linking the antigen-binding domains may be an ionic bond, hydrogen bond, or hydrophobic bond.

[0177] Whether the number of the bonds between the antigen-binding domains is larger than that of a control antigen-binding molecule (e.g., an antigen-binding molecule having a structure substantially similar to a naturally-occurring antibody structure) can be assessed by, for example, the following method. First, an antigen-binding molecule of interest and a control antigen-binding molecule are treated with a protease that cuts out

the antigen-binding domain (for example, a protease that cleaves the N-terminal side of the crosslinkage site of the hinge regions such as papain and Lys-C), and then subjected to non-reducing electrophoresis. Next, an antibody that recognizes a part of the antigen-binding domain (for example, anti-kappa chain HRP-labelled antibody) is used to detect fragments which are present after the protease treatment. When only a monomer of the antigen-binding domain (for example, Fab monomer) is detected for the control antigen-binding molecule, and a multimer of the antigen-binding domain (for example, Fab dimer) is detected for the antigen-binding molecule of interest, then it can be assessed that the number of the bonds between the antigen-binding domains of the antigen-binding molecule of interest is larger than that of the control antigen-binding molecule.

The formation of a disulfide bond between cysteines in a modified antigen-binding molecule produced by introducing cysteines into a control antigen-binding molecule can be assessed by, for example, the following method. First, an antigen-binding molecule of interest is incubated with chymotrypsin in 20 mM phosphate buffer (pH7.0), and then the mass of peptides expected to be generated from the amino acid sequence of each antibody is detected by LC/MS. If a component corresponding to the theoretical mass of a peptide that should be generated when the newly-introduced cysteines form a disulfide bond is detected, the introduced cysteines can be assessed as having formed a disulfide bond. Moreover, if this component becomes undetectable when the sample containing the above-mentioned antigen-binding molecule is analyzed after adding an agent for reducing disulfide bonds (for example, tris(2-carboxyethyl)phosphine) to the sample, the correctness of the above assessment will be further strongly verified.

[0178] < Resistance to protease cleavage >

In a non-limiting embodiment, the antigen-binding molecule of the present disclosure has resistance to protease cleavage. In certain embodiments, the resistance to protease cleavage of the antigen-binding molecule of the present disclosure is increased compared with a control antigen-binding molecule (for example, an antigen-binding molecule having a structure substantially similar to a naturally-occurring antibody structure) where the number of bonds between the antigen-binding domains is lesser by one or more compared to the antigen-binding molecule. In a further embodiment, the one less bond can be selected from bonds in which the amino acid residues from which the bonds between the antigen-binding domains originate are derived from mutated amino acid residues which are not present in a wild-type Fab or hinge region (for example, cysteine residues which are not present in the wild-type Fab or hinge region). If the proportion of the full-length molecule (for example, full-length IgG molecule) remaining after protease treatment is increased, or the proportion of a particular

fragment (for example, Fab monomer) produced after protease treatment is reduced for an antigen-binding molecule compared to a control antigen-binding molecule, then it can be assessed that the resistance to protease cleavage is increased (protease resistance is improved).

In certain embodiments, the proportion of the full-length molecule remaining after protease treatment may be, relative to all antigen-binding molecules, for example, 0.5% or more, 1% or more, 1.5% or more, 2% or more, 2.5% or more, 3% or more, 3.5% or more, 4% or more, 4.5% or more, 5% or more, 7.5% or more, 10% or more, 12.5% or more, 15% or more, 20% or more, 25% or more, 30% or more, 35% or more, 40% or more, 45% or more, or 50% or more. In certain other embodiments, the proportion of a monomer of an antigen-binding domain (for example, Fab) produced after protease treatment may be, relative to all antigen-binding molecules, for example, 99% or less, 98% or less, 97% or less, 96% or less, 95% or less, 94% or less, 93% or less, 92% or less, 91% or less, 90% or less, 85% or less, 80% or less, 75% or less, 70% or less, 60% or less, 50% or less, 40% or less, 30% or less, 20% or less, or 10% or less. In certain other embodiments, the proportion of a dimer of an antigen-binding domain (for example, Fab) produced after protease treatment may be, relative to all antigen-binding molecules, for example, 0.5% or more, 1% or more, 1.5% or more, 2% or more, 2.5% or more, 3% or more, 3.5% or more, 4% or more, 4.5% or more, 5% or more, 7.5% or more, 10% or more, 12.5% or more, 15% or more, 20% or more, 25% or more, 30% or more, 35% or more, 40% or more, 45% or more, or 50% or more. Examples of proteases include, but are not limited to, Lys-C, plasmin, human neutrophil elastase (HNE), and papain.

[0179] In a further aspect, an antigen-binding molecule according to any of the above embodiments may incorporate any of the features, singly or in combination, as described in Sections 1-7 below:

[0180] 1. Antigen-Binding Molecule Affinity

In certain embodiments, an antigen-binding molecule provided herein has a dissociation constant (KD) of 1 micro M or less, 100 nM or less, 10 nM or less, 1 nM or less, 0.1 nM or less, 0.01 nM or less, or 0.001 nM or less (e.g., 10^{-8} M or less, e.g., from 10^{-8} M to 10^{-13} M, e.g., from 10^{-9} M to 10^{-13} M).

[0181] 2. Antibody Fragments

In certain embodiments, an antigen-binding molecule provided herein is an antibody fragment. Antibody fragments include, but are not limited to, Fab, Fab', Fab'-SH, $F(ab')_2$, Fv, and scFv fragments, and other fragments described herein. For a review of certain antibody fragments, see Hudson et al. Nat. Med. 9:129-134 (2003). For a review of scFv fragments, see, e.g., Pluckthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., (Springer-Verlag, New York), pp.

269-315 (1994); see also WO 93/16185; and U.S. Patent Nos. 5,571,894 and 5,587,458. For discussion of Fab and $F(ab')_2$ fragments comprising salvage receptor binding epitope residues and having increased in vivo half-life, see U.S. Patent No. 5,869,046.

[0182] Diabodies are antibody fragments with two antigen-binding sites that may be bivalent or bispecific. See, for example, EP 404,097; WO 1993/01161; Hudson et al., Nat. Med. 9:129-134 (2003); and Hollinger et al., Proc. Natl. Acad. Sci. USA 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., Nat. Med. 9:129-134 (2003).

[0183] 3. Chimeric and Humanized Antibodies

In certain embodiments, an antigen-binding molecule provided herein is a chimeric antibody. Certain chimeric antibodies are described, e.g., in U.S. Patent No. 4,816,567; and Morrison et al., Proc. Natl. Acad. Sci. USA, 81:6851-6855 (1984)). In one example, a chimeric antibody comprises a non-human variable region (e.g., a variable region derived from a mouse, rat, hamster, rabbit, or non-human primate, such as a monkey) and a human constant region. In a further example, a chimeric antibody is a "class switched" antibody in which the class or subclass has been changed from that of the parent antibody. Chimeric antibodies include antigen-binding fragments thereof.

[0184] In certain embodiments, a chimeric antibody is a humanized antibody. Typically, a non-human antibody is humanized to reduce immunogenicity to humans, while retaining the specificity and affinity of the parental non-human antibody. Generally, a humanized antibody comprises one or more variable domains in which HVRs, e.g., CDRs, (or portions thereof) are derived from a non-human antibody, and FRs (or portions thereof) are derived from human antibody sequences. A humanized antibody optionally will also comprise at least a portion of a human constant region. In some embodiments, some FR residues in a humanized antibody are substituted with corresponding residues from a non-human antibody (e.g., the antibody from which the HVR residues are derived), e.g., to restore or improve antibody specificity or affinity.

[0185] 4. Human Antibodies

In certain embodiments, an antigen-binding molecule provided herein is a human antibody. Human antibodies can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk and van de Winkel, Curr. Opin. Pharmacol. 5: 368-74 (2001) and Lonberg, Curr. Opin. Immunol. 20:450-459 (2008).

[0186] 5. Library-Derived Antigen-Binding Molecules

Antigen-binding molecules of the invention may be isolated by screening combinatorial libraries for antigen-binding molecules with the desired activity or activities. For example, a variety of methods are known in the art for generating phage display

libraries and screening such libraries for antigen-binding molecules possessing the desired binding characteristics. Such methods are reviewed, e.g., in Hoogenboom et al. in Methods in Molecular Biology 178:1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, 2001) and further described, e.g., in the McCafferty et al., Nature 348:552-554; Clackson et al., Nature 352: 624-628 (1991); Marks et al., J. Mol. Biol. 222: 581-597 (1992); Marks and Bradbury, in Methods in Molecular Biology 248:161-175 (Lo, ed., Human Press, Totowa, NJ, 2003); Sidhu et al., J. Mol. Biol. 338(2): 299-310 (2004); Lee et al., J. Mol. Biol. 340(5): 1073-1093 (2004); Fellouse, Proc. Natl. Acad. Sci. USA 101(34): 12467-12472 (2004); and Lee et al., J. Immunol. Methods 284(1-2): 119-132(2004).

[0187] 6. Multispecific Antigen-Binding Molecules

In certain embodiments, an antigen-binding molecule provided herein is a multi-specific antigen-binding molecule, e.g. a bispecific antigen-binding molecule. Multi-specific antigen-binding molecules are monoclonal antigen-binding molecules that have binding specificities for at least two different sites. In certain embodiments, one of the binding specificities is for a particular antigen (e.g., CD3) and the other is for any other antigen (e.g., CD28 or cancer antigen). In certain embodiments, bispecific antigen-binding molecules may bind to two different epitopes on a single antigen. Bispecific antigen-binding molecules can be prepared as full-length antibodies or antibody fragments.

[0188] Techniques for making multispecific antigen-binding molecules include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein and Cuello, Nature 305: 537 (1983)), WO 93/08829, and Traunecker et al., EMBO J. 10: 3655 (1991)), and "knob-in-hole" (also called "knobs-in-holes" or "KiH") engineering (see, e.g., U.S. Patent No. 5,731,168). Multi-specific antigen-binding molecules may also be made by engineering electrostatic steering effects for making antibody Fc-heterodimeric molecules (WO 2009/089004A1); cross-linking two or more antibodies or fragments (see, e.g., US Patent No. 4,676,980, and Brennan et al., Science, 229: 81 (1985)); using leucine zippers to produce bi-specific antibodies (see, e.g., Kostelny et al., J. Immunol., 148(5):1547-1553 (1992)); using "diabody" technology for making bispecific antibody fragments (see, e.g., Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993)); and using single-chain Fv (scFv) dimers (see, e.g. Gruber et al., J. Immunol., 152:5368 (1994)); and preparing trispecific antibodies as described, e.g., in Tutt et al. J. Immunol. 147: 60 (1991).

[0189] Engineered antibodies with three or more functional antigen binding sites, including "Octopus antibodies," are also included herein (see, e.g. US 2006/0025576A1).

[0190] 7. Antigen-Binding Molecule Variants

In certain embodiments, amino acid sequence variants of the antigen-binding molecules provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antigen-binding molecule. Amino acid sequence variants of an antigen-binding molecule may be prepared by introducing appropriate modifications into the nucleotide sequence encoding the antigen-binding molecule, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of residues within the amino acid sequences of the antigen-binding molecule. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, e.g., antigen-binding.

[0191] a) Substitution, Insertion, and Deletion Variants

In certain embodiments, antigen-binding molecule variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the HVRs and FRs. Conservative substitutions are shown the table below under the heading of "preferred substitutions." More substantial changes are provided in the table under the heading of "exemplary substitutions," and as further described below in reference to amino acid side chain classes. Amino acid substitutions may be introduced into an antigen-binding molecule of interest and the products screened for a desired activity, e.g., retained/improved antigen binding, decreased immunogenicity, or improved ADCC or CDC.

[0192]

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

[0193] Amino acids may be grouped according to common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

[0194] One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antigen-binding molecule (e.g. a humanized or human antibody). Generally, the resulting variant(s) selected for further study will have modifications (e.g., improvements) in certain biological properties (e.g., increased affinity, reduced immunogenicity) relative to the parent antigen-binding molecule and/or will

have substantially retained certain biological properties of the parent antigen-binding molecule. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated, e.g., using phage display-based affinity maturation techniques such as those described herein. Briefly, one or more HVR residues are mutated and the variant antibodies displayed on phage and screened for a particular biological activity (e.g. binding affinity).

[0195] Alterations (e.g., substitutions) may be made in HVRs, e.g., to improve antigen-binding molecule affinity. Such alterations may be made in HVR "hotspots," i.e., residues encoded by codons that undergo mutation at high frequency during the somatic maturation process (see, e.g., Chowdhury, Methods Mol. Biol. 207:179-196 (2008)), and/or residues that contact antigen, with the resulting variant VH or VL being tested for binding affinity. Affinity maturation by constructing and reselecting from secondary libraries has been described, e.g., in Hoogenboom et al. in Methods in Molecular Biology 178:1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, (2001).) In some embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (e.g., error-prone PCR, chain shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any antigen-binding molecule variants with the desired affinity. Another method to introduce diversity involves HVR-directed approaches, in which several HVR residues (e.g., 4-6 residues at a time) are randomized. HVR residues involved in antigen binding may be specifically identified, e.g., using alanine scanning mutagenesis or modeling. CDR-H3 and CDR-L3 in particular are often targeted.

[0196] In certain embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the antigen-binding molecule to bind antigen. For example, conservative alterations (e.g., conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in HVRs. Such alterations may, for example, be outside of antigen contacting residues in the HVRs. In certain embodiments of the variant VH and VL sequences provided above, each HVR either is unaltered, or contains no more than one, two or three amino acid substitutions.

[0197] A useful method for identification of residues or regions of an antigen-binding molecule that may be targeted for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (1989) Science, 244:1081-1085. In this method, a residue or group of target residues (e.g., charged residues such as arg, asp, his, lys, and glu) are identified and replaced by a neutral or negatively charged amino acid (e.g., alanine or polyalanine) to determine whether the interaction of the antigen-binding molecule with antigen is affected. Further substitutions may be in-

troduced at the amino acid locations demonstrating functional sensitivity to the initial substitutions. Alternatively, or additionally, a crystal structure of a complex of antigens and an antigen-binding molecule may be analyzed to identify contact points between the antigen-binding molecule and antigen. Such contact residues and neighboring residues may be targeted or eliminated as candidates for substitution. Variants may be screened to determine whether they contain the desired properties.

[0198] Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antigen-binding molecule with an N-terminal methionyl residue. Other insertional variants of the antigen-binding molecule include the fusion of an enzyme (e.g. for ADEPT) or a polypeptide which increases the plasma half-life of the antigen-binding molecule to the N- or C-terminus of the antigen-binding molecule.

[0199] b) Glycosylation variants

In certain embodiments, an antigen-binding molecule provided herein is altered to increase or decrease the extent to which the antigen-binding molecule is glycosylated. Addition or deletion of glycosylation sites to an antigen-binding molecule may be conveniently accomplished by altering the amino acid sequence such that one or more glycosylation sites is created or removed.

[0200] Where the antigen-binding molecule comprises an Fc region, the carbohydrate attached thereto may be altered. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region. See, e.g., Wright et al. TIBTECH 15:26-32 (1997). The oligosaccharide may include various carbohydrates, e.g., mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the "stem" of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an antigen-binding molecule of the invention may be made in order to create antigen-binding molecule variants with certain improved properties.

[0201] In one embodiment, antigen-binding molecule variants are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. For example, the amount of fucose in such antigen-binding molecule may be from 1% to 80%, from 1% to 65%, from 5% to 65% or from 20% to 40%. The amount of fucose is determined by calculating the average amount of fucose within the sugar chain at Asn297, relative to the sum of all glycostructures attached to Asn 297 (e. g. complex, hybrid and high mannose structures) as measured by MALDI-TOF mass spectrometry, as described in WO 2008/077546, for example. Asn297 refers to the asparagine residue

located at about position 297 in the Fc region (EU numbering of Fc region residues); however, Asn297 may also be located about +/- 3 amino acids upstream or downstream of position 297, i.e., between positions 294 and 300, due to minor sequence variations in antigen-binding molecules. Such fucosylation variants may have improved ADCC function. See, e.g., US Patent Publication Nos. US 2003/0157108 (Presta, L.); US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Examples of publications related to "defucosylated" or "fucose-deficient" antigen-binding molecule variants include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/0110704; US 2004/0110282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO2005/053742; WO2002/031140; Okazaki et al. J. Mol. Biol. 336:1239-1249 (2004); Yamane-Ohnuki et al. Biotech. Bioeng. 87: 614 (2004). Examples of cell lines capable of producing defucosylated antigen-binding molecules include Lec13 CHO cells deficient in protein fucosylation (Ripka et al. Arch. Biochem. Biophys. 249:533-545 (1986); US Pat Appl No US 2003/0157108 A1, Presta, L; and WO 2004/056312 A1, Adams et al., especially at Example 11), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, FUT8, knockout CHO cells (see, e.g., Yamane-Ohnuki et al. Biotech. Bioeng. 87: 614 (2004); Kanda, Y. et al., Biotechnol. Bioeng., 94(4):680-688 (2006); and WO2003/085107).

[0202] Antigen-binding molecule variants are further provided with bisected oligosaccharides, e.g., in which a biantennary oligosaccharide attached to the Fc region of the antigen-binding molecule is bisected by GlcNAc. Such antigen-binding molecule variants may have reduced fucosylation and/or improved ADCC function. Examples of such antibody variants are described, e.g., in WO 2003/011878 (Jean-Mairet et al.); US Patent No. 6,602,684 (Umana et al.); and US 2005/0123546 (Umana et al.). Antigen-binding molecule variants with at least one galactose residue in the oligosaccharide attached to the Fc region are also provided. Such antigen-binding molecule variants may have improved CDC function. Such antigen-binding molecule variants are described, e.g., in WO 1997/30087 (Patel et al.); WO 1998/58964 (Raju, S.); and WO 1999/22764 (Raju, S.).

[0203] c) Fc region variants

In certain embodiments, one or more amino acid modifications may be introduced into the Fc region of an antigen-binding molecule provided herein, thereby generating an Fc region variant. The Fc region variant may comprise a human Fc region sequence (e.g., a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (e.g. a substitution) at one or more amino acid positions.

[0204] In certain embodiments, the invention contemplates an antigen-binding molecule

variant that possesses some but not all effector functions, which make it a desirable candidate for applications in which the half life of the antigen-binding molecule in vivo is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. In vitro and/or in vivo cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antigen-binding molecule lacks Fc gamma R binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express Fc gamma RIII only, whereas monocytes express Fc gamma RI, Fc gamma RII and Fc gamma RIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol. 9:457-492 (1991). Non-limiting examples of in vitro assays to assess ADCC activity of a molecule of interest is described in U.S. Patent No. 5,500,362 (see, e.g. Hellstrom, I. et al. Proc. Nat'l Acad. Sci. USA 83:7059-7063 (1986)) and Hellstrom, I et al., Proc. Nat'l Acad. Sci. USA 82:1499-1502 (1985); 5,821,337 (see Bruggemann, M. et al., J. Exp. Med. 166:1351-1361 (1987)). Alternatively, non-radioactive assays methods may be employed (see, for example, ACT1™ non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, CA; and CytoTox 96 (registered trademark) non-radioactive cytotoxicity assay (Promega, Madison, WI). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in an animal model such as that disclosed in Clynes et al. Proc. Nat'l Acad. Sci. USA 95:652-656 (1998). C1q binding assays may also be carried out to confirm that the antigen-binding molecule is unable to bind C1q and hence lacks CDC activity. See, e.g., C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess complement activation, a CDC assay may be performed (see, for example, Gazzano-Santoro et al., J. Immunol. Methods 202:163 (1996); Cragg, M.S. et al., Blood 101:1045-1052 (2003); and Cragg, M.S. and M.J. Glennie, Blood 103:2738-2743 (2004)). FcRn binding and in vivo clearance/half life determinations can also be performed using methods known in the art (see, e.g., Petkova, S.B. et al., Int'l. Immunol. 18(12):1759-1769 (2006)).

[0205] Antigen-binding molecules with reduced effector function include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Patent No. 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (US Patent No. 7,332,581).

[0206] Certain antigen-binding molecule variants with increased or decreased binding to

FcRs are described. (See, e.g., U.S. Patent No. 6,737,056; WO 2004/056312, and Shields et al., J. Biol. Chem. 9(2): 6591-6604 (2001).)

[0207] In certain embodiments, an antigen-binding molecule variant comprises an Fc region with one or more amino acid substitutions which improve ADCC, e.g., substitutions at positions 298, 333, and/or 334 of the Fc region (EU numbering of residues).

[0208] In some embodiments, alterations are made in the Fc region that result in altered (i.e., either increased or decreased) C1q binding and/or Complement Dependent Cytotoxicity (CDC), e.g., as described in US Patent No. 6,194,551, WO 99/51642, and Idusogie et al. J. Immunol. 164: 4178-4184 (2000).

[0209] Antibodies with increased half lives and increased binding to the neonatal Fc receptor (FcRn), which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., J. Immunol. 117:587 (1976) and Kim et al., J. Immunol. 24:249 (1994)), are described in US2005/0014934A1 (Hinton et al.). Those antibodies comprise an Fc region with one or more substitutions therein which increase binding of the Fc region to FcRn. Such Fc variants include those with substitutions at one or more of Fc region residues: 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434, e.g., substitution of Fc region residue 434 (US Patent No. 7,371,826).

[0210] See also Duncan & Winter, Nature 322:738-40 (1988); U.S. Patent No. 5,648,260; U.S. Patent No. 5,624,821; and WO 94/29351 concerning other examples of Fc region variants.

[0211] d) Cysteine engineered antigen-binding molecule variants

In certain embodiments, it may be desirable to create cysteine engineered antigen-binding molecules, e.g., "thioMAbs," in which one or more residues of an antigen-binding molecule are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the antigen-binding molecule. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antigen-binding molecule and may be used to conjugate the antigen-binding molecule to other moieties, such as drug moieties or linker-drug moieties, to create an immunoconjugate, as described further herein. In certain embodiments, any one or more of the following residues may be substituted with cysteine: V205 (Kabat numbering) of the light chain; A118 (EU numbering) of the heavy chain; and S400 (EU numbering) of the heavy chain Fc region. Cysteine engineered antigen-binding molecules may be generated as described, e.g., in U.S. Patent No. 7,521,541.

[0212] e) Antigen-Binding Molecule Derivatives

In certain embodiments, an antigen-binding molecule provided herein may be further modified to contain additional nonproteinaceous moieties that are known in the art and readily available. The moieties suitable for derivatization of the antigen-binding

molecule include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, polypropylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antigen-binding molecule may vary, and if more than one polymer are attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antigen-binding molecule to be improved, whether the antigen-binding molecule derivative will be used in a therapy under defined conditions, etc.

[0213] In connection with an antigen-binding molecule in the present disclosure, examples of the desired property (activity) can include, but are not particularly limited to, binding activity, neutralizing activity, cytotoxic activity, agonist activity, antagonist activity, and enzymatic activity. The agonist activity is an activity of intracellularly transducing signals, for example, through the binding of an antibody to an antigen such as a receptor to induce change in some physiological activity. Examples of the physiological activity can include, but are not limited to, proliferative activity, survival activity, differentiation activity, transcriptional activity, membrane transport activity, binding activity, proteolytic activity, phosphorylating/dephosphorylating activity, redox activity, transfer activity, nucleolytic activity, dehydration activity, cell death-inducing activity, and apoptosis-inducing activity.

[0214] In another embodiment, conjugates of an antigen-binding molecule and nonproteinaceous moiety that may be selectively heated by exposure to radiation are provided. In one embodiment, the nonproteinaceous moiety is a carbon nanotube (Kam et al., Proc. Natl. Acad. Sci. USA 102: 11600-11605 (2005)). The radiation may be of any wavelength, and includes, but is not limited to, wavelengths that do not harm ordinary cells, but which heat the nonproteinaceous moiety to a temperature at which cells proximal to the antigen binding molecule-nonproteinaceous moiety are killed.

[0215] B. Recombinant Methods and Compositions

Antigen-binding molecules may be produced using recombinant methods and compositions, e.g., as described in U.S. Patent No. 4,816,567. In one embodiment, isolated

nucleic acid encoding an antigen-binding molecule in the present disclosure (a polypeptide comprising an antigen-binding domain described herein) is provided. Such nucleic acid may encode an amino acid sequence comprising the VL and/or an amino acid sequence comprising the VH of the antigen-binding molecule (e.g., the light and/or heavy chains of the antigen-binding molecule). In a further embodiment, one or more vectors (e.g., expression vectors) comprising such nucleic acid are provided. In a further embodiment, a host cell comprising such nucleic acid is provided. In one such embodiment, a host cell comprises (e.g., has been transformed with): (1) a vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antigen-binding molecule and an amino acid sequence comprising the VH of the antigen-binding molecule, or (2) a first vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antigen-binding molecule and a second vector comprising a nucleic acid that encodes an amino acid sequence comprising the VH of the antigen-binding molecule. In one embodiment, the host cell is eukaryotic, e.g. a Chinese Hamster Ovary (CHO) cell or lymphoid cell (e.g., Y0, NS0, Sp2/0 cell). In one embodiment, a method of making an antigen-binding molecule in the present disclosure is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the antigen-binding molecule, as provided above, under conditions suitable for expression of the antigen-binding molecule, and optionally recovering the antigen-binding molecule from the host cell (or host cell culture medium).

[0216] For recombinant production of an antigen-binding molecule in the present disclosure, nucleic acid encoding an antigen-binding molecule, e.g., as described above, is isolated and inserted into one or more vectors for further cloning and/or expression in a host cell. Such nucleic acid may be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antigen-binding molecule).

[0217] Suitable host cells for cloning or expression of antigen-binding molecule-encoding vectors include prokaryotic or eukaryotic cells described herein. For example, antigen-binding molecules may be produced in bacteria, in particular when glycosylation and Fc effector function are not needed. For expression of antibody fragments and polypeptides in bacteria, see, e.g., U.S. Patent Nos. 5,648,237, 5,789,199, and 5,840,523. (See also Charlton, Methods in Molecular Biology, Vol. 248 (B.K.C. Lo, ed., Humana Press, Totowa, NJ, 2003), pp. 245-254, describing expression of antibody fragments in E. coli.) After expression, the antigen-binding molecule may be isolated from the bacterial cell paste in a soluble fraction and can be further purified.

[0218] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast

are suitable cloning or expression hosts for antigen-binding molecule-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been "humanized," resulting in the production of an antigen-binding molecule with a partially or fully human glycosylation pattern. See Gerngross, Nat. Biotech. 22:1409-1414 (2004), and Li et al., Nat. Biotech. 24:210-215 (2006).

[0219] Suitable host cells for the expression of glycosylated antigen-binding molecule are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of Spodoptera frugiperda cells.

[0220] Plant cell cultures can also be utilized as hosts. See, e.g., US Patent Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978, and 6,417,429 (describing PLANTIBODIES™ technology for producing antigen-binding molecules in transgenic plants).

[0221] Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293 cells as described, e.g., in Graham et al., J. Gen Virol. 36:59 (1977)); baby hamster kidney cells (BHK); mouse sertoli cells (TM4 cells as described, e.g., in Mather, Biol. Reprod. 23:243-251 (1980)); monkey kidney cells (CV1); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK); buffalo rat liver cells (BRL 3A); human lung cells (W138); human liver cells (Hep G2); mouse mammary tumor (MMT 060562); TRI cells, as described, e.g., in Mather et al., Annals N.Y. Acad. Sci. 383:44-68 (1982); MRC 5 cells; and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including DHFR$^-$ CHO cells (Urlaub et al., Proc. Natl. Acad. Sci. USA 77:4216 (1980)); and myeloma cell lines such as Y0, NS0 and Sp2/0. For a review of certain mammalian host cell lines suitable for antigen-binding molecule production, see, e.g., Yazaki and Wu, Methods in Molecular Biology, Vol. 248 (B.K.C. Lo, ed., Humana Press, Totowa, NJ), pp. 255-268 (2003).

[0222] C. Assays

Antigen-binding molecules provided herein may be identified, screened for, or characterized for their physical/chemical properties and/or biological activities by various assays known in the art.

[0223] 1. Binding assays and other assays

In one aspect, an antigen-binding molecule in the present disclosure is tested for its antigen binding activity, e.g., by known methods such as ELISA, Western blot, etc.

[0224] 2. Activity assays

In one aspect, assays are provided for identifying antigen-binding molecules thereof

having biological activity. Biological activity may include, e.g., activity of holding two antigen molecules at spatially close positions, activity of regulating interaction between two antigen molecules, activity of promoting activation of an receptor by a ligand, activity of promoting catalytic reaction of an enzyme with a substrate, promoting interaction between a cell expressing a first antigen and a cell expressing a second antigen, activity of promoting damage of a target cell by a cell with cytotoxic activity (e.g., a T cell, NK cell, monocyte, or macrophage), activity of regulating activation of two antigen molecules which are activated by association with each other, and resistance to protease cleavage . Antigen-binding molecules having such biological activity in vivo and/or in vitro are also provided.

[0225] Furthermore, an antigen-binding molecule in the present disclosure can exert various biological activities depending on the type of an antigen molecule to which the antigen-binding molecule binds. Examples of such antigen-binding molecules include an antigen-binding molecule which binds to a T cell receptor (TCR) complex (e.g., CD3) and has activity of inducing T cell activation (agonist activity); and an antigen-binding molecule which binds to a molecule of TNF receptor superfamily (e.g., OX40 or 4-1BB) or of other co-stimulatory molecules (e.g., CD28 or ICOS) and has activity of promoting the above-mentioned activation (agonist activity). In certain embodiments, such biological activity exerted through the binding to an antigen molecule is enhanced or diminished by the linking of two or more antigen-binding domains comprised in the antigen-binding molecule in the present disclosure. Without being limited by theory, in certain embodiments, such enhancement or diminishment may be achieved because the interaction between two or more antigen molecules is regulated through the binding to the antigen-binding molecule in the present disclosure (e.g., the association between two or more antigen molecules is promoted).

[0226] In certain embodiments, an antigen-binding molecule of the invention is tested for such biological activity. Whether two antigen molecules are held spatially close can be evaluated using techniques such as crystal structure analysis, electron microscopy, and electron tomography-based structural analysis of a complex composed of antigens and an antigen-binding molecule. Whether two antigen-binding domains are spatially close to each other or whether the mobility of two antigen-binding domains is reduced can also be evaluated by the above-mentioned techniques. In particular, as for techniques to analyze the three-dimensional structure of IgG molecules using electron tomography, see, for example, Zhang et al., Sci. Rep. 5:9803 (2015). In electron tomography, the frequency of occurrence of structures that a subject molecule may form can be shown by histograms, enabling distributional evaluation of structural changes such as reduced mobility of domains. For example, when the relationship between values that can be taken by structure-related parameters, such as distance and angle

between two domains, and their frequency of occurrence is shown by histograms, one can determine that the mobility of the two domains is decreased if their areas of distribution are decreased. Activity exerted through interaction and such of two antigen molecules can be evaluated by selecting and using an appropriate activity measurement system from known ones according to the type of target antigen molecules. The effect on protease cleavage can be evaluated using methods known to those skilled in the art, or methods described in the Examples below.

[0227] D. Pharmaceutical Formulations (Pharmaceutical Compositions)

Pharmaceutical formulations of an antigen-binding molecule as described herein are prepared by mixing such antigen-binding molecule having the desired degree of purity with one or more optional pharmaceutically acceptable carriers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG). Exemplary pharmaceutically acceptable carriers herein further include interstitial drug dispersion agents such as soluble neutral-active hyaluronidase glycoproteins (sHASEGP), for example, human soluble PH-20 hyaluronidase glycoproteins, such as rHuPH20 (HYLENEX (registered trademark), Baxter International, Inc.). Certain exemplary sHASEGPs and methods of use, including rHuPH20, are described in US Patent Publication Nos. 2005/0260186 and 2006/0104968. In one aspect, a sHASEGP is combined with one or more additional glycosaminoglycanases such as chondroitinases.

[0228] Exemplary lyophilized antigen-binding molecule formulations are described in US Patent No. 6,267,958. Aqueous antigen-binding molecule formulations include those described in US Patent No. 6,171,586 and WO2006/044908, the latter formulations including a histidine-acetate buffer.

[0229] The formulation herein may also contain more than one active ingredients as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such active ingredients are suitably present in combination in amounts that are effective for the purpose intended.

[0230] Active ingredients may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

[0231] Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antigen-binding molecule, which matrices are in the form of shaped articles, e.g. films, or microcapsules.

[0232] The formulations to be used for in vivo administration are generally sterile. Sterility may be readily accomplished, e.g., by filtration through sterile filtration membranes.

Examples

[0233] The following are examples of antigen-binding molecules and methods of the present disclosure. It will be understood that various other embodiments may be practiced, given the general description provided above.

[0234] [Example 1] Optimizing methods for producing, purification and assessment of antibodies having one or more disulfide bonds within Fab region

Preparation and assessment of antibodies having a single pair of cysteine substitution at various positions in the antibodies were described in Reference Examples 1-25. Based on the results of non-reducing SDS-PAGE (Reference Examples 8-2, 9-2, 10-2, and 11-2; see also Figures 1 to 4), it was found that some of the preparation of antibody having cysteine substitution comprises two or more structural variants/ isoforms which differ in electrophoretic mobility, i.e. Double, Triple or Several bands as observed from the non-reducing SDS-PAGE gel images. For example, two bands were observed in the G1T4.S191C-IgG1 variant (cysteine substitution at the position 191 of the CH1 region) with about 66.3 % percentage of the new band (corresponds to antibody preparation having one disulfide bond formed between two Fabs at position 191 of the CH1 region) relative to the band corresponding to that of the parent antibody. The results suggest that the antibody preparation of the G1T4.S191C-IgG1 variant comprises two or more structural isoforms which differ by one disulfide bond

formed between the engineered cysteines, in particular isoform having the "paired cysteines" or isoform having the "free or unpaired cysteines", can be generated during recombinant antibody production.

As described in further detail hereinbelow, the following non-limiting examples are directed to providing efficient and facile production, purification and analysis of the antibody having an engineered disulfide bond formed between the two Fabs of the antibody; methods for increasing structural homogeneity and relative abundance of the antibody in the "paired cysteines" form, i.e. having one or more engineered disulfide bond(s) formed between the two Fabs of the antibody; or methods for decreasing relative abundance of the antibody in the "free or unpaired cysteines" form, i.e. having no engineered disulfide bond formed between the two Fabs of the antibody.

[0235] Example 1-1 Production of antibodies having multiple additional disulfide bonds within the Fab region

To improve the percentage of antibody preparation of G1T4.S191C-IgG1 variant having an engineered disulfide bond formed at the position 191 of CH1 region of the antibody, additional one or two disulfide bonds were introduced into the heavy chain of an anti-human CD3 antibody, OKT3 (heavy chain: OKT3VH0000-G1T4 (SEQ ID NO: 1242), light chain: OKT3VL0000-KT0 (SEQ ID NO: 1243)) via cysteine substitution.

An amino acid residue structurally exposed to the surface of the OKT3 heavy chain constant region (G1T4, SEQ ID NO: 1244) was substituted with cysteine to produce the variant of OKT3 heavy chain constant region (G1T4.S191C, SEQ ID NO: 1245) shown in Table 1. In addition, other amino acid residues structurally exposed to the surface of G1T4.S191C were substituted with cysteine to produce the variants of G1T4.S191C shown in Table 2. These heavy chain constant regions were each linked with the OKT3 heavy chain variable region (OKT3VH0000, SEQ ID NO: 1246) to produce the OKT3 heavy chain variants, and expression vectors encoding the corresponding genes were produced by a method known in the art.

Similarly, amino acid residues structurally exposed to the surface of the OKT3 heavy chain constant region 1 (G1T4k, SEQ ID NO: 1263) and constant region 2 (G1T4h, SEQ ID NO: 1264) were substituted with cysteine to produce the variant of OKT3 heavy chain constant regions shown in Table 3, respectively. In addition, other amino acid residues structurally exposed to the surface of the variants shown in Table 3 were substituted with cysteine to produce the variants shown in Table 4. These heavy chain constant regions were each linked with the OKT3 heavy chain variable region (OKT3VH0000, SEQ ID NO: 1246) to produce the OKT3 heavy chain variants, and expression vectors encoding the corresponding genes were produced by a method known in the art. It is noted that the Knobs-into-Holes (KiH) mutations in the CH3

region are introduced into the heavy-chain constant regions 1 and 2 in this Example for promoting heterodimerization.

The OKT3 heavy chain variants produced as mentioned above were combined with the OKT3 light chain. The OKT3 variants shown in Table 5 and 6 were expressed by transient expression using Expi293 cells (Life technologies) by a method known to the person skilled in the art, and purified with Protein A by a method known to the person skilled in the art. In this Example, OKT3 and OKT3-KiH are called "parent antibodies", OKT3.S191C and OKT3-KiH.S191C are called "S191C variants", and their variants are called "additional variants", respectively.

[0236] [Table 1]

G1T4 variant with single cysteine substitution

Variant of heavy chain constant region	Position of cysteine substitution (EU numbering)	SEQ ID NO:
G1T4.S191C	191	1245

[0237] [Table 2]

G1T4.S191C variants with additional cysteine substitution

Variants of heavy chain constant region	Position of cysteine substitution (EU numbering)	SEQ ID NO:
G1T4.S191C.S131C.G194C	S131C/G194C	1247
G1T4.S191C.S132C.G194C	S132C/G194C	1248
G1T4.S191C.K133C.G194C	K133C/G194C	1249
G1T4.S191C.S134C.G194C	S134C/G194C	1250
G1T4.S191C.T135C.G194C	T135C/G194C	1251
G1T4.S191C.S136C.G194C	S136C/G194C	1252
G1T4.S191C.G137C.G194C	G137C/G194C	1253
G1T4.S191C.G138C.G194C	G138C/G194C	1254
G1T4.S191C.S131C.T195C	S131C/T195C	1255
G1T4.S191C.S132C.T195C	S132C/T195C	1256
G1T4.S191C.K133C.T195C	K133C/T195C	1257
G1T4.S191C.S134C.T195C	S134C/T195C	1258
G1T4.S191C.T135C.T195C	T135C/T195C	1259
G1T4.S191C.S136C.T195C	S136C/T195C	1260
G1T4.S191C.G137C.T195C	G137C/T195C	1261
G1T4.S191C.G138C.T195C	G138C/T195C	1262

[0238]

[Table 3]

G1T4k and G1T4h variants with single cysteine substitution

Variants of heavy chain constant region	Position of cysteine substitution (EU numbering)	SEQ ID NO:
G1T4k.S191C	S191C	1265
G1T4h.S191C	S191C	1266

[0239] [Table 4]

G1T4k.S191C and G1T4h.S191C variants with additional cysteine substitution

Variants of heavy chain constant region	Position of cysteine substitution (EU numbering)	SEQ ID NO:
G1T4k.S191C.S131C	S131C	1267
G1T4k.S191C.S132C	S132C	1268
G1T4k.S191C.K133C	K133C	1269
G1T4k.S191C.S134C	S134C	1270
G1T4k.S191C.T135C	T135C	1271
G1T4k.S191C.S136C	S136C	1272
G1T4k.S191C.G137C	G137C	1273
G1T4k.S191C.G138C	G138C	1274
G1T4k.S191C.S131C	S131C	1275
G1T4k.S191C.S132C	S132C	1276
G1T4k.S191C.K133C	K133C	1277
G1T4k.S191C.S134C	S134C	1278
G1T4k.S191C.T135C	T135C	1279
G1T4k.S191C.S136C	S136C	1280
G1T4k.S191C.G137C	G137C	1281
G1T4k.S191C.G138C	G138C	1282
G1T4h.S191C.G194C	G194C	1283
G1T4h.S191C.T195C	T195C	1284

[0240]

[Table 5]

OKT3 variants with cysteine substitution

Name of OKT3 variants	Short Name	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:	Light chain SEQ ID NO:
OKT3	OKT3	1246	1244	1243
OKT3.S191C	OKT3.S191C	1246	1245	1243
OKT3.S191C.S131C.G194C	OKT3.S191C_v1	1246	1247	1243
OKT3.S191C.S132C.G194C	OKT3.S191C_v2	1246	1248	1243
OKT3.S191C.K133C.G194C	OKT3.S191C_v3	1246	1249	1243
OKT3.S191C.S134C.G194C	OKT3.S191C_v4	1246	1250	1243
OKT3.S191C.T135C.G194C	OKT3.S191C_v5	1246	1251	1243
OKT3.S191C.S136C.G194C	OKT3.S191C_v6	1246	1252	1243
OKT3.S191C.G137C.G194C	OKT3.S191C_v7	1246	1253	1243
OKT3.S191C.G138C.G194C	OKT3.S191C_v8	1246	1254	1243
OKT3.S191C.S131C.T195C	OKT3.S191C_v9	1246	1255	1243
OKT3.S191C.S132C.T195C	OKT3.S191C_v10	1246	1256	1243
OKT3.S191C.K133C.T195C	OKT3.S191C_v11	1246	1257	1243
OKT3.S191C.S134C.T195C	OKT3.S191C_v12	1246	1258	1243
OKT3.S191C.T135C.T195C	OKT3.S191C_v13	1246	1259	1243
OKT3.S191C.S136C.T195C	OKT3.S191C_v14	1246	1260	1243
OKT3.S191C.G137C.T195C	OKT3.S191C_v15	1246	1261	1243
OKT3.S191C.G138C.T195C	OKT3.S191C_v16	1246	1262	1243

[0241]

[Table 6]

OKT3-KiH variants with cysteine substitution

Name of OKT3 variants	Short Name	Heavy chain 1 variable region SEQ ID NO:	Heavy chain 1 constant region SEQ ID NO:	Heavy chain 2 variable region SEQ ID NO:	Heavy chain 2 constant region SEQ ID NO:	Light chain SEQ ID NO:
OKT3-KiH	OKT3-KiH	1246	1263	1246	1264	1243
OKT3-KiH.S191C	OKT3-KiH.S191C	1246	1265	1246	1266	1243
OKT3-KiH.S191C.S131C /S191C.G194C	OKT3-KiH.S191C_v1	1246	1267	1246	1283	1243
OKT3-KiH.S191C.S132C /S191C.G194C	OKT3-KiH.S191C_v2	1246	1268	1246	1283	1243
OKT3-KiH.S191C.K133C /S191C.G194C	OKT3-KiH.S191C_v3	1246	1269	1246	1283	1243
OKT3-KiH.S191C.S134C /S191C.G194C	OKT3-KiH.S191C_v4	1246	1270	1246	1283	1243
OKT3-KiH.S191C.T135C /S191C.G194C	OKT3-KiH.S191C_v5	1246	1271	1246	1283	1243
OKT3-KiH.S191C.S136C /S191C.G194C	OKT3-KiH.S191C_v6	1246	1272	1246	1283	1243
OKT3-KiH.S191C.G137C /S191C.G194C	OKT3-KiH.S191C_v7	1246	1273	1246	1283	1243
OKT3-KiH.S191C.G138C /S191C.G194C	OKT3-KiH.S191C_v8	1246	1274	1246	1283	1243
OKT3-KiH.S191C.S131C /S191C.T195C	OKT3-KiH.S191C_v9	1246	1267	1246	1284	1243
OKT3-KiH.S191C.S132C /S191C.T195C	OKT3-KiH.S191C_v10	1246	1268	1246	1284	1243
OKT3-KiH.S191C.K133C /S191C.T195C	OKT3-KiH.S191C_v11	1246	1269	1246	1284	1243
OKT3-KiH.S191C.S134C /S191C.T195C	OKT3-KiH.S191C_v12	1246	1270	1246	1284	1243

OKT3-KiH.S191C.T135C /S191C.T195C	OKT3-KiH.S191C_v13	1246	1271	1246	1284	1243
OKT3-KiH.S191C.S136C /S191C.T195C	OKT3-KiH.S191C_v14	1246	1272	1246	1284	1243
OKT3-KiH.S191C.G137C /S191C.T195C	OKT3-KiH.S191C_v15	1246	1273	1246	1284	1243
OKT3-KiH.S191C.G138C /S191C.T195C	OKT3-KiH.S191C_v16	1246	1274	1246	1284	1243

[0242] Example 1-2 Assessment of electrophoretic mobility in polyacrylamide gel of antibodies having multiple additional disulfide bonds within the Fab region

It was examined whether the antibodies produced in Example 1-1 show a different electrophoretic mobility in polyacrylamide gel by non-reducing SDS-PAGE.

Sample Buffer Solution (2ME-) (x4) (Wako; 198-13282) was used for preparation of electrophoresis samples. The samples were treated for 10 minutes under the condition of specimen concentration 50 microgram/mL and 70 degrees C, and then subjected to non-reducing SDS-PAGE. In non-reducing SDS-PAGE, electrophoresis was carried out for 90 minutes at 125 V, using 4% SDS-PAGE mini 15well 1.0mm 15well (TEFCO; Cat#01-052-6). Then, the gel was stained with CBB stain, the gel image was captured with ChemiDocTouchMP (BIORAD), and the bands were quantified with Image Lab (BIORAD).

The gel images are shown in Figures 1 to 4. In the gel images, two bands ("upper band" and "lower band") were observed in the S191C variants, and the molecular weight of the upper bands correspond to that of the parent antibodies. It is highly likely that structural changes such as crosslinking via disulfide bonds of Fabs were caused by cysteine substitution, which resulted in the change in electrophoretic mobility. Thus, the lower band can be considered to correspond to the antibody having one or more engineered disulfide bond(s) formed between the CH1 regions. Among the antibody variant samples with additional cysteine substitutions, most of them showed a higher lower band to upper band ratio, compared to S191C variants. Thus, the results suggest that additional cysteine substitutions to the S191C variants as listed in Table 6 are likely to enhance/promote disulfide bond crosslinking of Fabs, and additional cysteine substitutions could be an effective way to improve or increase the percentage or structural homogeneity of antibody preparation of the S191C variants having an engineered disulfide bond formed at the position 191 of CH1 region of the antibody.

[0243] [Example 2] Assessment of antibodies having additional disulfide bond and charged mutations within the Fab region

Example 2-1 Production of antibodies having additional disulfide bond and charged mutations within the Fab region

One disulfide bond and charge mutations were introduced into the heavy chain of an anti-human CD3 antibody, OKT3 (heavy chain: OKT3VH0000-G1T4 (SEQ ID NO: 1242), light chain: OKT3VL0000-KT0 (SEQ ID NO: 1243)).
An amino acid residue structurally exposed to the surface of the OKT3 heavy chain constant region (G1T4, SEQ ID NO: 1244) was substituted with cysteine to produce the variant of OKT3 heavy chain constant region (G1T4.S191C, SEQ ID NO: 1245) shown in Table 1. In addition, other amino acid residues structurally exposed to the surface of G1T4.S191C were substituted with charged amino acids to produce the variants of G1T4.S191C shown in Table 7. These heavy chain constant regions were each linked with the OKT3 heavy chain variable region (OKT3VH0000, SEQ ID NO: 1246) to produce the OKT3 heavy chain variants, and expression vectors encoding the corresponding genes were produced by a method known in the art.

[0244]

[Table 7]

G1T4.S191C variants with additional charged amino acid substitution

Variants of heavy chain constant region	Charged mutations (EU numbering)	SEQ ID NO:
G1T4.S191C0004	S136K/G137K/G138K/L193D/G194D/T195D	1285
G1T4.S191C0005	S136K/G137K/G138K/L193E/G194E/T195E	1286
G1T4.S191C0006	S136R/G137R/G138R/L193D/G194D/T195D	1287
G1T4.S191C0007	S136R/G137R/G138R/L193E/G194E/T195E	1288
G1T4.S191C0019	G137K/G138K/L193D/G194D/T195D	1289
G1T4.S191C0020	S136K/G138K/L193D/G194D/T195D	1290
G1T4.S191C0021	S136K/G137K/L193D/G194D/T195D	1291
G1T4.S191C0022	S136K/G137K/G138K/G194D/T195D	1292
G1T4.S191C0023	S136K/G137K/G138K/L193D/T195D	1293
G1T4.S191C0024	S136K/G137K/G138K/L193D/G194D	1294
G1T4.S191C0025	G137K/G138K/L193E/G194E/T195E	1295
G1T4.S191C0026	S136K/G138K/L193E/G194E/T195E	1296
G1T4.S191C0027	S136K/G137K/L193E/G194E/T195E	1297
G1T4.S191C0028	S136K/G137K/G138K/G194E/T195E	1298
G1T4.S191C0029	S136K/G137K/G138K/L193E/T195E	1299
G1T4.S191C0030	S136K/G137K/G138K/L193E/G194E	1300
G1T4.S191C0031	G137R/G138R/L193D/G194D/T195D	1301
G1T4.S191C0032	S136R/G138R/L193D/G194D/T195D	1302
G1T4.S191C0033	S136R/G137R/L193D/G194D/T195D	1303
G1T4.S191C0034	S136R/G137R/G138R/G194D/T195D	1304
G1T4.S191C0035	S136R/G137R/G138R/L193D/T195D	1305
G1T4.S191C0036	S136R/G137R/G138R/L193D/G194D	1306
G1T4.S191C0052	S136K/L193D/G194D/T195D	1307
G1T4.S191C0053	G137K/L193D/G194D/T195D	1308
G1T4.S191C0054	G138K/L193D/G194D/T195D	1309
G1T4.S191C0055	S136K/L193E/G194E/T195E	1310
G1T4.S191C0056	G137K/L193E/G194E/T195E	1311
G1T4.S191C0057	G138K/L193E/G194E/T195E	1312
G1T4.S191C0058	S136R/L193D/G194D/T195D	1313

G1T4.S191C0059	G137R/L193D/G194D/T195D	1314
G1T4.S191C0060	G138R/L193D/G194D/T195D	1315
G1T4.S191C0061	S136R/L193E/G194E/T195E	1316
G1T4.S191C0062	G137R/L193E/G194E/T195E	1317
G1T4.S191C0063	G138R/L193E/G194E/T195E	1318
G1T4.S191C0078	G138K/T195D	1319
G1T4.S191C0079	G138K/T195E	1320
G1T4.S191C0080	G138R/T195D	1321
G1T4.S191C0081	G138R/T195E	1322
G1T4.S191C0082	G138D/T195K	1323
G1T4.S191C0083	G138D/T195R	1324
G1T4.S191C0084	G138E/T195K	1325
G1T4.S191C0085	G138E/T195R	1326
G1T4.S191C0086	G138K/G194D	1327
G1T4.S191C0087	G138K/G194E	1328
G1T4.S191C0088	G138R/G194D	1329
G1T4.S191C0089	G138R/G194E	1330
G1T4.S191C0090	G138D/G194K	1331
G1T4.S191C0091	G138D/G194R	1332
G1T4.S191C0092	G138E/G194K	1333
G1T4.S191C0093	G138E/G194R	1334
G1T4.S191C0094	G137K/T195D	1335
G1T4.S191C0095	G137K/T195E	1336
G1T4.S191C0096	G137R/T195D	1337
G1T4.S191C0097	G137R/T195E	1338
G1T4.S191C0098	G137D/T195K	1339
G1T4.S191C0099	G137D/T195R	1340
G1T4.S191C0100	G137E/T195K	1341
G1T4.S191C0101	G137E/T195R	1342
G1T4.S191C0102	G137K/G194D	1343
G1T4.S191C0103	G137K/G194E	1344
G1T4.S191C0104	G137R/G194D	1345
G1T4.S191C0105	G137R/G194E	1346

G1T4.S191C0106	G137D/G194K	1347
G1T4.S191C0107	G137D/G194R	1348
G1T4.S191C0108	G137E/G194K	1349
G1T4.S191C0109	G137E/G194R	1350
G1T4.S191C0110	G137K/G138K/G194E/T195E	1351
G1T4.S191C0111	G137K/G138K/L193E/T195E	1352
G1T4.S191C0112	S136K/G138K/G194E/T195E	1353
G1T4.S191C0113	S136K/G138K/L193E/T195E	1354
G1T4.S191C0114	S136K/G137K/G194E/T195E	1355
G1T4.S191C0115	S136K/G137K/L193E/T195E	1356
G1T4.S191C0116	S136K/G194E/T195E	1357
G1T4.S191C0117	S136K/L193E/T195E	1358
G1T4.S191C0118	G137K/G194E/T195E	1359
G1T4.S191C0119	G137K/L193E/T195E	1360
G1T4.S191C0120	G138K/G194E/T195E	1361
G1T4.S191C0121	G138K/L193E/T195E	1362
G1T4.S191C0122	G137R/G138R/G194D/T195D	1363
G1T4.S191C0123	G137R/G138R/L193D/T195D	1364
G1T4.S191C0124	S136R/G138R/G194D/T195D	1365
G1T4.S191C0125	S136R/G138R/L193D/T195D	1366
G1T4.S191C0126	S136R/G137R/G194D/T195D	1367
G1T4.S191C0127	S136R/G137R/L193D/T195D	1368
G1T4.S191C0128	S136R/G194D/T195D	1369
G1T4.S191C0129	S136R/L193D/T195D	1370
G1T4.S191C0130	G137R/G194D/T195D	1371
G1T4.S191C0131	G137R/L193D/T195D	1372
G1T4.S191C0132	G138R/G194D/T195D	1373
G1T4.S191C0133	G138R/L193D/T195D	1374
G1T4.S191C0134	S136K/T195D	1375
G1T4.S191C0135	S136K/T195E	1376
G1T4.S191C0136	S136R/T195D	1377
G1T4.S191C0137	S136R/T195E	1378
G1T4.S191C0138	S136D/T195K	1379

G1T4.S191C0139	S136D/T195R	1380
G1T4.S191C0140	S136E/T195K	1381
G1T4.S191C0141	S136E/T195R	1382
G1T4.S191C0142	S136K/G194D	1383
G1T4.S191C0143	S136K/G194E	1384
G1T4.S191C0144	S136R/G194D	1385
G1T4.S191C0145	S136R/G194E	1386
G1T4.S191C0146	S136D/G194K	1387
G1T4.S191C0147	S136D/G194R	1388
G1T4.S191C0148	S136E/G194K	1389
G1T4.S191C0149	S136E/G194R	1390

[0245] The OKT3 heavy chain variants produced as mentioned above were combined with the OKT3 light chain. The OKT3 variants shown in Table 8 were expressed by transient expression using Expi293 cells (Life technologies) by a method known in the art, and purified with Protein A by a method known in the art. In this Example, OKT3 is called "parent antibody", OKT3.S191C is called "S191C variant", and its variants are called "charged variants".

[0246]

[Table 8]

OKT3 variants with cysteine and charged amino acid substitution

Name of OKT3 variants	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:	Light chain SEQ ID NO:
OKT3	1246	1244	1243
OKT3.S191C	1246	1245	1243
OKT3.S191C0004	1246	1285	1243
OKT3.S191C0005	1246	1286	1243
OKT3.S191C0006	1246	1287	1243
OKT3.S191C0007	1246	1288	1243
OKT3.S191C0019	1246	1289	1243
OKT3.S191C0020	1246	1290	1243
OKT3.S191C0021	1246	1291	1243
OKT3.S191C0022	1246	1292	1243
OKT3.S191C0023	1246	1293	1243
OKT3.S191C0024	1246	1294	1243
OKT3.S191C0025	1246	1295	1243
OKT3.S191C0026	1246	1296	1243
OKT3.S191C0027	1246	1297	1243
OKT3.S191C0028	1246	1298	1243
OKT3.S191C0029	1246	1299	1243
OKT3.S191C0030	1246	1300	1243
OKT3.S191C0031	1246	1301	1243
OKT3.S191C0032	1246	1302	1243
OKT3.S191C0033	1246	1303	1243
OKT3.S191C0034	1246	1304	1243
OKT3.S191C0035	1246	1305	1243
OKT3.S191C0036	1246	1306	1243
OKT3.S191C0052	1246	1307	1243

OKT3.S191C0053	1246	1308	1243
OKT3.S191C0054	1246	1309	1243
OKT3.S191C0055	1246	1310	1243
OKT3.S191C0056	1246	1311	1243
OKT3.S191C0057	1246	1312	1243
OKT3.S191C0058	1246	1313	1243
OKT3.S191C0059	1246	1314	1243
OKT3.S191C0060	1246	1315	1243
OKT3.S191C0061	1246	1316	1243
OKT3.S191C0062	1246	1317	1243
OKT3.S191C0063	1246	1318	1243
OKT3.S191C0078	1246	1319	1243
OKT3.S191C0079	1246	1320	1243
OKT3.S191C0080	1246	1321	1243
OKT3.S191C0081	1246	1322	1243
OKT3.S191C0082	1246	1323	1243
OKT3.S191C0083	1246	1324	1243
OKT3.S191C0084	1246	1325	1243
OKT3.S191C0085	1246	1326	1243
OKT3.S191C0086	1246	1327	1243
OKT3.S191C0087	1246	1328	1243
OKT3.S191C0088	1246	1329	1243
OKT3.S191C0089	1246	1330	1243
OKT3.S191C0090	1246	1331	1243
OKT3.S191C0091	1246	1332	1243
OKT3.S191C0092	1246	1333	1243
OKT3.S191C0093	1246	1334	1243
OKT3.S191C0094	1246	1335	1243
OKT3.S191C0095	1246	1336	1243
OKT3.S191C0096	1246	1337	1243
OKT3.S191C0097	1246	1338	1243
OKT3.S191C0098	1246	1339	1243
OKT3.S191C0099	1246	1340	1243

OKT3.S191C0100	1246	1341	1243
OKT3.S191C0101	1246	1342	1243
OKT3.S191C0102	1246	1343	1243
OKT3.S191C0103	1246	1344	1243
OKT3.S191C0104	1246	1345	1243
OKT3.S191C0105	1246	1346	1243
OKT3.S191C0106	1246	1347	1243
OKT3.S191C0107	1246	1348	1243
OKT3.S191C0108	1246	1349	1243
OKT3.S191C0109	1246	1350	1243
OKT3.S191C0110	1246	1351	1243
OKT3.S191C0111	1246	1352	1243
OKT3.S191C0112	1246	1353	1243
OKT3.S191C0113	1246	1354	1243
OKT3.S191C0114	1246	1355	1243
OKT3.S191C0115	1246	1356	1243
OKT3.S191C0116	1246	1357	1243
OKT3.S191C0117	1246	1358	1243
OKT3.S191C0118	1246	1359	1243
OKT3.S191C0119	1246	1360	1243
OKT3.S191C0120	1246	1361	1243
OKT3.S191C0121	1246	1362	1243
OKT3.S191C0122	1246	1363	1243
OKT3.S191C0123	1246	1364	1243
OKT3.S191C0124	1246	1365	1243
OKT3.S191C0125	1246	1366	1243
OKT3.S191C0126	1246	1367	1243
OKT3.S191C0127	1246	1368	1243
OKT3.S191C0128	1246	1369	1243
OKT3.S191C0129	1246	1370	1243
OKT3.S191C0130	1246	1371	1243
OKT3.S191C0131	1246	1372	1243
OKT3.S191C0132	1246	1373	1243

OKT3.S191C0133	1246	1374	1243
OKT3.S191C0134	1246	1375	1243
OKT3.S191C0135	1246	1376	1243
OKT3.S191C0136	1246	1377	1243
OKT3.S191C0137	1246	1378	1243
OKT3.S191C0138	1246	1379	1243
OKT3.S191C0139	1246	1380	1243
OKT3.S191C0140	1246	1381	1243
OKT3.S191C0141	1246	1382	1243
OKT3.S191C0142	1246	1383	1243
OKT3.S191C0143	1246	1384	1243
OKT3.S191C0144	1246	1385	1243
OKT3.S191C0145	1246	1386	1243
OKT3.S191C0146	1246	1387	1243
OKT3.S191C0147	1246	1388	1243
OKT3.S191C0148	1246	1389	1243
OKT3.S191C0149	1246	1390	1243

[0247] Example 2-2 Assessment of electrophoretic mobility in polyacrylamide gel of antibodies having additional disulfide bond and charged mutations within the Fab region

Similarly to Example 1-2, non-reducing SDS-PAGE was carried out with the charged variants produced in Example 2-1, the gel image was captured, and intensities of bands were quantified.

In the gel images, two bands were observed in the S191C variant, and the molecular weight of the upper bands correspond to that of the parent antibody. It is highly likely that structural changes such as crosslinking via disulfide bonds of Fabs were caused by cysteine substitution, which resulted in the change in electrophoretic mobility. Thus, the lower band can be considered to correspond to the antibody having one or more engineered disulfide bond(s) formed between the CH1 regions. The ratio of the lower band to upper band are shown in Table 9. Among charged variants, most of them showed a higher lower band to upper band ratio, compared to that of S191C variants. Thus, the results suggest that additional charged amino acid mutations to the S191C variants as listed in Table 7 are likely to enhance/promote disulfide bond crosslinking of Fabs, and additional charged amino acid mutations could be an effective way to improve or increase the percentage or structural homogeneity of antibody preparation of the S191C variants having an engineered disulfide bond formed at the position 191 of CH1 region of the antibody.

[0248]

[Table 9]

The ratio of the lower band to upper band of OKT3 variants with cysteine and charged amino acid substitution

Name of OKT3 variants	Ratio of lower band to upper band (%)
OKT3	0
OKT3.S191C	65.4
OKT3.S191C0004	86.3
OKT3.S191C0005	87.2
OKT3.S191C0006	87.4
OKT3.S191C0007	83.8
OKT3.S191C0019	85.1
OKT3.S191C0020	82.6
OKT3.S191C0021	83.7
OKT3.S191C0022	77
OKT3.S191C0023	78.1
OKT3.S191C0024	75.2
OKT3.S191C0025	84
OKT3.S191C0026	85.9
OKT3.S191C0027	85.6
OKT3.S191C0028	70.3
OKT3.S191C0029	77.4
OKT3.S191C0030	79.4
OKT3.S191C0031	87.9
OKT3.S191C0032	84.4
OKT3.S191C0033	85.4
OKT3.S191C0034	79.9
OKT3.S191C0035	82
OKT3.S191C0036	82.3
OKT3.S191C0052	75.5
OKT3.S191C0053	85.7
OKT3.S191C0054	77.7
OKT3.S191C0055	79

OKT3.S191C0056	87.2
OKT3.S191C0057	83
OKT3.S191C0058	84.7
OKT3.S191C0059	78.6
OKT3.S191C0060	74.7
OKT3.S191C0061	88.4
OKT3.S191C0062	87.2
OKT3.S191C0063	85.5
OKT3.S191C0078	74.6
OKT3.S191C0079	69.7
OKT3.S191C0080	76.5
OKT3.S191C0081	72.9
OKT3.S191C0082	56.2
OKT3.S191C0083	69.7
OKT3.S191C0084	46.5
OKT3.S191C0085	67.1
OKT3.S191C0086	58.2
OKT3.S191C0087	49.7
OKT3.S191C0088	63.7
OKT3.S191C0089	65.5
OKT3.S191C0090	43.9
OKT3.S191C0091	56.9
OKT3.S191C0092	43.9
OKT3.S191C0093	53.5
OKT3.S191C0094	79.2
OKT3.S191C0095	77.6
OKT3.S191C0096	79.3
OKT3.S191C0097	71.1
OKT3.S191C0098	45.3
OKT3.S191C0099	60
OKT3.S191C0100	45.6
OKT3.S191C0101	55.9
OKT3.S191C0102	72

OKT3.S191C0103	73.2
OKT3.S191C0104	76.3
OKT3.S191C0105	74.3
OKT3.S191C0106	42.1
OKT3.S191C0107	52.1
OKT3.S191C0108	38.1
OKT3.S191C0109	44.8
OKT3.S191C0110	72.1
OKT3.S191C0111	79.2
OKT3.S191C0112	72.5
OKT3.S191C0113	78.8
OKT3.S191C0114	71.5
OKT3.S191C0115	78.5
OKT3.S191C0116	66.4
OKT3.S191C0117	78.2
OKT3.S191C0118	86.3
OKT3.S191C0119	83.4
OKT3.S191C0120	no data
OKT3.S191C0121	82.1
OKT3.S191C0122	82.3
OKT3.S191C0123	76.6
OKT3.S191C0124	76.8
OKT3.S191C0125	78.7
OKT3.S191C0126	85.5
OKT3.S191C0127	88.1
OKT3.S191C0128	77.7
OKT3.S191C0129	79.2
OKT3.S191C0130	80
OKT3.S191C0131	85.3
OKT3.S191C0132	87.6
OKT3.S191C0133	87.6
OKT3.S191C0134	66.7
OKT3.S191C0135	72.5

OKT3.S191C0136	73.7
OKT3.S191C0137	71.9
OKT3.S191C0138	45.1
OKT3.S191C0139	58.6
OKT3.S191C0140	41.4
OKT3.S191C0141	57
OKT3.S191C0142	70.5
OKT3.S191C0143	66
OKT3.S191C0144	75.9
OKT3.S191C0145	68.3
OKT3.S191C0146	43.5
OKT3.S191C0147	53.8
OKT3.S191C0148	40
OKT3.S191C0149	49.8

[0249] [Example 3] Assessment of antibodies having additional disulfide bond and hydrophobic mutations within the Fab region

Example 3-1 Production of antibodies having additional disulfide bond and hydrophobic mutations within Fab region

One disulfide bond and charge mutations were introduced into the heavy chain of an anti-human CD3 antibody, OKT3 (heavy chain: OKT3VH0000-G1T4 (SEQ ID NO: 1242), light chain: OKT3VL0000-KT0 (SEQ ID NO: 1243)).

An amino acid residue structurally exposed to the surface of the OKT3 heavy chain constant region (G1T4, SEQ ID NO: 1244) was substituted with cysteine to produce the variant of OKT3 heavy chain constant region (G1T4.S191C, SEQ ID NO: 1245) shown in Table 1. In addition, other amino acid residues structurally exposed to the surface of G1T4.S191C were substituted with hydrophobic amino acids to produce the variants of G1T4.S191C shown in Table 10. These heavy chain constant regions were each linked with the OKT3 heavy chain variable region (OKT3VH0000, SEQ ID NO: 1246) to produce the OKT3 heavy chain variants, and expression vectors encoding the corresponding genes were produced by a method known in the art.

[0250]

[Table 10]

G1T4.S191C variants with hydrophobic amino acid substitution

Variants of heavy chain constant region	Hydrophobic mutations (EU numbering)	SEQ ID NO:
G1T4.S191C0001	S136W/G137W/G138W/L193W/G194W/T195W	1391
G1T4.S191C0002	S136L/G137L/G138L/G194L/T195L	1392
G1T4.S191C0003	S136V/G137V/G138V/L193V/G194V/T195V	1393
G1T4.S191C0008	S136A/G137A/G138A/L193A/G194A/T195A	1394
G1T4.S191C0009	S136V/G137V/G138V/L193W/G194W/T195W	1395
G1T4.S191C0010	S136W/G137W/G138W/L193V/G194V/T195V	1396
G1T4.S191C0011	S136V/G137V/G138V/G194L/T195L	1397
G1T4.S191C0012	S136L/G137L/G138L/L193V/G194V/T195V	1398
G1T4.S191C0013	G137V/G138V/L193V/G194V/T195V	1399
G1T4.S191C0014	S136V/G138V/L193V/G194V/T195V	1400
G1T4.S191C0015	S136V/G137V/L193V/G194V/T195V	1401
G1T4.S191C0016	S136V/G137V/G138V/G194V/T195V	1402
G1T4.S191C0017	S136V/G137V/G138V/L193V/T195V	1403
G1T4.S191C0018	S136V/G137V/G138V/L193V/G194V	1404
G1T4.S191C0037	S136A/G137A/G138A/L193W/G194W/T195W	1405
G1T4.S191C0038	S136A/G137A/G138A/G194L/T195L	1406
G1T4.S191C0039	S136A/G137A/G138A/L193V/G194V/T195V	1407
G1T4.S191C0040	S136W/G137W/G138W/L193A/G194A/T195A	1408
G1T4.S191C0041	G137W/G138W/L193A/G194A/T195A	1409
G1T4.S191C0042	S136W/G138W/L193A/G194A/T195A	1410
G1T4.S191C0043	S136W/G137W/L193A/G194A/T195A	1411
G1T4.S191C0044	S136W/L193A/G194A/T195A	1412
G1T4.S191C0045	G137W/L193A/G194A/T195A	1413
G1T4.S191C0046	G138W/L193A/G194A/T195A	1414
G1T4.S191C0047	S136L/G137L/G138L/L193A/G194A/T195A	1415
G1T4.S191C0048	S136V/G137V/G138V/L193A/G194A/T195A	1416
G1T4.S191C0049	S136V/L193V/G194V/T195V	1417
G1T4.S191C0050	G137V/L193V/G194V/T195V	1418
G1T4.S191C0051	G138V/L193V/G194V/T195V	1419
G1T4.S191C0072	S136W/L193S/G194V/T195A	1420
G1T4.S191C0073	G137W/L193S/G194V/T195A	1421
G1T4.S191C0074	G138W/L193S/G194V/T195A	1422
G1T4.S191C0075	G137V/G138A/L193W	1423
G1T4.S191C0076	G137V/G138A/G194W	1424
G1T4.S191C0077	G137V/G138A/T195W	1425

[0251] The OKT3 heavy chain variants produced as mentioned above were combined with the OKT3 light chain. The OKT3 variants shown in Table 11 were expressed by transient expression using Expi293 cells (Life technologies) by a method known in the art, and purified with Protein A by a method known in the art. In this Example, OKT3 is called "parent antibody", OKT3.S191C is called "S191C variant", and its variants are called "hydrophobic variants".

[0252] [Table 11]

OKT3 variants with cysteine and hydrophobic amino acid substitution

Name of OKT3 variants	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:	Light chain SEQ ID NO:
OKT3	1246	1244	1243
OKT3.S191C	1246	1245	1243
OKT3.S191C0001	1246	1391	1243
OKT3.S191C0002	1246	1392	1243
OKT3.S191C0003	1246	1393	1243
OKT3.S191C0008	1246	1394	1243
OKT3.S191C0009	1246	1395	1243
OKT3.S191C0010	1246	1396	1243
OKT3.S191C0011	1246	1397	1243
OKT3.S191C0012	1246	1398	1243
OKT3.S191C0013	1246	1399	1243
OKT3.S191C0014	1246	1400	1243
OKT3.S191C0015	1246	1401	1243
OKT3.S191C0016	1246	1402	1243
OKT3.S191C0017	1246	1403	1243
OKT3.S191C0018	1246	1404	1243
OKT3.S191C0037	1246	1405	1243
OKT3.S191C0038	1246	1406	1243
OKT3.S191C0039	1246	1407	1243
OKT3.S191C0040	1246	1408	1243
OKT3.S191C0041	1246	1409	1243
OKT3.S191C0042	1246	1410	1243
OKT3.S191C0043	1246	1411	1243
OKT3.S191C0044	1246	1412	1243
OKT3.S191C0045	1246	1413	1243
OKT3.S191C0046	1246	1414	1243
OKT3.S191C0047	1246	1415	1243
OKT3.S191C0048	1246	1416	1243

OKT3.S191C0049	1246	1417	1243
OKT3.S191C0050	1246	1418	1243
OKT3.S191C0051	1246	1419	1243
OKT3.S191C0072	1246	1420	1243
OKT3.S191C0073	1246	1421	1243
OKT3.S191C0074	1246	1422	1243
OKT3.S191C0075	1246	1423	1243
OKT3.S191C0076	1246	1424	1243
OKT3.S191C0077	1246	1425	1243

[0253] Example 3-2 Assessment of electrophoretic mobility in polyacrylamide gel of antibodies having additional disulfide bond and hydrophobic mutations within the Fab region

Similarly to Example 1-2, non-reducing SDS-PAGE was carried out with the hydrophobic variants produced in Example 3-1, the gel image was captured, and bands were quantified.

In the gel images, two bands were observed in S191C variant, and the molecular weight of the upper bands correspond to that of the parent antibody. It is highly likely that structural changes such as crosslinking via disulfide bonds of Fabs were caused by cysteine substitution, which resulted in the change in electrophoretic mobility. Thus, the lower band can be considered to correspond to the antibody having one or more engineered disulfide bond(s) formed between the CH1 regions. The ratio of the lower bands to upper bands are shown in Table 12. Among hydrophobic variants, most of them showed a higher lower band to upper band ratio, compared to that of S191C variants. It is highly likely that structural changes such as crosslinking via disulfide bonds of Fabs were caused by cysteine substitution, which resulted in the change in electrophoretic mobility. Thus, the results suggest that additional hydrophobic amino acid mutations to the S191C variants as listed in Table 10 are likely to enhance/promote disulfide bond crosslinking of Fabs, and additional hydrophobic amino acid mutations could be an effective way to improve or increase the percentage or structural homogeneity of antibody preparation of the S191C variants having an engineered disulfide bond formed at the position 191 of CH1 region of the antibody.

[0254]

[Table 12]

The ratio of the lower band to upper band of OKT3 variants with cysteine and hydrophobic amino acid substitution

Name of OKT3 variants	Ratio of lower band to upper band (%)
OKT3	0
OKT3.S191C	65.4
OKT3.S191C0001	no data
OKT3.S191C0002	no data
OKT3.S191C0003	no data
OKT3.S191C0008	75.7
OKT3.S191C0009	no data
OKT3.S191C0010	no data
OKT3.S191C0011	no data
OKT3.S191C0012	91.3
OKT3.S191C0013	no data
OKT3.S191C0014	83.5
OKT3.S191C0015	82.7
OKT3.S191C0016	no data
OKT3.S191C0017	69.9
OKT3.S191C0018	76.1
OKT3.S191C0037	87.3
OKT3.S191C0038	77.3
OKT3.S191C0039	81
OKT3.S191C0040	93.9
OKT3.S191C0041	87.7
OKT3.S191C0042	95.7
OKT3.S191C0043	94.1
OKT3.S191C0044	94.8
OKT3.S191C0045	82
OKT3.S191C0046	93.7
OKT3.S191C0047	95.1
OKT3.S191C0048	82.5

OKT3.S191C0049	81.5
OKT3.S191C0050	77.7
OKT3.S191C0051	no data
OKT3.S191C0072	83.9
OKT3.S191C0073	85.5
OKT3.S191C0074	87.6
OKT3.S191C0075	63.2
OKT3.S191C0076	88.3
OKT3.S191C0077	84.1

[0255] [Example 4] Assessment of effect of de-cysteinylation by a reducing agent such as 2-MEA to promote formation of disulfide bonds in Fabs

Example 4-1 Production of antibodies having cysteine substitution in the heavy chain

Amino acid residue at position 191 (EU numbering) in the heavy chain of an anti-human IL6R neutralizing antibody, MRA, was substituted with cysteine (heavy chain: MRAH-G1T4.S191C (SEQ ID NO: 1426, light chain: MRAL-k0 (SEQ ID NO: 1427). Expression vectors encoding the corresponding genes were produced by a method known in the art.

This antibody was expressed by transient expression using Expi293 cells (Life technologies) by a method known in the art, and purified with Protein A by a method known in the art. It was concentrated to 24.1 mg/mL using Jumbosep Centrifugal Filter (PALL: OD030C65) for use in high concentrations.

[0256] Example 4-2 Preparation of antibody samples treated with 2-MEA

Using the antibody produced in Example 4-1, it was examined whether treatment/incubation with a reducing agent such as 2-MEA (2-Mercaptoethylamin) can promote formation of disulfide bonds in Fabs by inducing de-cysteinylation of capped-cysteine residues that do not form disulfide bond cross-linking.

2-MEA (Sigma-Aldrich: M6500) was dissolved in 25mM NaCl, 20mM Na-Phosphate buffer, pH7.0. The antibody and 2-MEA were mixed to the concentration shown in Table 13, and incubated in 5mM NaCl, 20mM Na-Phosphate buffer, pH7.0 at 37 degrees C for 2 hours. To stop the reduction reaction, the buffer of the mixtures with 2-MEA was changed to the buffer without 2-MEA. Then, the samples were incubated at room temperature overnight for re-oxidation.

[0257]

[Table 13]

Concentrations of antibody and 2-MEA in each sample

Sample No.	Antibody conc. (mM)	Reagents Conc. (mM)	Figure No.	Lane No.
1 [Control]	20	0	5, 6	3
2	20	0.01	5	4
3	20	0.05	5	5
4	20	0.1	5	6
5	20	0.25	6	4
6	20	0.5	6	5
7	20	1	6	6
8	20	2.5	6	7
9	20	5	6	8
10	20	10	6	9
11	20	25	6	10
12	20	50	6	11
13	20	100	6	12
14 [Control]	1	0	7, 8	3
15	1	0.01	7	4
16	1	0.05	7	5
17	1	0.1	7	6
18	1	0.25	8	4
19	1	0.5	8	5
20	1	1	8	6
21	1	2.5	8	7
22	1	5	8	8
23	1	10	8	9
24	1	25	8	10
25	1	50	8	11
26	1	100	8	12

[0258] Example 4-3 Assessment of electrophoretic mobility in polyacrylamide gel of the samples in each concentration with antibody and 2-MEA

It was examined whether the antibody samples treated with 2-MEA produced in Example 4-2 show a different electrophoretic mobility (i.e. different lower band to upper band ratio) in polyacrylamide gel by non-reducing SDS-PAGE.

Sample Buffer Solution (2ME-) (x4) (Wako; 198-13282) was used for preparation of electrophoresis samples. The samples were treated for 10 minutes under the condition of specimen concentration 100 microgram/mL and 70 degrees C, and then subjected to

non-reducing SDS-PAGE. In non-reducing SDS-PAGE, electrophoresis was carried out for 90 minutes at 125 V, using 4% SDS-PAGE mini 15well 1.0mm 15well (TEFCO; 01-052-6). Then, the gel was stained with CBB stain, the gel image was captured with ChemiDocTouchMP (BIORAD), and the bands were quantified with Image Lab (BIORAD).

The gel images are shown in Figures 5 to 8. In the gel images, two bands were observed in the sample without (0 mM of) a reducing agent (control; lane 3 in each figure), and the molecular weights of the upper bands correspond to that of the parent antibody. It is highly likely that structural changes such as crosslinking via disulfide bonds of Fabs were caused by cysteine substitution, which resulted in the change in electrophoretic mobility. Thus, the lower band can be considered to correspond to the antibody having one or more engineered disulfide bond(s) formed between the CH1 regions. The results show that most of antibody samples treated/incubated with 2-MEA showed a higher lower band to higher band ratio, compared to antibody samples without 2-MEA treatment. The results suggest that incubation of the antibody with a reducing agent such as 2-MEA could be an effective way to improve or increase the percentage or structural homogeneity of antibody preparation of the S191C variants having an engineered disulfide bond formed at the position 191 of the CH1 region of the antibody.

[0259] [Example 5] Assessment of effect of de-cysteinylation by a reducing agent such as TCEP to promote formation of disulfide bonds in Fabs

Example 5-1 Preparation of antibody samples treated with TCEP

Using the antibody produced in Example 4-1, it was examined whether treatment/ incubation with a reducing agent such as TCEP can promote formation of disulfide bonds in Fabs by inducing de-cysteinylation of capped-cysteine residues that do not form disulfide bond cross-linking.

TCEP (Sigma-Aldrich: C4706) was dissolved in ultra pure water and adjusted to pH 7 with NaOH. The antibody and TCEP were mixed to the concentration shown in Table 14, and incubated in 5mM NaCl, 20mM Na-Phosphate buffer, pH7.0 at 37 degrees C for 2 hours. To stop the reduction reaction, the buffer of the mixtures with TCEP was changed to the buffer without TCEP. Then, the samples were incubated at room temperature (RT) overnight for re-oxidation.

[0260]

[Table 14]

Concentrations of antibody and TCEP in each sample

Sample No.	Antibody conc. (mM)	Reagents Conc. (mM)	Figure No.	Lane No.
1 [Control]	20	0	9, 10	3
2	20	0.01	9	4
3	20	0.05	9	5
4	20	0.1	9	6
5	20	0.25	10	4
6	20	0.5	10	5
7	20	1	10	6
8	20	2.5	10	7
9	20	5	10	8
10	20	10	10	9
11	20	25	10	10
12	20	50	10	11
13	20	100	10	12
14 [Control]	1	0	11	3
15	1	0.25	11	4
16	1	0.5	11	5
17	1	1	11	6
18	1	2.5	11	7
19	1	5	11	8
20	1	10	11	9
21	1	25	11	10
22	1	50	11	11
23	1	100	11	12

[0261] Example 5-2 Assessment of electrophoretic mobility in polyacrylamide gel of the samples in each concentration with antibody and TCEP

Similarly to Example 4-3, non-reducing SDS-PAGE was carried out with the antibody samples treated with TCEP in Example 5-1, the gel image was captured, and bands were quantified.

The gel images are shown in Figures 9 to 11. In the gel images, two bands were observed in the sample without (0 mM of) a reducing agent (control; lane 3 in each figure), and the molecular weights of the upper bands correspond to that of the parent antibody. It is highly likely that structural changes such as crosslinking via disulfide bonds of Fabs were caused by cysteine substitution, which resulted in the change in electrophoretic mobility. Thus, the lower band can be considered to correspond to the

antibody having one or more engineered disulfide bond(s) formed between the CH1 regions. The results show that most of samples incubated/treated with TCEP showed a higher lower band to upper band ratio, compared to that of an antibody sample without TCEP treatment. The results suggest that incubation of the antibody with a reducing agent such as TCEP could be an effective way to improve or increase the percentage or structural homogeneity of antibody preparation of the S191C variants having an engineered disulfide bond formed at the position 191 of the CH1 region of the antibody.

[0262] [Example 6] Assessment of effect of de-cysteinylation by other reducing agents to promote formation of disulfide bonds in Fabs

Example 6-1 Preparation of reaction samples using 4 reducing agents

Using the antibody produced in Example 1-1, four different reducing agents, namely DTT, Cysteine, GSH, Na_2SO_3, were examined for whether they can promote formation of disulfide bonds in Fabs by inducing de-cysteinylation of capped-cysteine residues that do not form disulfide bond cross-linking.

DTT (Wako: 040-29223), L-Cysteine (Sigma-Aldrich: 168149), Glutathione (Wako: 077-02011) and Na_2SO_3 (Wako: 198-03412) were dissolved in 25mM NaCl, 20mM Na-Phosphate buffer, pH7.0. Na_2SO_3 was adjusted to pH 7 with HCl. The antibody and each reducing agent were mixed to the concentration shown in Table 15, and incubated in 5mM NaCl, 20mM Na-Phosphate buffer, pH7.0 at room temperature (RT) overnight. To stop the reduction reaction, the buffer of the mixtures with each reducing agent was changed to the buffer without the reducing agent. Then, the samples were incubated at room temperature overnight for re-oxidation.

[0263]

[Table 15]

Concentrations of antibody and reducing reagents in each sample

Sample No.	Antibody conc. (mM)	Reagents name	Reagents Conc. (mM)	Figure No.	Lane No.
1 [Control]	20	None	0	12, 13	3
2	20	DTT	0.1	12	4
3	20	DTT	1	12	5
4	20	DTT	10	12	6
5	20	DTT	100	12	7
6	20	Cysteine	0.1	12	8
7	20	Cysteine	1	12	9
8	20	Cysteine	10	12	10
9	20	Cysteine	100	12	11
10	20	GSH	0.1	13	4
11	20	GSH	1	13	5
12	20	GSH	10	13	6
13	20	GSH	50	13	7
14	20	Na_2SO_3	0.01	13	8
15	20	Na_2SO_3	0.1	13	9
16	20	Na_2SO_3	1	13	10
17	20	Na_2SO_3	10	13	11

[0264] Example 6-2 Assessment of electrophoretic mobility in polyacrylamide gel of the samples in each concentration with antibody and 4 reducing agents

Similarly to Example 4-3, non-reducing SDS-PAGE was carried out with the antibody samples produced in Example 6-1, the gel image was captured, and bands were quantified.

The gel images are shown in Figures 12 and 13. In the gel images, two bands were observed in the sample without (0 mM of) a reducing agent (control ; lane 3 in each figure), and the molecular weights of the upper bands correspond to that of the parent antibody. It is highly likely that structural changes such as crosslinking via disulfide bonds of Fabs were caused by cysteine substitution, which resulted in the change in electrophoretic mobility. Thus, the lower band can be considered to correspond to the antibody having one or more engineered disulfide bond(s) formed between the CH1 regions.

The results show that samples incubated/treated with the different reducing agents (DTT, Cysteine, GSH, and Na_2SO_3) all showed a higher lower band to upper band ratio, compared to that of an antibody sample without reducing agent treatment. The results suggest that incubation of the antibody with the reducing agent could be an

effective way to improve or increase the percentage or structural homogeneity of antibody preparation of the S191C variants having an engineered disulfide bond formed at the position 191 of the CH1 region of the antibody.

[0265] [Example 7] Assessment of effect of de-cysteinylation by a reducing agent such as 2-MEA and TCEP in different pH buffers

Example 7-1 Preparation of antibody samples treated with 2-MEA and TCEP

Using the antibody produced in Example 4-1, 2-MEA and TCEP were examined for whether they can promote formation of disulfide bonds in Fabs under various pH conditions.

2-MEA (Sigma-Aldrich: M6500) and TCEP (Sigma-Aldrich: C4706) were dissolved in 25mM NaCl, 20mM Na-Phosphate buffer, pH7.0. Especially, TCEP was adjusted to pH 7 with NaOH. 20 mg/mL of the antibody was mixed with 1 mM 2-MEA or 0.25 mM TCEP under each pH condition shown in Table 16. The composition of the pH buffer is as follows: 50mM Acetic Acid pH3.1, 50mM Acetic Acid adjust to pH4.0 with 1M Tris base, 50mM Acetic Acid adjust to pH5.0 with 1M Tris base, 25mM NaCl, 20mM Na-Phosphate buffer pH6.0, 25mM NaCl, 20mM Na-Phosphate buffer pH7.0, 25mM NaCl, 20mM Na-Phosphate buffer pH8.0. Mixed samples were incubated in each pH buffer at 37 degrees C for 2 hours. To stop the reduction reaction, the buffers of the mixtures with reducing agents were changed to the buffers without the reducing agents. Then, the samples were incubated at RT overnight for re-oxidation.

[0266]

[Table 16]

pH of reaction buffers comprising the antibody and reducing reagent in each sample

Sample No.	Antibody conc. (mM)	Reagents name	pH of reaction buffer	Figure No.	Lane No.
1 [Control]	20	None	3	14	3
2	20	2-MEA	3	14	4
3	20	TCEP	3	14	5
4 [Control]	20	None	4	14	6
5	20	2-MEA	4	14	7
6	20	TCEP	4	14	8
7 [Control]	20	None	5	14	9
8	20	2-MEA	5	14	10
9	20	TCEP	5	14	11
10 [Control]	20	None	6	15	3
11	20	2-MEA	6	15	4
12	20	TCEP	6	15	5
13 [Control]	20	None	7	15	6
8	20	2-MEA	7	15	7
15	20	TCEP	7	15	8
16 [Control]	20	None	8	15	9
17	20	2-MEA	8	15	10
18	20	TCEP	8	15	11

[0267] Example 7-2 Assessment of electrophoretic mobility in polyacrylamide gel of the samples in each pH buffer

Similarly to Example 4-3, non-reducing SDS-PAGE was carried out with the reaction samples produced in Example 7-1, the gel image was captured, and bands were quantified.

The gel images are shown in Figures 14 to 16. In the gel images, two bands were observed in the sample without (0 mM of) a reducing agent (control; lane 3, 6 and 9 in each figure), and the molecular weights of the upper bands correspond to that of the parent antibody. It is highly likely that structural changes such as crosslinking via disulfide bonds of Fabs were caused by cysteine substitution, which resulted in the change in electrophoretic mobility. Thus, the lower band can be considered to correspond to the antibody having one or more engineered disulfide bond(s) formed between the CH1 regions.

The results show that antibody samples incubated/treated with reducing agents at different pH conditions showed a higher lower band to upper band ratio, compared to that of an antibody sample without reducing agent treatment.

[0268] [Example 8] Separation of crosslinked OKT3.S191C and its variants by cation exchange chromatography

Example 8-1 Fractionation of OKT3.S191C by cation exchange chromatography

Cation exchange chromatography (CIEX) was conducted on a ProPac™ WCX-10 BioLC column, 4 mm x 250 mm (Thermo) at a flow rate of 0.5 ml/min on an UltiMate 3000 UHPLC system (Thermo Scientific Dionex). The column temperature was set at 40 degrees C. Eighty microgram of OKT3.S191C (heavy chain: OKT3VH0000-G1T4.S191C (SEQ ID NO: 1428), light chain: OKT3VL0000-KT0 (SEQ ID NO: 1243)) were loaded after the column was equilibrated with 35% mobile phase A (CX-1 pH Gradient Buffer A, pH5.6, Thermo) mixed with 65% mobile phase B (CX-1 pH Gradient Buffer B, pH10.2, Thermo). Then the column was eluted with linear gradient from 65 to 85% mobile phase B for 20 min. Detection was done by UV detector (280 nm). Four times of injections were carried out and a total of 12 fractions were collected between 11 and 17 min, with samples taken at 30-sec intervals (Figure 17). Each fraction was concentrated and evaluated using non-reducing SDS-PAGE (as described in Example 7-2). Chromatograms were analyzed using Chromeleon™ 6.8 (Thermo Scientific Dionex).

As shown in the non-reducing SDS-PAGE data (Figure 18), the acidic peaks contained the non-crosslinked Fabs (upper band), whereas the main peak contained only crosslinked Fabs (lower band). This indicated that the non-crosslinked species were eluted faster (in fraction RA3-6) and the crosslinked Fab can be separated from them using cation exchange chromatography.

[0269] Example 8-2 Fractionation of OKT3.S191C0110 by cation exchange chromatography

Cation exchange chromatography (CIEX) was conducted on a ProPac™ WCX-10 BioLC column, 4 mm x 250 mm (Thermo) at a flow rate of 0.5 ml/min on an UltiMate 3000 UHPLC system (Thermo Scientific Dionex). The column temperature was set at 40 degrees C. Approximately 100 microgram of OKT3.S191C0110 (heavy chain: OKT3VH0000-G1T4.S191C0110 (SEQ ID NO: 1429), light chain: OKT3VL0000-KT0 (SEQ ID NO: 1243)) was loaded after the column was equilibrated with 35% mobile phase A (CX-1 pH Gradient Buffer A, pH5.6, Thermo) mixed with 65% mobile phase B (CX-1 pH Gradient Buffer B, pH10.2, Thermo). Then the column was eluted with linear gradient from 65 to 100% mobile phase B for 20 min. Detection was done by UV detector (280 nm). Three times of injections were carried out and a total of 40 fractions were collected between 10 and 30 min, with samples taken at 30-sec intervals (Figure 19). Each fraction was concentrated and evaluated using non-reducing SDS-PAGE (described in Example 7-2). Chromatograms were analyzed using Chromeleon™ 6.8 (Thermo Scientific Dionex).

As shown in the SDS-PAGE data (Figure 20), the antibody species with non-crosslinked Fabs (upper band) was observed in the acidic peaks and the basic peaks, whereas the main peak contained only the antibody species with crosslinked Fabs (lower band). This indicates that additional charge mutations affected the surface charge in the antibody species with non-crosslinked Fab. The cation exchange chromatography is a useful tool to purify the antibody with crosslinked Fabs.

[0270] [Example 9] Assessment of antibodies having additional disulfide bond and charged mutations within Fab region

Example 9-1 Production of antibodies having additional disulfide bond and charged mutations within Fab region

One disulfide bond and charged mutations were introduced in the heavy chain of an anti-human CD3 antibody, OKT3 (heavy chain: OKT3VH0000-G1T4 (SEQ ID NO: 1242), light chain: OKT3VL0000-KT0 (SEQ ID NO: 1243)).

An amino acid residue structurally exposed to the surface of the OKT3 heavy chain constant region (G1T4, SEQ ID NO: 1244) was substituted with cysteine to produce the variant of OKT3 heavy chain constant region (G1T4.S191C, SEQ ID NO: 1245). In addition, CH1-CH1 interface amino acid residues structurally exposed to the surface of G1T4.S191C were substituted with charged amino acids (Figure 62A) to produce the variants of G1T4.S191C shown in Table 82. These heavy chain constant regions were each linked with the OKT3 heavy chain variable region (OKT3VH0000, SEQ ID NO: 1246) to produce the OKT3 heavy chain variants, and expression vectors encoding the corresponding genes were produced by a method known in the art.

[0271]

[Table 82]

G1T4.S191C variants with charged amino acid substitution

Variants of heavy chain constant region	Charged mutations made to G1T4.S191C (EU numbering)
G1T4.S191C0150	G137K/G138K/L193E
G1T4.S191C0151	G137K/G138K/G194E
G1T4.S191C0152	G137K/G138K/T195E
G1T4.S191C0153	S136K/G138K/L193E
G1T4.S191C0154	S136K/G138K/G194E
G1T4.S191C0155	S136K/G138K/T195E
G1T4.S191C0156	S136K/G137K/L193E
G1T4.S191C0157	S136K/G137K/G194E
G1T4.S191C0158	S136K/G137K/T195E
G1T4.S191C0159	G137R/G138R/L193D
G1T4.S191C0160	G137R/G138R/G194D
G1T4.S191C0161	G137R/G138R/T195D
G1T4.S191C0162	S136R/G138R/L193D
G1T4.S191C0163	S136R/G138R/G194D
G1T4.S191C0164	S136R/G138R/T195D
G1T4.S191C0165	S136R/G137R/L193D
G1T4.S191C0166	S136R/G137R/G194D
G1T4.S191C0167	S136R/G137R/T195D
G1T4.S191C0168	G137K/G138K/L193D
G1T4.S191C0169	G137K/G138K/G194D
G1T4.S191C0170	G137K/G138K/T195D
G1T4.S191C0171	S136K/G138K/L193D
G1T4.S191C0172	S136K/G138K/G194D
G1T4.S191C0173	S136K/G138K/T195D
G1T4.S191C0174	S136K/G137K/L193D
G1T4.S191C0175	S136K/G137K/G194D
G1T4.S191C0176	S136K/G137K/T195D

G1T4.S191C0177	G137R/G138R/L193E
G1T4.S191C0178	G137R/G138R/G194E
G1T4.S191C0179	G137R/G138R/T195E
G1T4.S191C0180	S136R/G138R/L193E
G1T4.S191C0181	S136R/G138R/G194E
G1T4.S191C0182	S136R/G138R/T195E
G1T4.S191C0183	S136R/G137R/L193E
G1T4.S191C0184	S136R/G137R/G194E
G1T4.S191C0185	S136R/G137R/T195E
G1T4.S191C0186	S136K/L193D
G1T4.S191C0187	S136K/L193E
G1T4.S191C0188	S136R/L193D
G1T4.S191C0189	S136R/L193E
G1T4.S191C0190	G137K/L193D
G1T4.S191C0191	G137K/L193E
G1T4.S191C0192	G137R/L193D
G1T4.S191C0193	G137R/L193E
G1T4.S191C0194	G138K/L193D
G1T4.S191C0195	G138K/L193E
G1T4.S191C0196	G138R/L193D
G1T4.S191C0197	G138R/L193E

[0272] The OKT3 heavy chain variants produced above were combined with the OKT3 light chain. The OKT3 variants shown in Table 83 were expressed by transient expression using Expi293 cells (Life technologies) by a method known in the art, and purified with Protein A by a method known in the art. In this Example, OKT3 is called "parent antibody", OKT3.S191C is called "S191C variant", and its variants are called "charged variants".

[0273]

[Table 83]

OKT3 variants with cysteine and charged amino acid substitution

Name of OKT3 variants	Heavy chain variable region SEQ ID NO:	Heavy chain constant region (SEQ ID NO)	Light chain SEQ ID NO:
OKT3	1246	G1T4 (SEQ ID NO: 1244)	1243
OKT3.S191C	1246	G1T4.S191C (SEQ ID NO: 1245)	1243
OKT3.S191C0150	1246	G1T4.S191C0150	1243
OKT3.S191C0151	1246	G1T4.S191C0151	1243
OKT3.S191C0152	1246	G1T4.S191C0152	1243
OKT3.S191C0153	1246	G1T4.S191C0153	1243
OKT3.S191C0154	1246	G1T4.S191C0154	1243
OKT3.S191C0155	1246	G1T4.S191C0155	1243
OKT3.S191C0156	1246	G1T4.S191C0156	1243
OKT3.S191C0157	1246	G1T4.S191C0157	1243
OKT3.S191C0158	1246	G1T4.S191C0158	1243
OKT3.S191C0159	1246	G1T4.S191C0159	1243
OKT3.S191C0160	1246	G1T4.S191C0160	1243
OKT3.S191C0161	1246	G1T4.S191C0161	1243
OKT3.S191C0162	1246	G1T4.S191C0162	1243
OKT3.S191C0163	1246	G1T4.S191C0163	1243
OKT3.S191C0164	1246	G1T4.S191C0164	1243
OKT3.S191C0165	1246	G1T4.S191C0165	1243
OKT3.S191C0166	1246	G1T4.S191C0166	1243
OKT3.S191C0167	1246	G1T4.S191C0167	1243
OKT3.S191C0168	1246	G1T4.S191C0168	1243
OKT3.S191C0169	1246	G1T4.S191C0169	1243
OKT3.S191C0170	1246	G1T4.S191C0170	1243
OKT3.S191C0171	1246	G1T4.S191C0171	1243
OKT3.S191C0172	1246	G1T4.S191C0172	1243

OKT3.S191C0173	1246	G1T4.S191C0173	1243
OKT3.S191C0174	1246	G1T4.S191C0174	1243
OKT3.S191C0175	1246	G1T4.S191C0175	1243
OKT3.S191C0176	1246	G1T4.S191C0176	1243
OKT3.S191C0177	1246	G1T4.S191C0177	1243
OKT3.S191C0178	1246	G1T4.S191C0178	1243
OKT3.S191C0179	1246	G1T4.S191C0179	1243
OKT3.S191C0180	1246	G1T4.S191C0180	1243
OKT3.S191C0181	1246	G1T4.S191C0181	1243
OKT3.S191C0182	1246	G1T4.S191C0182	1243
OKT3.S191C0183	1246	G1T4.S191C0183	1243
OKT3.S191C0184	1246	G1T4.S191C0184	1243
OKT3.S191C0185	1246	G1T4.S191C0185	1243
OKT3.S191C0186	1246	G1T4.S191C0186	1243
OKT3.S191C0187	1246	G1T4.S191C0187	1243
OKT3.S191C0188	1246	G1T4.S191C0188	1243
OKT3.S191C0189	1246	G1T4.S191C0189	1243
OKT3.S191C0190	1246	G1T4.S191C0190	1243
OKT3.S191C0191	1246	G1T4.S191C0191	1243
OKT3.S191C0192	1246	G1T4.S191C0192	1243
OKT3.S191C0193	1246	G1T4.S191C0193	1243
OKT3.S191C0194	1246	G1T4.S191C0194	1243
OKT3.S191C0195	1246	G1T4.S191C0195	1243
OKT3.S191C0196	1246	G1T4.S191C0196	1243
OKT3.S191C0197	1246	G1T4.S191C0197	1243

[0274] Example 9-2 Assessment of electrophoretic mobility in polyacrylamide gel of antibodies having additional disulfide bond and charged mutations within Fab region

Similarly to Example 1-2, non-reducing SDS-PAGE was carried out with the charged variants produced in Example 9-1, the gel image was captured, and bands were quantified.

In the gel images, two bands were observed in S191C variant, and the molecular weight of the upper bands was similar to that of the parent antibody. The ratio of the lower bands to upper bands are shown in Table 84. Among charged variants, most of them showed higher ratio of lower bands to upper bands, compared to S191C variants. It is highly likely that structural changes such as crosslinking via disulfide bond of Fabs were caused by cysteine substitution, which resulted in the change in electrophoretic mobility. Thus, additional charged mutations to S191C variant are likely to enhance crosslinking of Fabs.

[0275] [Table 84]

The ratio of the lower bands to upper bands of OKT3 variants with cysteine and charged amino acid substitution

Name of OKT3 variants	Ratio of lower band to upper band (%)
OKT3	0
OKT3.S191C	65.4
OKT3.S191C0150	70.0
OKT3.S191C0151	58.5
OKT3.S191C0152	66.5
OKT3.S191C0153	70.7
OKT3.S191C0154	56.7
OKT3.S191C0155	65.9
OKT3.S191C0156	70.2
OKT3.S191C0157	71.6
OKT3.S191C0158	68.5
OKT3.S191C0159	68.9
OKT3.S191C0160	68.1
OKT3.S191C0161	79.1
OKT3.S191C0162	69.6
OKT3.S191C0163	63.3
OKT3.S191C0164	70.8
OKT3.S191C0165	76.7
OKT3.S191C0166	75.6
OKT3.S191C0167	68.0
OKT3.S191C0168	66.2
OKT3.S191C0169	58.8
OKT3.S191C0170	74.5
OKT3.S191C0171	72.9
OKT3.S191C0172	64.0
OKT3.S191C0173	69.1
OKT3.S191C0174	62.1

OKT3.S191C0175	70.4
OKT3.S191C0176	89.8
OKT3.S191C0177	76.7
OKT3.S191C0178	86.3
OKT3.S191C0179	82.2
OKT3.S191C0180	80.5
OKT3.S191C0181	76.0
OKT3.S191C0182	78.8
OKT3.S191C0183	78.5
OKT3.S191C0184	80.6
OKT3.S191C0185	83.5
OKT3.S191C0186	75.7
OKT3.S191C0187	81.6
OKT3.S191C0188	68.5
OKT3.S191C0189	83.4
OKT3.S191C0190	78.0
OKT3.S191C0191	72.5
OKT3.S191C0192	80.6
OKT3.S191C0193	76.3
OKT3.S191C0194	84.8
OKT3.S191C0195	79.3
OKT3.S191C0196	82.7
OKT3.S191C0197	85.5

[0276] Example 9-3 Assessment of peak separation of antibodies having additional disulfide bond and charged mutations within Fab region by cation exchange chromatography

Cation exchange chromatography (CIEX) was conducted on a ProPac™ WCX-10 BioLC column, 4 mm X 250 mm (Thermo) at a flow rate of 0.5 ml/min on an Alliance HPLC system (Waters). Column temperature was set at 40 degrees C. Eighty microgram of charged variants produced in Example 9-1 were loaded after column was equilibrated with 35% mobile phase A (CX-1 pH Gradient Buffer A, pH5.6, Thermo) mixed with 65% mobile phase B (CX-1 pH Gradient Buffer B, pH10.2, Thermo). Then the column was eluted with linear gradient from 65 to 100% mobile phase B for 35 min. Detection was done by UV detector (280 nm). Chromatograms of CIEX are shown in Figure 58 and 59.

In Figure 58 and 59, similar peak patterns to Figure 19, which could separate crosslinked and non-crosslinked Fabs, were observed in some charged variants. It is highly likely that additional charged mutations to S191C variant can enhance not only crosslinking of Fabs but also separation between crosslinked and non-crosslinked Fabs

by CIEX. See also Figure 62B.

[0277] [Example 10] Assessment of different antibodies having additional disulfide bond and charged mutations within Fab region

Example 10-1 Production of different antibodies having additional disulfide bond and charged mutations within Fab region

One disulfide bond and charged mutations were introduced in the heavy chain of an anti-human CD3 antibody, OKT3 (heavy chain: OKT3VH0000-G1T4 (SEQ ID NO: 1242), light chain: OKT3VL0000-KT0 (SEQ ID NO: 1243)). Similarly, one disulfide bond and charge mutations were introduced in the heavy chain of an anti-human IL-6R antibody, MRA (heavy chain: MRAH-G1T4 (SEQ ID NO: 15), light chain: MRAL-k0 (SEQ ID NO: 16)).

An amino acid residue structurally exposed to the surface of the OKT3 and MRA heavy chain constant region (G1T4, SEQ ID NO: 1244) was substituted with cysteine to produce the variant of OKT3 heavy chain constant region (G1T4.S191C, SEQ ID NO: 1245). In addition, CH1-CH1 interface amino acid residues structurally exposed to the surface of G1T4.S191C were substituted with charged amino acids (Figure 62A) to produce the variants of G1T4.S191C shown in Table 85. These heavy chain constant regions were each linked with the OKT3 heavy chain variable region (OKT3VH0000, SEQ ID NO: 1246) and MRA heavy chain variable region (MRAH, SEQ ID NO: P17) respectively to produce the OKT3 and MRA heavy chain variants, and expression vectors encoding the corresponding genes were produced by a method known in the art.

[0278]

[Table 85]

G1T4.S191C variants with charged amino acid substitution

Variants of heavy chain constant region	Charged mutations made to G1T4.S191C (EU numbering)
G1T4.S191C0159	G137R/G138R/L193D
G1T4.S191C0161	G137R/G138R/T195D
G1T4.S191C0162	S136R/G138R/L193D
G1T4.S191C0164	S136R/G138R/T195D
G1T4.S191C0165	S136R/G137R/L193D
G1T4.S191C0167	S136R/G137R/T195D
G1T4.S191C0177	G137R/G138R/L193E
G1T4.S191C0179	G137R/G138R/T195E
G1T4.S191C0180	S136R/G138R/L193E
G1T4.S191C0182	S136R/G138R/T195E
G1T4.S191C0183	S136R/G137R/L193E
G1T4.S191C0185	S136R/G137R/T195E

[0279] The OKT3 and MRA heavy chain variants produced above were combined with the OKT3 and MRA light chains respectively. The OKT3 and MRA variants shown in Table 86 were expressed by transient expression using Expi293 cells (Life technologies) by a method known in the art, and purified with Protein A by a method known in the art. In this Example, OKT3 and MRA are called "parent antibody", OKT3.S191C and MRA.S191C are called "S191C variant", and their variants are called "charged variants".

[0280]

[Table 86]

OKT3 and MRA variants with cysteine and charged amino acid substitution

Original antibody	Name of OKT3 variants	Heavy chain variable region SEQ ID NO:	Heavy chain constant region (SEQ ID NO)	Light chain SEQ ID NO:
OKT3	OKT3	1246	G1T4 (SEQ ID NO: 1244)	1243
OKT3	OKT3.S191C	1246	G1T4.S191C (SEQ ID NO: 1245)	1243
OKT3	OKT3.S191C0159	1246	G1T4.S191C0159	1243
OKT3	OKT3.S191C0161	1246	G1T4.S191C0161	1243
OKT3	OKT3.S191C0162	1246	G1T4.S191C0162	1243
OKT3	OKT3.S191C0164	1246	G1T4.S191C0164	1243
OKT3	OKT3.S191C0165	1246	G1T4.S191C0165	1243
OKT3	OKT3.S191C0167	1246	G1T4.S191C0167	1243
OKT3	OKT3.S191C0177	1246	G1T4.S191C0177	1243
OKT3	OKT3.S191C0179	1246	G1T4.S191C0179	1243
OKT3	OKT3.S191C0180	1246	G1T4.S191C0180	1243
OKT3	OKT3.S191C0182	1246	G1T4.S191C0182	1243
OKT3	OKT3.S191C0183	1246	G1T4.S191C0183	1243
OKT3	OKT3.S191C0185	1246	G1T4.S191C0185	1243
MRA	MRA	17	G1T4 (SEQ ID NO: 1244)	16
MRA	MRA.S191C	17	G1T4.S191C (SEQ ID NO: 1245)	16
MRA	MRA.S191C0159	17	G1T4.S191C0159	16
MRA	MRA.S191C0161	17	G1T4.S191C0161	16
MRA	MRA.S191C0162	17	G1T4.S191C0162	16
MRA	MRA.S191C0164	17	G1T4.S191C0164	16
MRA	MRA.S191C0165	17	G1T4.S191C0165	16
MRA	MRA.S191C0167	17	G1T4.S191C0167	16
MRA	MRA.S191C0177	17	G1T4.S191C0177	16
MRA	MRA.S191C0179	17	G1T4.S191C0179	16
MRA	MRA.S191C0180	17	G1T4.S191C0180	16
MRA	MRA.S191C0182	17	G1T4.S191C0182	16
MRA	MRA.S191C0183	17	G1T4.S191C0183	16
MRA	MRA.S191C0185	17	G1T4.S191C0185	16

[0281] Example 10-2 Assessment of electrophoretic mobility in polyacrylamide gel of different antibodies having additional disulfide bond and charged mutations within Fab region

It was examined whether the antibodies produced in Example 10-1 show a different electrophoretic mobility in polyacrylamide gel by non-reducing SDS-PAGE. Sample Buffer Solution (2ME-) (x4) (Wako; 198-13282) was used for preparation of electrophoresis samples. The samples were treated for 10 minutes under the condition of specimen concentration 75 microgram/mL and 70 degrees C, and then subjected to non-reducing SDS-PAGE. In non-reducing SDS-PAGE, electrophoresis was carried out for 90 minutes at 126 V, using 4% SDS-PAGE mini 15well 1.0mm 15well (TEFCO; Cat#01-052-6). Then, the gel was stained with CBB stain, the gel image was captured with ChemiDocTouchMP (BIORAD), and the bands were quantified with Image Lab (BIORAD).

In the gel images, two bands were observed in S191C variant, and the molecular weight of the upper bands was similar to that of the parent antibody. The ratio of the lower bands to upper bands are shown in Table 87 and plotted in a scatter diagram shown in Figure 60. Good correlation between the ratio of lower bands to upper bands in OKT3 and MRA was observed. Thus, additional charged mutations to S191C variant are likely to enhance crosslinking of Fabs of not only OKT3 but also other antibodies binding to other antigen such as MRA.

[0282]

[Table 87]

The ratio of the lower bands to upper bands of OKT3 and MRA variants with cysteine and charged amino acid substitution

Original antibody	Name of OKT3 variants	Ratio of lower band to upper band (%)
OKT3	OKT3	0
OKT3	OKT3.S191C	72.4
OKT3	OKT3.S191C0159	80.9
OKT3	OKT3.S191C0161	83.0
OKT3	OKT3.S191C0162	83.3
OKT3	OKT3.S191C0164	78.7
OKT3	OKT3.S191C0165	81.0
OKT3	OKT3.S191C0167	79.6
OKT3	OKT3.S191C0177	80.5
OKT3	OKT3.S191C0179	79.9
OKT3	OKT3.S191C0180	83.9
OKT3	OKT3.S191C0182	77.8
OKT3	OKT3.S191C0183	88.0
OKT3	OKT3.S191C0185	84.8
MRA	MRA	0
MRA	MRA.S191C	70.8
MRA	MRA.S191C0159	80.3
MRA	MRA.S191C0161	78.8
MRA	MRA.S191C0162	81.6
MRA	MRA.S191C0164	77.7
MRA	MRA.S191C0165	78.0
MRA	MRA.S191C0167	76.7
MRA	MRA.S191C0177	80.5
MRA	MRA.S191C0179	78.9
MRA	MRA.S191C0180	81.4
MRA	MRA.S191C0182	82.7
MRA	MRA.S191C0183	88.9
MRA	MRA.S191C0185	80.9

[0283] Example 10-3 Assessment of peak separation of different antibodies having additional disulfide bond and charged mutations within Fab region by cation exchange chromatography

Cation exchange chromatography (CIEX) was conducted on a ProPac™ WCX-10 BioLC column, 4 mm X 250 mm (Thermo) at a flow rate of 0.5 ml/min on an Alliance

HPLC system (Waters). Column temperature was set at 40 degrees C. Eighty microgram of charged variants produced in Example 10-1 were loaded after column was equilibrated with 45% mobile phase A (CX-1 pH Gradient Buffer A, pH5.6, Thermo) mixed with 55% mobile phase B (CX-1 pH Gradient Buffer B, pH10.2, Thermo). Then the column was eluted with linear gradient from 55 to 95% mobile phase B for 40 min. Detection was done by UV detector (280 nm). Chromatograms of CIEX are shown in Figure 61A and 61B.

In Figure 61A and 61B, similar peak patterns between OKT3 and MRA variants were observed. Thus, additional charged mutations to S191C variant are likely to enhance separation between crosslinked and non-crosslinked Fabs of not only OKT3 but also other antibodies binding to other antigen such as MRA by CIEX.

[0284] [Reference Example 1] Concept of Fab-crosslinked antibody

Agonist antibodies are superior in properties such as stability, pharmacokinetics, and production methods compared to natural ligands and their fusion proteins, and their pharmaceutical development is under way. However, in general, agonist antibodies with strong activity are more difficult to obtain than mere binding or neutralizing antibodies. A solution to this problem is therefore being wanted.

Properties needed for an agonist antibody may depend on the type of the ligand. For agonist antibodies against the TNF receptor superfamily, typified by Death receptor (DR), OX40, 4-1BB, CD40, and such, it has been reported that multimerization of antibody or ligand contributes to the activation. As techniques for increasing this effect, use of natural ligands, crosslinking by anti-Fc antibodies, crosslinking via Fc gamma Rs, multimerization of antibody binding domains, multimerization via antibody Fc, and such have been reported to enhance the agonist activity. It is also known that, for certain types of antigens, adjustment of the distance of antigen-binding sites using antibody Fab structure or scFv leads to enhancement of the agonist activity regardless of multimerization.

As another technique, an agonist antibody against a cytokine receptor which is a bispecific antibody capable of binding to different epitopes within the same antigen has been reported. Moreover, a method of improving agonist activity by using chemical conjugation to crosslink two different Fabs in a similar manner has been reported.

More methods besides those mentioned above for improving the activity of agonist antibodies are wanted. However, no simple method to achieve this has been reported. Thus, the inventors developed a method for crosslinking Fabs with each other through introducing minimum mutations, and demonstrated that this actually enhanced the agonist activity, thereby completing the invention. An exemplifying embodiment is shown in Fig. 21.

[0285] [Reference Example 2] Production of expression vectors for modified antibodies, and

expression and purification of modified antibodies

An antibody gene inserted in an expression vector for animal cells was subjected to amino acid residue sequence substitution by a method known to the person skilled in the art using PCR, the In-Fusion Advantage PCR cloning kit (TAKARA), or such, to construct an expression vector for a modified antibody. The nucleotide sequence of the resulting expression vector was determined by a method known to the person skilled in the art. The produced expression vector was transiently introduced into FreeStyle293 (registered trademark) or Expi293 (registered trademark) cells (Invitrogen) and the cells were allowed to express the modified antibody into culture supernatant. The modified antibody was purified from the obtained culture supernatant by a method known to the person skilled in the art using rProtein A Sepharose (registered trademark) Fast Flow (GE Healthcare). Absorbance at 280 nm was measured using a spectrophotometer. An absorption coefficient was calculated from the measured value using the PACE method and used to calculate the antibody concentration (Protein Science 1995;4:2411-2423).

The amount of aggregates of the modified antibody was analyzed by a method known to the person skilled in the art using Agilent 1260 Infinity (registered trademark) (Agilent Technologies) for HPLC and G3000SW_{XL} (TOSOH) as a gel filtration chromatography column. The concentration of the purified antibody was 0.1 mg/mL, and 10 microliter of the antibody was injected.

Antibodies prepared by this method (anti-CD3 epsilon antibodies, anti-CD28 antibodies, and anti-CD3 epsilon x anti-CD28 bispecific antibodies) are shown in Table 17.

[0286]

[Table 17]

Antibody names, SEQ ID NOs

Antibody name	SEQ ID NO:			
	Heavy chain 1	Light chain 1	Heavy chain 2	Light chain 2
CD3-G4s	1	10	-	-
CD3-G4sHH	2	10	-	-
CD3-G4sLL	1	11	-	-
CD3-G1s	4	10	-	-
OKT3-G1s	5	12	-	-
CD28-G1	6	13	-	-
CD3-G1sLL	4	11	-	-
CD3-G1sHH	7	10	-	-
CD3//CD28-G1s	4	10	6	13
CD3//CD28-G1sLL	4	11	6	14
CD3//CD28-G1sHH	7	10	9	13
CD3//CD28-G1sLH	4	11	9	13
CD3//CD28-G1sHL	7	10	6	14
OKT3//CD28-G1s	5	12	6	13
OKT3//CD28-G1sHH	8	12	9	13
OKT3//CD28-G1sHL	8	12	6	14

HH: position 191 (EU numbering) was altered to Cys in the two H chain constant regions

LL: position 126 (EU numbering) was altered to Cys in the two L chain constant regions

HL, LH: position 191 (EU numbering) was altered to Cys in one H chain constant region, and position 126 (EU numbering) was altered to Cys in one L chain constant region

[0287] [Reference Example 3] Preparation of bispecific antibodies

The purified antibody was dialyzed into TBS (WAKO) buffer and its concentration was adjusted to 1 mg /mL. As a 10x reaction buffer, 250 mM 2-MEA (SIGMA) was prepared. Two different homodimeric antibodies prepared in Reference Example 2 were mixed in equal amount. To this mixture, a 1/10 volume of the 10x reaction buffer was added and mixed. The mixture was allowed to stand at 37 degrees C for 90 minutes. After the reaction, the mixture was dialyzed into TBS to obtain a solution of a bispecific antibody in which the above two different antibodies were heterodimerized.

The antibody concentration was measured by the above-mentioned method, and the antibody was subjected to subsequent experiments.

[0288] [Reference Example 4] Assessment of agonist activity

Reference Example 4-1 Preparation of Jurkat cell suspension

Jurkat cells (TCR/CD3 Effector Cells (NFAT), Promega) were collected from flasks. The cells were washed with Assay Buffer (RPMI 1640 medium (Gibco), 10% FBS (HyClone), 1% MEM Non Essential Amino Acids (Invitrogen), and 1 mM Sodium Pyruvate (Invitrogen)), and then suspended at 3 x 10^6 cells/mL in Assay Buffer. This suspension of Jurkat cells was subjected to subsequent experiments .

[0289] Reference Example 4-2 Preparation of luminescence reagent solution

100 mL of Bio-Glo Luciferase Assay Buffer (Promega) was added to the bottle of Bio-Glo Luciferase Assay Substrate (Promega), and mixed by inversion. The bottle was protected from light and frozen at -20 degrees C. This luminescence reagent solution was subjected to subsequent experiments.

[0290] Reference Example 4-3 T cell activation assay

T cell activation by agonist signaling was assessed based on the fold change of luciferase luminescence. The aforementioned Jurkat cells are cells transformed with a luciferase reporter gene having a NFAT responsive sequence. When the cells are stimulated by an anti-TCR/CD3 antibody, the NFAT pathway is activated via intracellular signaling, thereby inducing luciferase expression. The Jurkat cells suspension prepared as described above was added to a 384-well flat-bottomed white plate at 10 microliter per well (3 x 10^4 cells/well). Next, the antibody solution prepared at each concentration (150, 15, 1.5, 0.15, 0.015, 0.0015, 0.00015, 0.000015 nM) was added at 20 microliter per well. This plate was allowed to stand in a 5% CO_2 incubator at 37 degrees C for 24 hours. After the incubation, the luminescence reagent solution was thawed, and 30 microliter of the solution was added to each well. The plate was then allowed to stand at room temperature for 10 minutes. Luciferase luminescence in each well of the plate was measured using a luminometer.

As a result, modified molecules with an additional disulfide bond linking the Fab-Fab of anti-CD3 epsilon antibody showed varied CD3-mediated signaling compared to the wild-type molecule (unmodified molecule) as shown in Figs. 22 and 23. Furthermore, as shown in Figs. 24 and 25, modified molecules of a bispecific antibody composed of an anti-CD3 epsilon antibody and an anti-CD28 antibody with an additional disulfide bond linking the Fab-Fab also showed largely varied CD3- and/or CD28-mediated signaling compared to the wild-type molecule.

These results suggest that introducing modifications of the present invention can enhance or diminish agonist activity possessed by antigen-binding molecules such as antibodies.

[0291] [Reference Example 5] Assessment of antibodies having cysteine substitution at various positions in the heavy chain

Reference Example 5-1 Assessment of antibodies having cysteine substitution at various positions in the heavy chain

The heavy chain variable region and constant region of an anti-human IL6R neutralizing antibody, MRA (heavy chain: MRAH-G1T4 (SEQ ID NO: 15), light chain: MRAL-k0 (SEQ ID NO: 16)) were subjected to a study in which an arbitrary amino acid residue structurally exposed to the surface was substituted with cysteine.

Amino acid residues within the heavy chain variable region of MRA (MRAH, SEQ ID NO: 17) were substituted with cysteine to produce variants of the heavy chain variable region of MRA shown in Table 18. These variants of the heavy chain variable region of MRA were each linked with the heavy chain constant region of MRA (G1T4, SEQ ID NO: 18) to produce variants of the heavy chain of MRA, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art.

In addition, amino acid residues within the heavy chain constant region of MRA (G1T4, SEQ ID NO: 18) were substituted with cysteine to produce variants of the heavy chain constant region of MRA shown in Table 19. These variants of the heavy chain constant region of MRA were each linked with the heavy chain variable region of MRA (MRAH, SEQ ID NO: 17) to produce variants of the heavy chain of MRA, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art.

The MRA heavy chain variants produced above were combined with the MRA light chain. The resultant MRA variants shown in Table 20 were expressed by transient expression using FreeStyle293 cells (Invitrogen) or Expi293 cells (Life technologies) by a method known to the person skilled in the art, and purified with Protein A by a method known to the person skilled in the art.

[0292]

[Table 18]

Variants of MRA heavy chain variable region and position of cysteine substitution

Variant of MRA heavy chain variable region	Position of cysteine substitution (Kabat numbering)	SEQ ID NO:
MRAH.Q5C	5	21
MRAH.E6C	6	22
MRAH.S7C	7	23
MRAH.G8C	8	24
MRAH.P9C	9	25
MRAH.G10C	10	26
MRAH.L11C	11	27
MRAH.V12C	12	28
MRAH.R13C	13	29
MRAH.P14C	14	30
MRAH.S15C	15	31
MRAH.Q16C	16	32
MRAH.T17C	17	33
MRAH.L18C	18	34
MRAH.S19C	19	35
MRAH.L20C	20	36
MRAH.T21C	21	37
MRAH.T23C	23	38
MRAH.S25C	25	39
MRAH.G26C	26	40
MRAH.S28C	28	41
MRAH.T30C	30	42
MRAH.R66C	66	43
MRAH.V67C	67	44
MRAH.T68C	68	45
MRAH.L70C	70	46
MRAH.D72C	72	47
MRAH.T73C	73	48

MRAH.S74C	74	49
MRAH.K75C	75	50
MRAH.N76C	76	51
MRAH.Q77C	77	52
MRAH.S79C	79	53
MRAH.L80C	80	54
MRAH.R81C	81	55
MRAH.L82C	82	56
MRAH.S82aC	82a	57
MRAH.S82bC	82b	58
MRAH.V82cC	82c	59
MRAH.S112C	112	60
MRAH.S113C	113	61
MRAH.S31C	31	62
MRAH.W35C	35	63
MRAH.S35aC	35a	64
MRAH.Y50C	50	65
MRAH.I51C	51	66
MRAH.S52C	52	67
MRAH.S62C	62	68
MRAH.L63C	63	69
MRAH.K64C	64	70
MRAH.S65C	65	71
MRAH.D101C	101	72
MRAH.Y102C	102	73

[0293]

[Table 19]

Variants of MRA heavy chain constant region and position of cysteine substitution

Variant of MRA heavy chain constant region	Position of cysteine substitution (EU numbering)	SEQ ID NO:
G1T4.A118C	118	74
G1T4.S119C	119	75
G1T4.T120C	120	76
G1T4.K121C	121	77
G1T4.G122C	122	78
G1T4.P123C	123	79
G1T4.S124C	124	80
G1T4.V125C	125	81
G1T4.F126C	126	82
G1T4.P127C	127	83
G1T4.S131C	131	84
G1T4.S132C	132	85
G1T4.K133C	133	86
G1T4.S134C	134	87
G1T4.T135C	135	88
G1T4.S136C	136	89
G1T4.G137C	137	90
G1T4.G138C	138	91
G1T4.T139C	139	92
G1T4.A140C	140	93
G1T4.A141C	141	94
G1T4.D148C	148	95
G1T4.Y149C	149	96
G1T4.F150C	150	97
G1T4.P151C	151	98
G1T4.E152C	152	99
G1T4.P153C	153	100
G1T4.V154C	154	101

G1T4.T155C	155	102
G1T4.V156C	156	103
G1T4.S157C	157	104
G1T4.W158C	158	105
G1T4.N159C	159	106
G1T4.S160C	160	107
G1T4.G161C	161	108
G1T4.A162C	162	109
G1T4.L163C	163	110
G1T4.T164C	164	111
G1T4.S165C	165	112
G1T4.G166C	166	113
G1T4.V167C	167	114
G1T4.V173C	173	115
G1T4.L174C	174	116
G1T4.Q175C	175	117
G1T4.S176C	176	118
G1T4.S177C	177	119
G1T4.G178C	178	120
G1T4.L179C	179	121
G1T4.Y180C	180	122
G1T4.V186C	186	123
G1T4.T187C	187	124
G1T4.V188C	188	125
G1T4.P189C	189	126
G1T4.S190C	190	127
G1T4.S191C	191	128
G1T4.S192C	192	129
G1T4.L193C	193	130
G1T4.G194C	194	131
G1T4.T195C	195	132
G1T4.Q196C	196	133
G1T4.T197C	197	134

G1T4.Y198C	198	135
G1T4.I199C	199	136
G1T4.N201C	201	137
G1T4.V202C	202	138
G1T4.N203C	203	139
G1T4.H204C	204	140
G1T4.K205C	205	141
G1T4.P206C	206	142
G1T4.S207C	207	143
G1T4.N208C	208	144
G1T4.T209C	209	145
G1T4.K210C	210	146
G1T4.V211C	211	147
G1T4.D212C	212	148
G1T4.K213C	213	149
G1T4.R214C	214	150
G1T4.V215C	215	151
G1T4.E216C	216	152
G1T4.P217C	217	153
G1T4.K218C	218	154
G1T4.S219C	219	155

[0294]

[Table 20]

MRA variants

	SEQ ID NO:			
Antibody name	Heavy chain variable region	Heavy chain constant region	Light chain variable region	Light chain constant region
MRAH.Q5C-G1T4	21	18	19	20
MRAH.E6C-G1T4	22	18	19	20
MRAH.S7C-G1T4	23	18	19	20
MRAH.G8C-G1T4	24	18	19	20
MRAH.P9C-G1T4	25	18	19	20
MRAH.G10C-G1T4	26	18	19	20
MRAH.L11C-G1T4	27	18	19	20
MRAH.V12C-G1T4	28	18	19	20
MRAH.R13C-G1T4	29	18	19	20
MRAH.P14C-G1T4	30	18	19	20
MRAH.S15C-G1T4	31	18	19	20
MRAH.Q16C-G1T4	32	18	19	20
MRAH.T17C-G1T4	33	18	19	20
MRAH.L18C-G1T4	34	18	19	20
MRAH.S19C-G1T4	35	18	19	20
MRAH.L20C-G1T4	36	18	19	20
MRAH.T21C-G1T4	37	18	19	20
MRAH.T23C-G1T4	38	18	19	20
MRAH.S25C-G1T4	39	18	19	20
MRAH.G26C-G1T4	40	18	19	20
MRAH.S28C-G1T4	41	18	19	20
MRAH.T30C-G1T4	42	18	19	20
MRAH.R66C-G1T4	43	18	19	20
MRAH.V67C-G1T4	44	18	19	20
MRAH.T68C-G1T4	45	18	19	20
MRAH.L70C-G1T4	46	18	19	20
MRAH.D72C-G1T4	47	18	19	20

MRAH.T73C-G1T4	48	18	19	20
MRAH.S74C-G1T4	49	18	19	20
MRAH.K75C-G1T4	50	18	19	20
MRAH.N76C-G1T4	51	18	19	20
MRAH.Q77C-G1T4	52	18	19	20
MRAH.S79C-G1T4	53	18	19	20
MRAH.L80C-G1T4	54	18	19	20
MRAH.R81C-G1T4	55	18	19	20
MRAH.L82C-G1T4	56	18	19	20
MRAH.S82aC-G1T4	57	18	19	20
MRAH.S82bC-G1T4	58	18	19	20
MRAH.V82cC-G1T4	59	18	19	20
MRAH.S112C-G1T4	60	18	19	20
MRAH.S113C-G1T4	61	18	19	20
MRAH.S31C-G1T4	62	18	19	20
MRAH.W35C-G1T4	63	18	19	20
MRAH.S35aC-G1T4	64	18	19	20
MRAH.Y50C-G1T4	65	18	19	20
MRAH.I51C-G1T4	66	18	19	20
MRAH.S52C-G1T4	67	18	19	20
MRAH.S62C-G1T4	68	18	19	20
MRAH.L63C-G1T4	69	18	19	20
MRAH.K64C-G1T4	70	18	19	20
MRAH.S65C-G1T4	71	18	19	20
MRAH.D101C-G1T4	72	18	19	20
MRAH.Y102C-G1T4	73	18	19	20
MRAH-G1T4.A118C	17	74	19	20
MRAH-G1T4.S119C	17	75	19	20
MRAH-G1T4.T120C	17	76	19	20
MRAH-G1T4.K121C	17	77	19	20
MRAH-G1T4.G122C	17	78	19	20
MRAH-G1T4.P123C	17	79	19	20
MRAH-G1T4.S124C	17	80	19	20

MRAH-G1T4.V125C	17	81	19	20
MRAH-G1T4.F126C	17	82	19	20
MRAH-G1T4.P127C	17	83	19	20
MRAH-G1T4.S131C	17	84	19	20
MRAH-G1T4.S132C	17	85	19	20
MRAH-G1T4.K133C	17	86	19	20
MRAH-G1T4.S134C	17	87	19	20
MRAH-G1T4.T135C	17	88	19	20
MRAH-G1T4.S136C	17	89	19	20
MRAH-G1T4.G137C	17	90	19	20
MRAH-G1T4.G138C	17	91	19	20
MRAH-G1T4.T139C	17	92	19	20
MRAH-G1T4.A140C	17	93	19	20
MRAH-G1T4.A141C	17	94	19	20
MRAH-G1T4.D148C	17	95	19	20
MRAH-G1T4.Y149C	17	96	19	20
MRAH-G1T4.F150C	17	97	19	20
MRAH-G1T4.P151C	17	98	19	20
MRAH-G1T4.E152C	17	99	19	20
MRAH-G1T4.P153C	17	100	19	20
MRAH-G1T4.V154C	17	101	19	20
MRAH-G1T4.T155C	17	102	19	20
MRAH-G1T4.V156C	17	103	19	20
MRAH-G1T4.S157C	17	104	19	20
MRAH-G1T4.W158C	17	105	19	20
MRAH-G1T4.N159C	17	106	19	20
MRAH-G1T4.S160C	17	107	19	20
MRAH-G1T4.G161C	17	108	19	20
MRAH-G1T4.A162C	17	109	19	20
MRAH-G1T4.L163C	17	110	19	20
MRAH-G1T4.T164C	17	111	19	20
MRAH-G1T4.S165C	17	112	19	20
MRAH-G1T4.G166C	17	113	19	20

MRAH-G1T4.V167C	17	114	19	20
MRAH-G1T4.V173C	17	115	19	20
MRAH-G1T4.L174C	17	116	19	20
MRAH-G1T4.Q175C	17	117	19	20
MRAH-G1T4.S176C	17	118	19	20
MRAH-G1T4.S177C	17	119	19	20
MRAH-G1T4.G178C	17	120	19	20
MRAH-G1T4.L179C	17	121	19	20
MRAH-G1T4.Y180C	17	122	19	20
MRAH-G1T4.V186C	17	123	19	20
MRAH-G1T4.T187C	17	124	19	20
MRAH-G1T4.V188C	17	125	19	20
MRAH-G1T4.P189C	17	126	19	20
MRAH-G1T4.S190C	17	127	19	20
MRAH-G1T4.S191C	17	128	19	20
MRAH-G1T4.S192C	17	129	19	20
MRAH-G1T4.L193C	17	130	19	20
MRAH-G1T4.G194C	17	131	19	20
MRAH-G1T4.T195C	17	132	19	20
MRAH-G1T4.Q196C	17	133	19	20
MRAH-G1T4.T197C	17	134	19	20
MRAH-G1T4.Y198C	17	135	19	20
MRAH-G1T4.I199C	17	136	19	20
MRAH-G1T4.N201C	17	137	19	20
MRAH-G1T4.V202C	17	138	19	20
MRAH-G1T4.N203C	17	139	19	20
MRAH-G1T4.H204C	17	140	19	20
MRAH-G1T4.K205C	17	141	19	20
MRAH-G1T4.P206C	17	142	19	20
MRAH-G1T4.S207C	17	143	19	20
MRAH-G1T4.N208C	17	144	19	20
MRAH-G1T4.T209C	17	145	19	20
MRAH-G1T4.K210C	17	146	19	20

MRAH-G1T4.V211C	17	147	19	20
MRAH-G1T4.D212C	17	148	19	20
MRAH-G1T4.K213C	17	149	19	20
MRAH-G1T4.R214C	17	150	19	20
MRAH-G1T4.V215C	17	151	19	20
MRAH-G1T4.E216C	17	152	19	20
MRAH-G1T4.P217C	17	153	19	20
MRAH-G1T4.K218C	17	154	19	20
MRAH-G1T4.S219C	17	155	19	20

[0295] Reference Example 5-2 Assessment of protease-mediated Fab fragmentation of antibodies having cysteine substitution at various positions in the heavy chain

Using a protease that cleaves the heavy chain hinge region of antibody to cause Fab fragmentation, the MRA variants produced in Reference Example 5-1 were examined for whether they acquired protease resistance so that their fragmentation would be inhibited. The protease used was Lys-C (Endoproteinase Lys-C Sequencing Grade) (SIGMA; 11047825001). Reaction was performed under the conditions of 2 ng/microliter protease, 100 microgram/mL antibody, 80% 25 mM Tris-HCl pH 8.0, 20% PBS, and 35 degrees C for two hours, or under the conditions of 2 ng/microliter protease, 20 microgram/mL antibody, 80% 25 mM Tris-HCl pH 8.0, 20% PBS, and 35 degrees C for one hour. The sample was then subjected to non-reducing capillary electrophoresis. Wes (Protein Simple) was used for capillary electrophoresis, and an HRP-labeled anti-kappa chain antibody (abcam; ab46527) was used for detection. The results are shown in Figs. 26 to 33. Lys-C treatment of MRA caused cleavage of the heavy chain hinge region, resulting in disappearance of the band of IgG at around 150 kDa and appearance of the band of Fab at around 50 kDa. For the MRA variants produced in Reference Example 5-1, some showed the band of Fab dimer appearing at around 96 kDa and some showed the band of undigested IgG detected at around 150 kDa after the protease treatment. The area of each band obtained after the protease treatment was outputted using software dedicated for Wes (Compass for SW; Protein Simple) to calculate the percentage of the band areas of undigested IgG, Fab dimer, etc. The calculated percentage of each band is shown in Table 21.

[0296]

[Table 21]

Antibody name	IgG (%)	Fab-Fab (%)	Fab (%)	Heavy chain SEQ ID NO:	Light chain SEQ ID NO:
MRAH.Q5C-G1T4	0.2	1.5	97.6	21	16
MRAH.E6C-G1T4	0	0.3	80.7	22	16
MRAH.S7C-G1T4	0.4	1.9	96.9	23	16
MRAH.G8C-G1T4	16.6	1.1	76.7	24	16
MRAH.P9C-G1T4	0.2	1.5	97.2	25	16
MRAH.G10C-G1T4	0.6	1.9	96.9	26	16
MRAH.L11C-G1T4	0	1.2	98.3	27	16
MRAH.V12C-G1T4	0.2	1	97.6	28	16
MRAH.R13C-G1T4	0.6	1.9	96.6	29	16
MRAH.P14C-G1T4	0.3	1.7	97.7	30	16
MRAH.S15C-G1T4	0.9	1.3	81.4	31	16
MRAH.Q16C-G1T4	92.5	0	2	32	16
MRAH.T17C-G1T4	0.4	1.4	97.8	33	16
MRAH.L18C-G1T4	0.3	0.6	96.1	34	16
MRAH.S19C-G1T4	0.3	1.2	98.1	35	16
MRAH.L20C-G1T4	1	0.3	93.3	36	16
MRAH.T21C-G1T4	0.5	1	98.3	37	16
MRAH.T23C-G1T4	no data	no data	no data	38	16
MRAH.S25C-G1T4	0.3	2.8	87	39	16
MRAH.G26C-G1T4	0.4	1.7	85.5	40	16
MRAH.S28C-G1T4	98.6	0	0.2	41	16
MRAH.T30C-G1T4	0.5	0.7	97.8	42	16
MRAH.R66C-G1T4	0.2	1.2	97.9	43	16
MRAH.V67C-G1T4	0.3	0.4	97.8	44	16
MRAH.T68C-G1T4	0.2	1.4	97.7	45	16
MRAH.L70C-G1T4	0.2	0.9	98	46	16
MRAH.D72C-G1T4	0.3	0.8	97.6	47	16
MRAH.T73C-G1T4	0.5	0.9	97.7	48	16
MRAH.S74C-G1T4	97.1	0	0.3	49	16
MRAH.K75C-G1T4	0.1	1.5	97	50	16

MRAH.N76C-G1T4	0.4	0.4	93.1	51	16
MRAH.Q77C-G1T4	0.1	0.2	99.6	52	16
MRAH.S79C-G1T4	0.1	1.6	96.7	53	16
MRAH.L80C-G1T4	0.2	0	96.5	54	16
MRAH.R81C-G1T4	0	1.4	98	55	16
MRAH.L82C-G1T4	0	0	96.8	56	16
MRAH.S82aC-G1T4	0.6	1	96.7	57	16
MRAH.S82bC-G1T4	97.5	0	0.3	58	16
MRAH.V82cC-G1T4	0.1	0.3	95.6	59	16
MRAH.S112C-G1T4	0.1	1.1	97.6	60	16
MRAH.S113C-G1T4	0.1	2.8	95.9	61	16
MRAH.S31C-G1T4	0.5	2	75.7	62	16
MRAH.W35C-G1T4	0.1	0.3	91.1	63	16
MRAH.S35aC-G1T4	0	0.6	90.7	64	16
MRAH.Y50C-G1T4	0.2	1.5	95.8	65	16
MRAH.I51C-G1T4	0.2	0.8	94.4	66	16
MRAH.S52C-G1T4	0.3	1.7	96.4	67	16
MRAH.S62C-G1T4	0.2	1.1	97.6	68	16
MRAH.L63C-G1T4	0.4	1.4	94.2	69	16
MRAH.K64C-G1T4	0	1.6	91.7	70	16
MRAH.S65C-G1T4	0.3	1.7	95.6	71	16
MRAH.D101C-G1T4	0	1.2	97	72	16
MRAH.Y102C-G1T4	0.2	1.3	96.8	73	16
MRAH-G1T4.A118C	1.2	1	89	74	16
MRAH-G1T4.S119C	2.3	14	77.7	75	16
MRAH-G1T4.T120C	0	0.1	0.1	76	16
MRAH-G1T4.K121C	2.4	1.1	82.2	77	16
MRAH-G1T4.G122C	8	1.4	79.8	78	16
MRAH-G1T4.P123C	7.1	0	45.7	79	16
MRAH-G1T4.S124C	0.8	1.7	94.5	80	16
MRAH-G1T4.V125C	2.3	0	62	81	16
MRAH-G1T4.F126C	2.1	1	85.5	82	16
MRAH-G1T4.P127C	2.9	1.4	77.4	83	16

MRAH-G1T4.S131C	68.4	0	0	84	16
MRAH-G1T4.S132C	13.9	0.8	54.6	85	16
MRAH-G1T4.K133C	66.8	0	0	86	16
MRAH-G1T4.S134C	63.5	0	21.9	87	16
MRAH-G1T4.T135C	44.7	13.2	23.6	88	16
MRAH-G1T4.S136C	22.9	27.3	35.1	89	16
MRAH-G1T4.G137C	8.4	18.1	62.1	90	16
MRAH-G1T4.G138C	no data	no data	no data	91	16
MRAH-G1T4.T139C	7.4	1.4	82.1	92	16
MRAH-G1T4.A140C	20.2	0	47.2	93	16
MRAH-G1T4.A141C	0.3	0	31.9	94	16
MRAH-G1T4.D148C	21	0	64.8	95	16
MRAH-G1T4.Y149C	0.5	0	58.1	96	16
MRAH-G1T4.F150C	79.2	0	0.4	97	16
MRAH-G1T4.P151C	2	0	56.1	98	16
MRAH-G1T4.E152C	0.9	0.3	84.8	99	16
MRAH-G1T4.P153C	4.4	0.8	86.6	100	16
MRAH-G1T4.V154C	4	0	45.7	101	16
MRAH-G1T4.T155C	20.2	1.4	67.6	102	16
MRAH-G1T4.V156C	7	0	39.2	103	16
MRAH-G1T4.S157C	13.5	3.2	75.9	104	16
MRAH-G1T4.W158C	4.2	0	66.1	105	16
MRAH-G1T4.N159C	13.9	1.9	76.1	106	16
MRAH-G1T4.S160C	7.7	20.9	66.2	107	16
MRAH-G1T4.G161C	14.1	12	68.6	108	16
MRAH-G1T4.A162C	9.6	17.9	65.8	109	16
MRAH-G1T4.L163C	10.2	6.1	75.9	110	16
MRAH-G1T4.T164C	3.8	3.2	88.7	111	16
MRAH-G1T4.S165C	7.8	4.1	81.5	112	16
MRAH-G1T4.G166C	4.5	2.2	89.4	113	16
MRAH-G1T4.V167C	5.5	2.5	81.2	114	16
MRAH-G1T4.V173C	2.1	1.6	92.2	115	16
MRAH-G1T4.L174C	19.8	0	67.1	116	16

MRAH-G1T4.Q175C	4.4	1.1	86.6	117	16
MRAH-G1T4.S176C	2.3	7.7	85.5	118	16
MRAH-G1T4.S177C	7.1	12.4	71.6	119	16
MRAH-G1T4.G178C	6.2	2.4	85.5	120	16
MRAH-G1T4.L179C	0.2	0	0	121	16
MRAH-G1T4.Y180C	0	0	72.7	122	16
MRAH-G1T4.V186C	0	0	73.3	123	16
MRAH-G1T4.T187C	0.8	2.5	90.3	124	16
MRAH-G1T4.V188C	0.3	4	82.7	125	16
MRAH-G1T4.P189C	0.9	4.7	89.6	126	16
MRAH-G1T4.S190C	10.9	0	74.4	127	16
MRAH-G1T4.S191C	2.3	46.4	45.1	128	16
MRAH-G1T4.S192C	1.3	11	83	129	16
MRAH-G1T4.L193C	3.6	0	70.5	130	16
MRAH-G1T4.G194C	13.8	0	0	131	16
MRAH-G1T4.T195C	29.6	0	57.3	132	16
MRAH-G1T4.Q196C	1.5	0	92.6	133	16
MRAH-G1T4.T197C	81.5	0	4.5	134	16
MRAH-G1T4.Y198C	0.1	0.3	17.1	135	16
MRAH-G1T4.I199C	1	1.7	91.6	136	16
MRAH-G1T4.N201C	0.7	4	90.3	137	16
MRAH-G1T4.V202C	0	0.1	6.6	138	16
MRAH-G1T4.N203C	0.6	2.4	89.8	139	16
MRAH-G1T4.H204C	0.4	2.2	77.7	140	16
MRAH-G1T4.K205C	0.2	2.3	85.5	141	16
MRAH-G1T4.P206C	0.4	2.1	86.9	142	16
MRAH-G1T4.S207C	no data	no data	no data	143	16
MRAH-G1T4.N208C	0.4	0	86.2	144	16
MRAH-G1T4.T209C	0.7	0	83.1	145	16
MRAH-G1T4.K210C	0.6	0	81.7	146	16
MRAH-G1T4.V211C	0.3	1	67.6	147	16
MRAH-G1T4.D212C	1.1	1.8	80.9	148	16
MRAH-G1T4.K213C	6.5	0	41.9	149	16

MRAH-G1T4.R214C	18.6	0	42.7	150	16
MRAH-G1T4.V215C	0	0	11.8	151	16
MRAH-G1T4.E216C	7.4	0	64.8	152	16
MRAH-G1T4.P217C	4.5	0.2	43.3	153	16
MRAH-G1T4.K218C	30.8	0	29.5	154	16
MRAH-G1T4.S219C	46.9	0.1	18	155	16

[0297] From this result, it was found that cysteine substitution in the heavy chain variable region or heavy chain constant region improved the protease resistance of the heavy chain hinge region in the MRA variants shown in Table 22. Alternatively, the result suggested that a Fab dimer was formed by a covalent bond between the Fab-Fab.

[0298]

[Table 22]

MRA variants

	SEQ ID NO:			
Antibody name	Heavy chain variable region	Heavy chain constant region	Light chain variable region	Light chain constant region
MRAH.G8C-G1T4	24	18	19	20
MRAH.Q16C-G1T4	32	18	19	20
MRAH.S28C-G1T4	41	18	19	20
MRAH.S74C-G1T4	49	18	19	20
MRAH.S82bC-G1T4	58	18	19	20
MRAH-G1T4.S119C	17	75	19	20
MRAH-G1T4.G122C	17	78	19	20
MRAH-G1T4.P123C	17	79	19	20
MRAH-G1T4.S131C	17	84	19	20
MRAH-G1T4.S132C	17	85	19	20
MRAH-G1T4.K133C	17	86	19	20
MRAH-G1T4.S134C	17	87	19	20
MRAH-G1T4.T135C	17	88	19	20
MRAH-G1T4.S136C	17	89	19	20
MRAH-G1T4.G137C	17	90	19	20
MRAH-G1T4.T139C	17	92	19	20
MRAH-G1T4.A140C	17	93	19	20
MRAH-G1T4.D148C	17	95	19	20
MRAH-G1T4.F150C	17	97	19	20
MRAH-G1T4.T155C	17	102	19	20
MRAH-G1T4.V156C	17	103	19	20
MRAH-G1T4.S157C	17	104	19	20
MRAH-G1T4.N159C	17	106	19	20
MRAH-G1T4.S160C	17	107	19	20
MRAH-G1T4.G161C	17	108	19	20
MRAH-G1T4.A162C	17	109	19	20
MRAH-G1T4.L163C	17	110	19	20

MRAH-G1T4.S165C	17	112	19	20
MRAH-G1T4.V167C	17	114	19	20
MRAH-G1T4.L174C	17	116	19	20
MRAH-G1T4.S176C	17	118	19	20
MRAH-G1T4.S177C	17	119	19	20
MRAH-G1T4.G178C	17	120	19	20
MRAH-G1T4.S190C	17	127	19	20
MRAH-G1T4.S191C	17	128	19	20
MRAH-G1T4.S192C	17	129	19	20
MRAH-G1T4.G194C	17	131	19	20
MRAH-G1T4.T195C	17	132	19	20
MRAH-G1T4.T197C	17	134	19	20
MRAH-G1T4.K213C	17	149	19	20
MRAH-G1T4.R214C	17	150	19	20
MRAH-G1T4.E216C	17	152	19	20
MRAH-G1T4.K218C	17	154	19	20
MRAH-G1T4.S219C	17	155	19	20

[0299] [Reference Example 6] Assessment of antibodies having cysteine substitution at various positions in the light chain

Reference Example 6-1 Assessment of antibodies having cysteine substitution at various positions in the light chain

The light chain variable region and constant region of an anti-human IL6R neutralizing antibody, MRA (heavy chain: MRAH-G1T4 (SEQ ID NO: 15), light chain: MRAL-k0 (SEQ ID NO: 16)) were subjected to a study in which an arbitrary amino acid residue structurally exposed to the surface was substituted with cysteine.

Amino acid residues within the light chain variable region of MRA (MRAL, SEQ ID NO: 19) were substituted with cysteine to produce variants of the light chain variable region of MRA shown in Table 23. These variants of the light chain variable region of MRA were each linked with the light chain constant region of MRA (k0, SEQ ID NO: 20) to produce variants of the light chain of MRA, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art.

In addition, amino acid residues within the light chain constant region of MRA (k0, SEQ ID NO: 20) were substituted with cysteine to produce variants of the light chain constant region of MRA shown in Table 24. These variants of the light chain constant region of MRA were each linked with the light chain variable region of MRA (MRAL, SEQ ID NO: 19) to produce variants of the light chain of MRA, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art.

The MRA light chain variants produced above were combined with the MRA heavy chain. The resultant MRA variants shown in Table 25 were expressed by transient expression using FreeStyle293 cells (Invitrogen) or Expi293 cells (Life technologies) by a method known to the person skilled in the art, and purified with Protein A by a method known to the person skilled in the art.

[0300] [Table 23]

Variants of MRA light chain variable region and position of cysteine substitution

Variant of MRA light chain variable region	Position of cysteine substitution (Kabat numbering)	SEQ ID NO:
MRAL.T5C	5	156
MRAL.Q6C	6	157
MRAL.S7C	7	158
MRAL.P8C	8	159
MRAL.S9C	9	160
MRAL.S10C	10	161
MRAL.L11C	11	162
MRAL.S12C	12	163
MRAL.A13C	13	164
MRAL.S14C	14	165
MRAL.V15C	15	166
MRAL.G16C	16	167
MRAL.D17C	17	168
MRAL.R18C	18	169
MRAL.V19C	19	170
MRAL.T20C	20	171
MRAL.I21C	21	172
MRAL.T22C	22	173
MRAL.G57C	57	174
MRAL.V58C	58	175
MRAL.P59C	59	176
MRAL.S60C	60	177
MRAL.R61C	61	178
MRAL.F62C	62	179
MRAL.S63C	63	180
MRAL.S65C	65	181
MRAL.S67C	67	182
MRAL.G68C	68	183

MRAL.T69C	69	184
MRAL.D70C	70	185
MRAL.T72C	72	186
MRAL.F73C	73	187
MRAL.T74C	74	188
MRAL.I75C	75	189
MRAL.S76C	76	190
MRAL.S77C	77	191
MRAL.L78C	78	192
MRAL.Q79C	79	193
MRAL.F98C	98	194
MRAL.G99C	99	195
MRAL.Q100C	100	196
MRAL.G101C	101	197
MRAL.T102C	102	198
MRAL.K103C	103	199
MRAL.V104C	104	200
MRAL.E105C	105	201
MRAL.I106C	106	202
MRAL.K107C	107	203
MRAL.A25C	25	204
MRAL.S26C	26	205
MRAL.Q27C	27	206
MRAL.Y32C	32	207
MRAL.L33C	33	208
MRAL.N34C	34	209
MRAL.Y50C	50	210
MRAL.T51C	51	211
MRAL.H55C	55	212
MRAL.S56C	56	213
MRAL.Y96C	96	214
MRAL.T97C	97	215

[0301]

[Table 24]

Variants of MRA light chain constant region and position of cysteine substitution

Variant of MRA light chain constant region	Position of cysteine substitution (EU numbering)	SEQ ID NO:
k0.R108C	108	216
k0.T109C	109	217
k0.V110C	110	218
k0.A111C	111	219
k0.A112C	112	220
k0.P113C	113	221
k0.S114C	114	222
k0.V115C	115	223
k0.F116C	116	224
k0.P120C	120	225
k0.S121C	121	226
k0.D122C	122	227
k0.E123C	123	228
k0.Q124C	124	229
k0.L125C	125	230
k0.K126C	126	231
k0.S127C	127	232
k0.G128C	128	233
k0.T129C	129	234
k0.A130C	130	235
k0.S131C	131	236
k0.L136C	136	237
k0.N137C	137	238
k0.N138C	138	239
k0.F139C	139	240
k0.Y140C	140	241
k0.P141C	141	242
k0.R142C	142	243

k0.E143C	143	244
k0.A144C	144	245
k0.K145C	145	246
k0.V146C	146	247
k0.Q147C	147	248
k0.W148C	148	249
k0.K149C	149	250
k0.V150C	150	251
k0.D151C	151	252
k0.N152C	152	253
k0.A153C	153	254
k0.L154C	154	255
k0.Q155C	155	256
k0.S156C	156	257
k0.G157C	157	258
k0.N158C	158	259
k0.S159C	159	260
k0.Q160C	160	261
k0.E161C	161	262
k0.S162C	162	263
k0.V163C	163	264
k0.T164C	164	265
k0.E165C	165	266
k0.Q166C	166	267
k0.D167C	167	268
k0.S168C	168	269
k0.K169C	169	270
k0.D170C	170	271
k0.S171C	171	272
k0.T172C	172	273
k0.Y173C	173	274
k0.S174C	174	275
k0.L175C	175	276

k0.T180C	180	277
k0.L181C	181	278
k0.S182C	182	279
k0.K183C	183	280
k0.A184C	184	281
k0.D185C	185	282
k0.Y186C	186	283
k0.E187C	187	284
k0.K188C	188	285
k0.H189C	189	286
k0.K190C	190	287
k0.V191C	191	288
k0.Y192C	192	289
k0.A193C	193	290
k0.E195C	195	291
k0.V196C	196	292
k0.T197C	197	293
k0.H198C	198	294
k0.Q199C	199	295
k0.G200C	200	296
k0.L201C	201	297
k0.S202C	202	298
k0.S203C	203	299
k0.P204C	204	300
k0.V205C	205	301
k0.T206C	206	302
k0.K207C	207	303
k0.S208C	208	304
k0.F209C	209	305
k0.N210C	210	306
k0.R211C	211	307
k0.G212C	212	308
k0.E213C	213	309

[0302]

[Table 25]

MRA variants

Antibody name	SEQ ID NO:			
	Heavy chain variable region	Heavy chain constant region	Light chain variable region	Light chain constant region
MRAL.T5C-k0	17	18	156	20
MRAL.Q6C-k0	17	18	157	20
MRAL.S7C-k0	17	18	158	20
MRAL.P8C-k0	17	18	159	20
MRAL.S9C-k0	17	18	160	20
MRAL.S10C-k0	17	18	161	20
MRAL.L11C-k0	17	18	162	20
MRAL.S12C-k0	17	18	163	20
MRAL.A13C-k0	17	18	164	20
MRAL.S14C-k0	17	18	165	20
MRAL.V15C-k0	17	18	166	20
MRAL.G16C-k0	17	18	167	20
MRAL.D17C-k0	17	18	168	20
MRAL.R18C-k0	17	18	169	20
MRAL.V19C-k0	17	18	170	20
MRAL.T20C-k0	17	18	171	20
MRAL.I21C-k0	17	18	172	20
MRAL.T22C-k0	17	18	173	20
MRAL.G57C-k0	17	18	174	20
MRAL.V58C-k0	17	18	175	20
MRAL.P59C-k0	17	18	176	20
MRAL.S60C-k0	17	18	177	20
MRAL.R61C-k0	17	18	178	20
MRAL.F62C-k0	17	18	179	20
MRAL.S63C-k0	17	18	180	20
MRAL.S65C-k0	17	18	181	20
MRAL.S67C-k0	17	18	182	20

MRAL.G68C-k0	17	18	183	20
MRAL.T69C-k0	17	18	184	20
MRAL.D70C-k0	17	18	185	20
MRAL.T72C-k0	17	18	186	20
MRAL.F73C-k0	17	18	187	20
MRAL.T74C-k0	17	18	188	20
MRAL.I75C-k0	17	18	189	20
MRAL.S76C-k0	17	18	190	20
MRAL.S77C-k0	17	18	191	20
MRAL.L78C-k0	17	18	192	20
MRAL.Q79C-k0	17	18	193	20
MRAL.F98C-k0	17	18	194	20
MRAL.G99C-k0	17	18	195	20
MRAL.Q100C-k0	17	18	196	20
MRAL.G101C-k0	17	18	197	20
MRAL.T102C-k0	17	18	198	20
MRAL.K103C-k0	17	18	199	20
MRAL.V104C-k0	17	18	200	20
MRAL.E105C-k0	17	18	201	20
MRAL.I106C-k0	17	18	202	20
MRAL.K107C-k0	17	18	203	20
MRAL.A25C-k0	17	18	204	20
MRAL.S26C-k0	17	18	205	20
MRAL.Q27C-k0	17	18	206	20
MRAL.Y32C-k0	17	18	207	20
MRAL.L33C-k0	17	18	208	20
MRAL.N34C-k0	17	18	209	20
MRAL.Y50C-k0	17	18	210	20
MRAL.T51C-k0	17	18	211	20
MRAL.H55C-k0	17	18	212	20
MRAL.S56C-k0	17	18	213	20
MRAL.Y96C-k0	17	18	214	20
MRAL.T97C-k0	17	18	215	20

MRAL-k0.R108C	17	18	19	216
MRAL-k0.T109C	17	18	19	217
MRAL-k0.V110C	17	18	19	218
MRAL-k0.A111C	17	18	19	219
MRAL-k0.A112C	17	18	19	220
MRAL-k0.P113C	17	18	19	221
MRAL-k0.S114C	17	18	19	222
MRAL-k0.V115C	17	18	19	223
MRAL-k0.F116C	17	18	19	224
MRAL-k0.P120C	17	18	19	225
MRAL-k0.S121C	17	18	19	226
MRAL-k0.D122C	17	18	19	227
MRAL-k0.E123C	17	18	19	228
MRAL-k0.Q124C	17	18	19	229
MRAL-k0.L125C	17	18	19	230
MRAL-k0.K126C	17	18	19	231
MRAL-k0.S127C	17	18	19	232
MRAL-k0.G128C	17	18	19	233
MRAL-k0.T129C	17	18	19	234
MRAL-k0.A130C	17	18	19	235
MRAL-k0.S131C	17	18	19	236
MRAL-k0.L136C	17	18	19	237
MRAL-k0.N137C	17	18	19	238
MRAL-k0.N138C	17	18	19	239
MRAL-k0.F139C	17	18	19	240
MRAL-k0.Y140C	17	18	19	241
MRAL-k0.P141C	17	18	19	242
MRAL-k0.R142C	17	18	19	243
MRAL-k0.E143C	17	18	19	244
MRAL-k0.A144C	17	18	19	245
MRAL-k0.K145C	17	18	19	246
MRAL-k0.V146C	17	18	19	247
MRAL-k0.Q147C	17	18	19	248

MRAL-k0.W148C	17	18	19	249
MRAL-k0.K149C	17	18	19	250
MRAL-k0.V150C	17	18	19	251
MRAL-k0.D151C	17	18	19	252
MRAL-k0.N152C	17	18	19	253
MRAL-k0.A153C	17	18	19	254
MRAL-k0.L154C	17	18	19	255
MRAL-k0.Q155C	17	18	19	256
MRAL-k0.S156C	17	18	19	257
MRAL-k0.G157C	17	18	19	258
MRAL-k0.N158C	17	18	19	259
MRAL-k0.S159C	17	18	19	260
MRAL-k0.Q160C	17	18	19	261
MRAL-k0.E161C	17	18	19	262
MRAL-k0.S162C	17	18	19	263
MRAL-k0.V163C	17	18	19	264
MRAL-k0.T164C	17	18	19	265
MRAL-k0.E165C	17	18	19	266
MRAL-k0.Q166C	17	18	19	267
MRAL-k0.D167C	17	18	19	268
MRAL-k0.S168C	17	18	19	269
MRAL-k0.K169C	17	18	19	270
MRAL-k0.D170C	17	18	19	271
MRAL-k0.S171C	17	18	19	272
MRAL-k0.T172C	17	18	19	273
MRAL-k0.Y173C	17	18	19	274
MRAL-k0.S174C	17	18	19	275
MRAL-k0.L175C	17	18	19	276
MRAL-k0.T180C	17	18	19	277
MRAL-k0.L181C	17	18	19	278
MRAL-k0.S182C	17	18	19	279
MRAL-k0.K183C	17	18	19	280
MRAL-k0.A184C	17	18	19	281

MRAL-k0.D185C	17	18	19	282
MRAL-k0.Y186C	17	18	19	283
MRAL-k0.E187C	17	18	19	284
MRAL-k0.K188C	17	18	19	285
MRAL-k0.H189C	17	18	19	286
MRAL-k0.K190C	17	18	19	287
MRAL-k0.V191C	17	18	19	288
MRAL-k0.Y192C	17	18	19	289
MRAL-k0.A193C	17	18	19	290
MRAL-k0.E195C	17	18	19	291
MRAL-k0.V196C	17	18	19	292
MRAL-k0.T197C	17	18	19	293
MRAL-k0.H198C	17	18	19	294
MRAL-k0.Q199C	17	18	19	295
MRAL-k0.G200C	17	18	19	296
MRAL-k0.L201C	17	18	19	297
MRAL-k0.S202C	17	18	19	298
MRAL-k0.S203C	17	18	19	299
MRAL-k0.P204C	17	18	19	300
MRAL-k0.V205C	17	18	19	301
MRAL-k0.T206C	17	18	19	302
MRAL-k0.K207C	17	18	19	303
MRAL-k0.S208C	17	18	19	304
MRAL-k0.F209C	17	18	19	305
MRAL-k0.N210C	17	18	19	306
MRAL-k0.R211C	17	18	19	307
MRAL-k0.G212C	17	18	19	308
MRAL-k0.E213C	17	18	19	309

[0303] Reference Example 6-2 Assessment of protease-mediated Fab fragmentation of antibodies having cysteine substitution at various positions in the light chain

Using a protease that cleaves the heavy chain hinge region of antibody to cause Fab fragmentation, the MRA variants produced in Reference Example 6-1 were examined for whether they acquired protease resistance so that their fragmentation would be inhibited. The protease used was Lys-C (Endoproteinase Lys-C Sequencing Grade) (SIGMA; 11047825001). Reaction was performed under the conditions of 2 ng/microliter protease, 100 microgram/mL antibody, 80% 25 mM Tris-HCl pH 8.0, 20% PBS, and 35 degrees C for two hours, or under the conditions of 2 ng/microliter protease, 20 microgram/mL antibody, 80% 25 mM Tris-HCl pH 8.0, 20% PBS, and 35

degrees C for one hour. The sample was then subjected to non-reducing capillary electrophoresis. Wes (Protein Simple) was used for capillary electrophoresis, and an HRP-labeled anti-kappa chain antibody (abcam; ab46527) was used for detection. The results are shown in Figs. 24 to 43. Lys-C treatment of MRA caused cleavage of the heavy chain hinge region, resulting in disappearance of the band of IgG at around 150 kDa and appearance of the band of Fab at around 50 kDa. For the MRA variants produced in Reference Example 6-1, some showed the band of Fab dimer appearing at around 96 kDa and some showed the band of undigested IgG detected at around 150 kDa after the protease treatment. The area of each band obtained after the protease treatment was outputted using software dedicated for Wes (Compass for SW; Protein Simple) to calculate the percentage of the band areas of undigested IgG, Fab dimer, etc. The calculated percentage of each band is shown in Table 26.

[0304]

[Table 26]

Antibody name	IgG (%)	Fab-Fab (%)	Fab (%)	Heavy chain SEQ ID NO:	Light chain SEQ ID NO:
MRAL.T5C-k0	0.1	0	71.1	15	156
MRAL.Q6C-k0	0.1	0	74.5	15	157
MRAL.S7C-k0	0.2	0	68.8	15	158
MRAL.P8C-k0	no data	no data	no data	15	159
MRAL.S9C-k0	0.3	0.4	82.9	15	160
MRAL.S10C-k0	0.2	0.4	85.8	15	161
MRAL.L11C-k0	0	0	83.4	15	162
MRAL.S12C-k0	0.9	0.4	87.2	15	163
MRAL.A13C-k0	0.1	0	88.6	15	164
MRAL.S14C-k0	0.3	0.6	85.9	15	165
MRAL.V15C-k0	0.2	0	84.8	15	166
MRAL.G16C-k0	0.8	0	82.3	15	167
MRAL.D17C-k0	0	0	92.3	15	168
MRAL.R18C-k0	0.2	0.4	87.1	15	169
MRAL.V19C-k0	0	0	63.3	15	170
MRAL.T20C-k0	0.5	0.6	83.6	15	171
MRAL.I21C-k0	0	0	5	15	172
MRAL.T22C-k0	0	0.3	89.5	15	173
MRAL.G57C-k0	0.2	0	91.7	15	174
MRAL.V58C-k0	0.4	0.7	88	15	175
MRAL.P59C-k0	0.7	1.5	94.6	15	176
MRAL.S60C-k0	0.1	0	86.9	15	177
MRAL.R61C-k0	0	0.3	86.9	15	178
MRAL.F62C-k0	0.2	0	60	15	179
MRAL.S63C-k0	0.5	0.6	88.1	15	180
MRAL.S65C-k0	0.4	0.8	83.3	15	181
MRAL.S67C-k0	1.5	0	72.8	15	182
MRAL.G68C-k0	0.7	0.9	83.9	15	183
MRAL.T69C-k0	1.1	0.6	86.4	15	184
MRAL.D70C-k0	0.8	0.9	88.2	15	185

MRAL.T72C-k0	0.6	0.7	90.1	15	186
MRAL.F73C-k0	0.3	0	59.5	15	187
MRAL.T74C-k0	0.2	0.6	95.6	15	188
MRAL.I75C-k0	no data	no data	no data	15	189
MRAL.S76C-k0	0.6	0.8	90.4	15	190
MRAL.S77C-k0	1.1	0	74.2	15	191
MRAL.L78C-k0	4.9	0	54.7	15	192
MRAL.Q79C-k0	1.2	0.6	93.1	15	193
MRAL.F98C-k0	0.6	0.8	71.8	15	194
MRAL.G99C-k0	0.6	0.4	88.2	15	195
MRAL.Q100C-k0	5	0.8	85	15	196
MRAL.G101C-k0	0.3	0.4	98.1	15	197
MRAL.T102C-k0	0.3	0	52.8	15	198
MRAL.K103C-k0	1.1	0.4	89.2	15	199
MRAL.V104C-k0	0.2	0.6	48.2	15	200
MRAL.E105C-k0	90.8	0	1.2	15	201
MRAL.I106C-k0	1.8	0	47.3	15	202
MRAL.K107C-k0	5.4	0	82.6	15	203
MRAL.A25C-k0	0.1	0.5	80	15	204
MRAL.S26C-k0	0.3	1.4	94	15	205
MRAL.Q27C-k0	0.3	1.3	94.6	15	206
MRAL.Y32C-k0	0	1.2	95.7	15	207
MRAL.L33C-k0	0	0	79.2	15	208
MRAL.N34C-k0	0.3	0.4	95.7	15	209
MRAL.Y50C-k0	0.4	1.3	97	15	210
MRAL.T51C-k0	0.2	1.2	96.9	15	211
MRAL.H55C-k0	0.2	1.5	95.7	15	212
MRAL.S56C-k0	0.1	0.8	97	15	213
MRAL.Y96C-k0	0.1	0.2	91.3	15	214
MRAL.T97C-k0	0.3	0.9	97.5	15	215
MRAL-k0.R108C	no data	no data	no data	15	216
MRAL-k0.T109C	0.5	16	74.5	15	217
MRAL-k0.V110C	1.2	4	75	15	218

MRAL-k0.A111C	0.2	0.7	85.9	15	219
MRAL-k0.A112C	3.3	6.1	80.3	15	220
MRAL-k0.P113C	no data	no data	no data	15	221
MRAL-k0.S114C	0.3	0.7	94	15	222
MRAL-k0.V115C	0	0.1	34.9	15	223
MRAL-k0.F116C	0.3	0.3	77.3	15	224
MRAL-k0.P120C	0	0	28.8	15	225
MRAL-k0.S121C	8.6	0	57.4	15	226
MRAL-k0.D122C	1.8	0.1	30.3	15	227
MRAL-k0.E123C	2.3	1.6	75.9	15	228
MRAL-k0.Q124C	1.3	0.9	50.4	15	229
MRAL-k0.L125C	0.4	0.1	66.6	15	230
MRAL-k0.K126C	59.3	9.9	16.5	15	231
MRAL-k0.S127C	0.3	0.9	79	15	232
MRAL-k0.G128C	0.2	7	71.5	15	233
MRAL-k0.T129C	0	0.4	76.2	15	234
MRAL-k0.A130C	0	0	49.9	15	235
MRAL-k0.S131C	0	0	16.7	15	236
MRAL-k0.L136C	0	0	15	15	237
MRAL-k0.N137C	0	0	47.5	15	238
MRAL-k0.N138C	0	0.5	86.8	15	239
MRAL-k0.F139C	0	0	0	15	240
MRAL-k0.Y140C	0	0	29.9	15	241
MRAL-k0.P141C	0.1	2.7	79.8	15	242
MRAL-k0.R142C	0	0.6	74.1	15	243
MRAL-k0.E143C	0	0.5	88.4	15	244
MRAL-k0.A144C	0	0.1	42.1	15	245
MRAL-k0.K145C	0	0.9	82.8	15	246
MRAL-k0.V146C	0	0	26.5	15	247
MRAL-k0.Q147C	0	1.8	78.5	15	248
MRAL-k0.W148C	no data	no data	no data	15	249
MRAL-k0.K149C	0	0.6	79.5	15	250
MRAL-k0.V150C	0	0	34.8	15	251

MRAL-k0.D151C	2.7	14.9	66.5	15	252
MRAL-k0.N152C	1.2	58.4	26.8	15	253
MRAL-k0.A153C	0	7.1	71.8	15	254
MRAL-k0.L154C	0	2.3	66.5	15	255
MRAL-k0.Q155C	0	0.6	73.3	15	256
MRAL-k0.S156C	0.3	32.3	40.5	15	257
MRAL-k0.G157C	0	1.4	71.8	15	258
MRAL-k0.N158C	0	0.7	76.2	15	259
MRAL-k0.S159C	0	1.1	74.7	15	260
MRAL-k0.Q160C	0	1.5	78.5	15	261
MRAL-k0.E161C	0	1	79.8	15	262
MRAL-k0.S162C	0.6	1.6	86.7	15	263
MRAL-k0.V163C	0	1.7	87.1	15	264
MRAL-k0.T164C	0	2.6	84.3	15	265
MRAL-k0.E165C	0	0.6	89.5	15	266
MRAL-k0.Q166C	0	2	86.2	15	267
MRAL-k0.D167C	0	0.5	90.5	15	268
MRAL-k0.S168C	0	0.8	94.1	15	269
MRAL-k0.K169C	0	0.4	95.3	15	270
MRAL-k0.D170C	0.2	0.1	96	15	271
MRAL-k0.S171C	0	0.1	93.8	15	272
MRAL-k0.T172C	0	0	77.4	15	273
MRAL-k0.Y173C	no data	no data	no data	15	274
MRAL-k0.S174C	0	0	65.8	15	275
MRAL-k0.L175C	0	0.2	59.3	15	276
MRAL-k0.T180C	0	0.3	93.3	15	277
MRAL-k0.L181C	1.3	0.6	86.4	15	278
MRAL-k0.S182C	0.9	1.9	95	15	279
MRAL-k0.K183C	4.4	0.9	90.7	15	280
MRAL-k0.A184C	1.6	27.9	67.7	15	281
MRAL-k0.D185C	0.5	1.1	96.5	15	282
MRAL-k0.Y186C	2.4	18.9	67.4	15	283
MRAL-k0.E187C	2.3	0	11.2	15	284

MRAL-k0.K188C	1.8	8.6	85.8	15	285
MRAL-k0.H189C	1	0.8	93	15	286
MRAL-k0.K190C	25.5	0.2	11.4	15	287
MRAL-k0.V191C	2.8	1.6	84	15	288
MRAL-k0.Y192C	0.4	1.1	67.5	15	289
MRAL-k0.A193C	1.7	1.4	94.5	15	290
MRAL-k0.E195C	0.9	1.7	95.5	15	291
MRAL-k0.V196C	1	1.1	67.5	15	292
MRAL-k0.T197C	0.8	1.5	94.8	15	293
MRAL-k0.H198C	0.7	1.3	85	15	294
MRAL-k0.Q199C	1.4	2.5	92.9	15	295
MRAL-k0.G200C	7.3	14.8	75.6	15	296
MRAL-k0.L201C	1.7	5	88	15	297
MRAL-k0.S202C	2.8	46.4	49.4	15	298
MRAL-k0.S203C	9.1	0	87.1	15	299
MRAL-k0.P204C	1	0	95.8	15	300
MRAL-k0.V205C	1.7	1	88.4	15	301
MRAL-k0.T206C	1.4	0.7	90.1	15	302
MRAL-k0.K207C	3.2	0.5	79.8	15	303
MRAL-k0.S208C	7.7	0.8	77.8	15	304
MRAL-k0.F209C	0	0	37.2	15	305
MRAL-k0.N210C	22.8	0	20.2	15	306
MRAL-k0.R211C	9.2	0	59.7	15	307
MRAL-k0.G212C	58.9	0	28.7	15	308
MRAL-k0.E213C	55.1	0	12.1	15	309

[0305] From this result, it was found that cysteine substitution in the light chain variable region or light chain constant region improved the protease resistance of the heavy chain hinge region in the MRA variants shown in Table 27. Alternatively, the result suggested that a Fab dimer was formed by a covalent bond between the Fab-Fab.

[0306]

[Table 27]

MRA variants

Antibody name	SEQ ID NO:			
	Heavy chain variable region	Heavy chain constant region	Light chain variable region	Light chain constant region
MRAL.Q100C-k0	17	18	196	20
MRAL.E105C-k0	17	18	201	20
MRAL.K107C-k0	17	18	203	20
MRAL-k0.T109C	17	18	19	217
MRAL-k0.A112C	17	18	19	220
MRAL-k0.S121C	17	18	19	226
MRAL-k0.K126C	17	18	19	231
MRAL-k0.G128C	17	18	19	233
MRAL-k0.D151C	17	18	19	252
MRAL-k0.N152C	17	18	19	253
MRAL-k0.A153C	17	18	19	254
MRAL-k0.S156C	17	18	19	257
MRAL-k0.A184C	17	18	19	281
MRAL-k0.Y186C	17	18	19	283
MRAL-k0.K188C	17	18	19	285
MRAL-k0.K190C	17	18	19	287
MRAL-k0.G200C	17	18	19	296
MRAL-k0.L201C	17	18	19	297
MRAL-k0.S202C	17	18	19	298
MRAL-k0.S203C	17	18	19	299
MRAL-k0.S208C	17	18	19	304
MRAL-k0.N210C	17	18	19	306
MRAL-k0.R211C	17	18	19	307
MRAL-k0.G212C	17	18	19	308
MRAL-k0.E213C	17	18	19	309

[0307] [Reference Example 7] Study of methods for assessing antibodies having cysteine substitution

Reference Example 7-1 Production of antibodies having cysteine substitution in the light chain

The amino acid residue at position 126 according to Kabat numbering in the light chain constant region (k0, SEQ ID NO: 20) of MRA, an anti-human IL6R neutralizing antibody (heavy chain: MRAH-G1T4 (SEQ ID NO: 15), light chain: MRAL-k0 (SEQ ID NO: 16)), was substituted with cysteine to produce a variant of the light chain

constant region of MRA, k0.K126C (SEQ ID No: 231). This variant of the light chain constant region of MRA was linked with the MRA light chain variable region (MRAL, SEQ ID NO: 19) to produce a variant of the light chain of MRA, and an expression vector encoding the corresponding gene was produced by a method known to the person skilled in the art.

The MRA light chain variant produced above was combined with the MRA heavy chain. The resultant MRA variant MRAL-k0.K126C (heavy chain: MRAH-G1T4 (SEQ ID NO: 15), light chain variable region: MRAL (SEQ ID NO: 19), light chain constant region: k0.K126C (SEQ ID NO: 231)) was expressed by transient expression using FreeStyle293 cells (Invitrogen) or Expi293 cells (Life technologies) by a method known to the person skilled in the art, and purified with Protein A by a method known to the person skilled in the art.

[0308] Reference Example 7-2 Assessment of protease-mediated capillary electrophoresis of antibodies having cysteine substitution in the light chain

Using a protease that cleaves the heavy chain hinge region of antibody to cause Fab fragmentation, the MRA light chain variant produced in Reference Example 7-1 was examined for whether it acquired protease resistance so that its fragmentation would be inhibited. The protease used was Lys-C (Endoproteinase Lys-C Sequencing Grade) (SIGMA; 11047825001). Reaction was performed under the conditions of 0.1, 0.4, 1.6, or 6.4 ng/microliter protease, 100 microgram/mL antibody, 80% 25 mM Tris-HCl pH 8.0, 20% PBS, and 35 degrees C for two hours. The sample was then subjected to non-reducing capillary electrophoresis. Wes (Protein Simple) was used for capillary electrophoresis, and an HRP-labeled anti-kappa chain antibody (abcam; ab46527) or an HRP-labeled anti-human Fc antibody (Protein Simple; 043-491) was used for detection. The result is shown in Fig. 44. For MRA treated with Lys-C, detection with the anti-kappa chain antibody showed disappearance of the band at around 150 kDa and appearance of a new band at around 50 kDa, and, at low Lys-C concentrations, also showed appearance of a slight band at 113 kDa. Detection with the anti-human Fc antibody showed disappearance of the band at around 150 kDa and appearance of a new band at around 61 kDa, and, at low Lys-C concentrations, also showed appearance of a slight band at 113 kDa. For the MRA variant produced in Reference Example 7-1, on the other hand, the band at around 150 kDa hardly disappeared, and a new band appeared at around 96 kDa. Detection with the anti-human Fc antibody showed that the band at around 150 kDa hardly disappeared and a new band appeared at around 61 kDa, and, at low Lys-C concentrations, a slight band also appeared at 113 kDa. The above results suggested that, as shown in Fig. 45, the band at around 150 kDa was IgG, the band at around 113 kDa was a one-arm form in which the heavy chain hinge was cleaved once, the band at around 96 kDa was a Fab dimer, the band at around 61 kDa

was Fc, and the band at around 50 kDa was Fab.

[0309] [Reference Example 8] Assessment of antibodies having cysteine substitution at various positions of IgG1

Reference Example 8-1 Production of antibodies having cysteine substitution at various positions of IgG1

The heavy chain and light chain of an anti-human IL6R neutralizing antibody, MRA-IgG1 (heavy chain: MRAH-G1T4 (SEQ ID NO: 15), light chain: MRAL-k0 (SEQ ID NO: 16)), were subjected to a study in which an arbitrary amino acid residue structurally exposed to the surface was substituted with cysteine.

Amino acid residues within the MRA-IgG1 heavy chain variable region (MRAH, SEQ ID NO: 17) were substituted with cysteine to produce variants of the MRA-IgG1 heavy chain variable region shown in Table 28. These variants of the MRA-IgG1 heavy chain variable region were each linked with the MRA-IgG1 heavy chain constant region (G1T4, SEQ ID NO: 18) to produce MRA-IgG1 heavy chain variants, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art. In addition, amino acid residues within the MRA-IgG1 heavy chain constant region (G1T4, SEQ ID NO: 18) were substituted with cysteine to produce variants of the MRA-IgG1 heavy chain constant region shown in Table 29. These variants of the MRA-IgG1 heavy chain constant region were each linked with the MRA-IgG1 heavy chain variable region (MRAH, SEQ ID NO: 17) to produce MRA-IgG1 heavy chain variants, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art.

[0310]

[Table 28]

Variant of MRA-lgG1 heavy chain variable region	Position of cysteine substitution (Kabat numbering)	SEQ ID NO:
MRAH.Q5C	5	322
MRAH.E6C	6	323
MRAH.S7C	7	324
MRAH.G8C	8	325
MRAH.P9C	9	326
MRAH.G10C	10	327
MRAH.L11C	11	328
MRAH.V12C	12	329
MRAH.R13C	13	330
MRAH.P14C	14	331
MRAH.S15C	15	332
MRAH.Q16C	16	333
MRAH.T17C	17	334
MRAH.L18C	18	335
MRAH.S19C	19	336
MRAH.L20C	20	337
MRAH.T21C	21	338
MRAH.T23C	23	339
MRAH.S25C	25	340
MRAH.G26C	26	341
MRAH.S28C	28	342
MRAH.T30C	30	343
MRAH.S31C	31	344
MRAH.W35C	35	345
MRAH.S35aC	35a	346
MRAH.Y50C	50	347
MRAH.I51C	51	348
MRAH.S52C	52	349
MRAH.S62C	62	350

MRAH.L63C	63	351
MRAH.K64C	64	352
MRAH.S65C	65	353
MRAH.R66C	66	354
MRAH.V67C	67	355
MRAH.T68C	68	356
MRAH.L70C	70	357
MRAH.D72C	72	358
MRAH.T73C	73	359
MRAH.S74C	74	360
MRAH.K75C	75	361
MRAH.N76C	76	362
MRAH.Q77C	77	363
MRAH.S79C	79	364
MRAH.L80C	80	365
MRAH.R81C	81	366
MRAH.L82C	82	367
MRAH.S82aC	82a	368
MRAH.S82bC	82b	369
MRAH.V82cC	82c	370
MRAH.D101C	101	371
MRAH.Y102C	102	372
MRAH.S112C	112	373
MRAH.S113C	113	374

[0311]

[Table 29]

Variant of MRA-IgG1 heavy chain constant region	Position of cysteine substitution (EU numbering)	SEQ ID NO:
G1T4.A118C	118	375
G1T4.S119C	119	376
G1T4.T120C	120	377
G1T4.K121C	121	378
G1T4.G122C	122	379
G1T4.P123C	123	380
G1T4.S124C	124	381
G1T4.V125C	125	382
G1T4.F126C	126	383
G1T4.P127C	127	384
G1T4.S131C	131	385
G1T4.S132C	132	386
G1T4.K133C	133	387
G1T4.S134C	134	388
G1T4.T135C	135	389
G1T4.S136C	136	390
G1T4.G137C	137	391
G1T4.G138C	138	392
G1T4.T139C	139	393
G1T4.A140C	140	394
G1T4.A141C	141	395
G1T4.D148C	148	396
G1T4.Y149C	149	397
G1T4.F150C	150	398
G1T4.P151C	151	399
G1T4.E152C	152	400
G1T4.P153C	153	401
G1T4.V154C	154	402
G1T4.T155C	155	403

G1T4.V156C	156	404
G1T4.S157C	157	405
G1T4.W158C	158	406
G1T4.N159C	159	407
G1T4.S160C	160	408
G1T4.G161C	161	409
G1T4.A162C	162	410
G1T4.L163C	163	411
G1T4.T164C	164	412
G1T4.S165C	165	413
G1T4.G166C	166	414
G1T4.V167C	167	415
G1T4.V173C	173	416
G1T4.L174C	174	417
G1T4.Q175C	175	418
G1T4.S176C	176	419
G1T4.S177C	177	420
G1T4.G178C	178	421
G1T4.L179C	179	422
G1T4.Y180C	180	423
G1T4.V186C	186	424
G1T4.T187C	187	425
G1T4.V188C	188	426
G1T4.P189C	189	427
G1T4.S190C	190	428
G1T4.S191C	191	429
G1T4.S192C	192	430
G1T4.L193C	193	431
G1T4.G194C	194	432
G1T4.T195C	195	433
G1T4.Q196C	196	434
G1T4.T197C	197	435
G1T4.Y198C	198	436

G1T4.I199C	199	437
G1T4.N201C	201	438
G1T4.V202C	202	439
G1T4.N203C	203	440
G1T4.H204C	204	441
G1T4.K205C	205	442
G1T4.P206C	206	443
G1T4.S207C	207	444
G1T4.N208C	208	445
G1T4.T209C	209	446
G1T4.K210C	210	447
G1T4.V211C	211	448
G1T4.D212C	212	449
G1T4.K213C	213	450
G1T4.R214C	214	451
G1T4.V215C	215	452
G1T4.E216C	216	453
G1T4.P217C	217	454
G1T4.K218C	218	455
G1T4.S219C	219	456

[0312] Similarly, amino acid residues within the MRA-IgG1 light chain variable region (MRAL, SEQ ID NO: 19) were substituted with cysteine to produce variants of the MRA-IgG1 light chain variable region shown in Table 30. These variants of the MRA-IgG1 light chain variable region were each linked with the MRA-IgG1 light chain constant region (k0, SEQ ID NO: 20) to produce MRA-IgG1 light chain variants, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art. In addition, amino acid residues within the MRA-IgG1 light chain constant region (k0, SEQ ID NO: 20) were substituted with cysteine to produce variants of the MRA-IgG1 light chain constant region shown in Table 31. These variants of the MRA-IgG1 heavy chain constant region were each linked with the MRA-IgG1 light chain variable region (MRAL, SEQ ID NO: 19) to produce MRA-IgG1 light chain variants, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art.

[0313]

[Table 30]

Variant of MRA-IgG1 light chain variable region	Position of cysteine substitution (Kabat numbering)	SEQ ID NO:
MRAL.T5C	5	457
MRAL.Q6C	6	458
MRAL.S7C	7	459
MRAL.P8C	8	460
MRAL.S9C	9	461
MRAL.S10C	10	462
MRAL.L11C	11	463
MRAL.S12C	12	464
MRAL.A13C	13	465
MRAL.S14C	14	466
MRAL.V15C	15	467
MRAL.G16C	16	468
MRAL.D17C	17	469
MRAL.R18C	18	470
MRAL.V19C	19	471
MRAL.T20C	20	472
MRAL.I21C	21	473
MRAL.T22C	22	474
MRAL.A25C	25	475
MRAL.S26C	26	476
MRAL.Q27C	27	477
MRAL.Y32C	32	478
MRAL.L33C	33	479
MRAL.N34C	34	480
MRAL.Y50C	50	481
MRAL.T51C	51	482
MRAL.H55C	55	483
MRAL.S56C	56	484
MRAL.G57C	57	485

MRAL.V58C	58	486
MRAL.P59C	59	487
MRAL.S60C	60	488
MRAL.R61C	61	489
MRAL.F62C	62	490
MRAL.S63C	63	491
MRAL.S65C	65	492
MRAL.S67C	67	493
MRAL.G68C	68	494
MRAL.T69C	69	495
MRAL.D70C	70	496
MRAL.T72C	72	497
MRAL.F73C	73	498
MRAL.T74C	74	499
MRAL.I75C	75	500
MRAL.S76C	76	501
MRAL.S77C	77	502
MRAL.L78C	78	503
MRAL.Q79C	79	504
MRAL.Y96C	96	505
MRAL.T97C	97	506
MRAL.F98C	98	507
MRAL.G99C	99	508
MRAL.Q100C	100	509
MRAL.G101C	101	510
MRAL.T102C	102	511
MRAL.K103C	103	512
MRAL.V104C	104	513
MRAL.E105C	105	514
MRAL.I106C	106	515
MRAL.K107C	107	516

[0314]

[Table 31]

Variant of MRA-IgG1 light chain constant region	Position of cysteine substitution (Kabat numbering)	SEQ ID NO:
k0.R108C	108	517
k0.T109C	109	518
k0.V110C	110	519
k0.A111C	111	520
k0.A112C	112	521
k0.P113C	113	522
k0.S114C	114	523
k0.V115C	115	524
k0.F116C	116	525
k0.P120C	120	526
k0.S121C	121	527
k0.D122C	122	528
k0.E123C	123	529
k0.Q124C	124	530
k0.L125C	125	531
k0.K126C	126	532
k0.S127C	127	533
k0.G128C	128	534
k0.T129C	129	535
k0.A130C	130	536
k0.S131C	131	537
k0.L136C	136	538
k0.N137C	137	539
k0.N138C	138	540
k0.F139C	139	541
k0.Y140C	140	542
k0.P141C	141	543
k0.R142C	142	544
k0.E143C	143	545

k0.A144C	144	546
k0.K145C	145	547
k0.V146C	146	548
k0.Q147C	147	549
k0.W148C	148	550
k0.K149C	149	551
k0.V150C	150	552
k0.D151C	151	553
k0.N152C	152	554
k0.A153C	153	555
k0.L154C	154	556
k0.Q155C	155	557
k0.S156C	156	558
k0.G157C	157	559
k0.N158C	158	560
k0.S159C	159	561
k0.Q160C	160	562
k0.E161C	161	563
k0.S162C	162	564
k0.V163C	163	565
k0.T164C	164	566
k0.E165C	165	567
k0.Q166C	166	568
k0.D167C	167	569
k0.S168C	168	570
k0.K169C	169	571
k0.D170C	170	572
k0.S171C	171	573
k0.T172C	172	574
k0.Y173C	173	575
k0.S174C	174	576
k0.L175C	175	577
k0.T180C	180	578

k0.L181C	181	579
k0.S182C	182	580
k0.K183C	183	581
k0.A184C	184	582
k0.D185C	185	583
k0.Y186C	186	584
k0.E187C	187	585
k0.K188C	188	586
k0.H189C	189	587
k0.K190C	190	588
k0.V191C	191	589
k0.Y192C	192	590
k0.A193C	193	591
k0.E195C	195	592
k0.V196C	196	593
k0.T197C	197	594
k0.H198C	198	595
k0.Q199C	199	596
k0.G200C	200	597
k0.L201C	201	598
k0.S202C	202	599
k0.S203C	203	600
k0.P204C	204	601
k0.V205C	205	602
k0.T206C	206	603
k0.K207C	207	604
k0.S208C	208	605
k0.F209C	209	606
k0.N210C	210	607
k0.R211C	211	608
k0.G212C	212	609
k0.E213C	213	610

[0315] The MRA-IgG1 heavy chain variants produced above were combined with the MRA-IgG1 light chain, or the MRA-IgG1 heavy chain was combined with the MRA-IgG1 light chain variants. The resultant MRA-IgG1 heavy chain variants and MRA-IgG1 light chain variants shown in Tables 32 and 33 were expressed by transient expression using FreeStyle293 cells (Invitrogen) or Expi293 cells (Life technologies) by a method known to the person skilled in the art, and purified with Protein A by a

method known to the person skilled in the art.

[0316] [Table 32]

MRA-IgG1 heavy chain variant name	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:	Light chain variable region SEQ ID NO:	Light chain constant region SEQ ID NO:
MRAH.Q5C-IgG1	322	18	19	20
MRAH.E6C-IgG1	323	18	19	20
MRAH.S7C-IgG1	324	18	19	20
MRAH.G8C-IgG1	325	18	19	20
MRAH.P9C-IgG1	326	18	19	20
MRAH.G10C-IgG1	327	18	19	20
MRAH.L11C-IgG1	328	18	19	20
MRAH.V12C-IgG1	329	18	19	20
MRAH.R13C-IgG1	330	18	19	20
MRAH.P14C-IgG1	331	18	19	20
MRAH.S15C-IgG1	332	18	19	20
MRAH.Q16C-IgG1	333	18	19	20
MRAH.T17C-IgG1	334	18	19	20
MRAH.L18C-IgG1	335	18	19	20
MRAH.S19C-IgG1	336	18	19	20
MRAH.L20C-IgG1	337	18	19	20
MRAH.T21C-IgG1	338	18	19	20
MRAH.T23C-IgG1	339	18	19	20
MRAH.S25C-IgG1	340	18	19	20
MRAH.G26C-IgG1	341	18	19	20
MRAH.S28C-IgG1	342	18	19	20
MRAH.T30C-IgG1	343	18	19	20
MRAH.S31C-IgG1	344	18	19	20
MRAH.W35C-IgG1	345	18	19	20
MRAH.S35aC-IgG1	346	18	19	20
MRAH.Y50C-IgG1	347	18	19	20
MRAH.I51C-IgG1	348	18	19	20
MRAH.S52C-IgG1	349	18	19	20

MRAH.S62C-IgG1	350	18	19	20
MRAH.L63C-IgG1	351	18	19	20
MRAH.K64C-IgG1	352	18	19	20
MRAH.S65C-IgG1	353	18	19	20
MRAH.R66C-IgG1	354	18	19	20
MRAH.V67C-IgG1	355	18	19	20
MRAH.T68C-IgG1	356	18	19	20
MRAH.L70C-IgG1	357	18	19	20
MRAH.D72C-IgG1	358	18	19	20
MRAH.T73C-IgG1	359	18	19	20
MRAH.S74C-IgG1	360	18	19	20
MRAH.K75C-IgG1	361	18	19	20
MRAH.N76C-IgG1	362	18	19	20
MRAH.Q77C-IgG1	363	18	19	20
MRAH.S79C-IgG1	364	18	19	20
MRAH.L80C-IgG1	365	18	19	20
MRAH.R81C-IgG1	366	18	19	20
MRAH.L82C-IgG1	367	18	19	20
MRAH.S82aC-IgG1	368	18	19	20
MRAH.S82bC-IgG1	369	18	19	20
MRAH.V82cC-IgG1	370	18	19	20
MRAH.D101C-IgG1	371	18	19	20
MRAH.Y102C-IgG1	372	18	19	20
MRAH.S112C-IgG1	373	18	19	20
MRAH.S113C-IgG1	374	18	19	20
G1T4.A118C-IgG1	17	375	19	20
G1T4.S119C-IgG1	17	376	19	20
G1T4.T120C-IgG1	17	377	19	20
G1T4.K121C-IgG1	17	378	19	20
G1T4.G122C-IgG1	17	379	19	20
G1T4.P123C-IgG1	17	380	19	20
G1T4.S124C-IgG1	17	381	19	20
G1T4.V125C-IgG1	17	382	19	20

G1T4.F126C-IgG1	17	383	19	20
G1T4.P127C-IgG1	17	384	19	20
G1T4.S131C-IgG1	17	385	19	20
G1T4.S132C-IgG1	17	386	19	20
G1T4.K133C-IgG1	17	387	19	20
G1T4.S134C-IgG1	17	388	19	20
G1T4.T135C-IgG1	17	389	19	20
G1T4.S136C-IgG1	17	390	19	20
G1T4.G137C-IgG1	17	391	19	20
G1T4.G138C-IgG1	17	392	19	20
G1T4.T139C-IgG1	17	393	19	20
G1T4.A140C-IgG1	17	394	19	20
G1T4.A141C-IgG1	17	395	19	20
G1T4.D148C-IgG1	17	396	19	20
G1T4.Y149C-IgG1	17	397	19	20
G1T4.F150C-IgG1	17	398	19	20
G1T4.P151C-IgG1	17	399	19	20
G1T4.E152C-IgG1	17	400	19	20
G1T4.P153C-IgG1	17	401	19	20
G1T4.V154C-IgG1	17	402	19	20
G1T4.T155C-IgG1	17	403	19	20
G1T4.V156C-IgG1	17	404	19	20
G1T4.S157C-IgG1	17	405	19	20
G1T4.W158C-IgG1	17	406	19	20
G1T4.N159C-IgG1	17	407	19	20
G1T4.S160C-IgG1	17	408	19	20
G1T4.G161C-IgG1	17	409	19	20
G1T4.A162C-IgG1	17	410	19	20
G1T4.L163C-IgG1	17	411	19	20
G1T4.T164C-IgG1	17	412	19	20
G1T4.S165C-IgG1	17	413	19	20
G1T4.G166C-IgG1	17	414	19	20
G1T4.V167C-IgG1	17	415	19	20

G1T4.V173C-IgG1	17	416	19	20
G1T4.L174C-IgG1	17	417	19	20
G1T4.Q175C-IgG1	17	418	19	20
G1T4.S176C-IgG1	17	419	19	20
G1T4.S177C-IgG1	17	420	19	20
G1T4.G178C-IgG1	17	421	19	20
G1T4.L179C-IgG1	17	422	19	20
G1T4.Y180C-IgG1	17	423	19	20
G1T4.V186C-IgG1	17	424	19	20
G1T4.T187C-IgG1	17	425	19	20
G1T4.V188C-IgG1	17	426	19	20
G1T4.P189C-IgG1	17	427	19	20
G1T4.S190C-IgG1	17	428	19	20
G1T4.S191C-IgG1	17	429	19	20
G1T4.S192C-IgG1	17	430	19	20
G1T4.L193C-IgG1	17	431	19	20
G1T4.G194C-IgG1	17	432	19	20
G1T4.T195C-IgG1	17	433	19	20
G1T4.Q196C-IgG1	17	434	19	20
G1T4.T197C-IgG1	17	435	19	20
G1T4.Y198C-IgG1	17	436	19	20
G1T4.I199C-IgG1	17	437	19	20
G1T4.N201C-IgG1	17	438	19	20
G1T4.V202C-IgG1	17	439	19	20
G1T4.N203C-IgG1	17	440	19	20
G1T4.H204C-IgG1	17	441	19	20
G1T4.K205C-IgG1	17	442	19	20
G1T4.P206C-IgG1	17	443	19	20
G1T4.S207C-IgG1	17	444	19	20
G1T4.N208C-IgG1	17	445	19	20
G1T4.T209C-IgG1	17	446	19	20
G1T4.K210C-IgG1	17	447	19	20
G1T4.V211C-IgG1	17	448	19	20

G1T4.D212C-IgG1	17	449	19	20
G1T4.K213C-IgG1	17	450	19	20
G1T4.R214C-IgG1	17	451	19	20
G1T4.V215C-IgG1	17	452	19	20
G1T4.E216C-IgG1	17	453	19	20
G1T4.P217C-IgG1	17	454	19	20
G1T4.K218C-IgG1	17	455	19	20
G1T4.S219C-IgG1	17	456	19	20

[0317]

[Table 33]

MRA-IgG1 light chain variant name	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:	Light chain variable region SEQ ID NO:	Light chain constant region SEQ ID NO:
MRAL.T5C-IgG1	17	18	457	20
MRAL.Q6C-IgG1	17	18	458	20
MRAL.S7C-IgG1	17	18	459	20
MRAL.P8C-IgG1	17	18	460	20
MRAL.S9C-IgG1	17	18	461	20
MRAL.S10C-IgG1	17	18	462	20
MRAL.L11C-IgG1	17	18	463	20
MRAL.S12C-IgG1	17	18	464	20
MRAL.A13C-IgG1	17	18	465	20
MRAL.S14C-IgG1	17	18	466	20
MRAL.V15C-IgG1	17	18	467	20
MRAL.G16C-IgG1	17	18	468	20
MRAL.D17C-IgG1	17	18	469	20
MRAL.R18C-IgG1	17	18	470	20
MRAL.V19C-IgG1	17	18	471	20
MRAL.T20C-IgG1	17	18	472	20
MRAL.I21C-IgG1	17	18	473	20
MRAL.T22C-IgG1	17	18	474	20
MRAL.A25C-IgG1	17	18	475	20
MRAL.S26C-IgG1	17	18	476	20
MRAL.Q27C-IgG1	17	18	477	20
MRAL.Y32C-IgG1	17	18	478	20
MRAL.L33C-IgG1	17	18	479	20
MRAL.N34C-IgG1	17	18	480	20
MRAL.Y50C-IgG1	17	18	481	20
MRAL.T51C-IgG1	17	18	482	20
MRAL.H55C-IgG1	17	18	483	20
MRAL.S56C-IgG1	17	18	484	20

MRAL.G57C-IgG1	17	18	485	20
MRAL.V58C-IgG1	17	18	486	20
MRAL.P59C-IgG1	17	18	487	20
MRAL.S60C-IgG1	17	18	488	20
MRAL.R61C-IgG1	17	18	489	20
MRAL.F62C-IgG1	17	18	490	20
MRAL.S63C-IgG1	17	18	491	20
MRAL.S65C-IgG1	17	18	492	20
MRAL.S67C-IgG1	17	18	493	20
MRAL.G68C-IgG1	17	18	494	20
MRAL.T69C-IgG1	17	18	495	20
MRAL.D70C-IgG1	17	18	496	20
MRAL.T72C-IgG1	17	18	497	20
MRAL.F73C-IgG1	17	18	498	20
MRAL.T74C-IgG1	17	18	499	20
MRAL.I75C-IgG1	17	18	500	20
MRAL.S76C-IgG1	17	18	501	20
MRAL.S77C-IgG1	17	18	502	20
MRAL.L78C-IgG1	17	18	503	20
MRAL.Q79C-IgG1	17	18	504	20
MRAL.Y96C-IgG1	17	18	505	20
MRAL.T97C-IgG1	17	18	506	20
MRAL.F98C-IgG1	17	18	507	20
MRAL.G99C-IgG1	17	18	508	20
MRAL.Q100C-IgG1	17	18	509	20
MRAL.G101C-IgG1	17	18	510	20
MRAL.T102C-IgG1	17	18	511	20
MRAL.K103C-IgG1	17	18	512	20
MRAL.V104C-IgG1	17	18	513	20
MRAL.E105C-IgG1	17	18	514	20
MRAL.I106C-IgG1	17	18	515	20
MRAL.K107C-IgG1	17	18	516	20
k0.R108C-IgG1	17	18	19	517

k0.T109C-IgG1	17	18	19	518
k0.V110C-IgG1	17	18	19	519
k0.A111C-IgG1	17	18	19	520
k0.A112C-IgG1	17	18	19	521
k0.P113C-IgG1	17	18	19	522
k0.S114C-IgG1	17	18	19	523
k0.V115C-IgG1	17	18	19	524
k0.F116C-IgG1	17	18	19	525
k0.P120C-IgG1	17	18	19	526
k0.S121C-IgG1	17	18	19	527
k0.D122C-IgG1	17	18	19	528
k0.E123C-IgG1	17	18	19	529
k0.Q124C-IgG1	17	18	19	530
k0.L125C-IgG1	17	18	19	531
k0.K126C-IgG1	17	18	19	532
k0.S127C-IgG1	17	18	19	533
k0.G128C-IgG1	17	18	19	534
k0.T129C-IgG1	17	18	19	535
k0.A130C-IgG1	17	18	19	536
k0.S131C-IgG1	17	18	19	537
k0.L136C-IgG1	17	18	19	538
k0.N137C-IgG1	17	18	19	539
k0.N138C-IgG1	17	18	19	540
k0.F139C-IgG1	17	18	19	541
k0.Y140C-IgG1	17	18	19	542
k0.P141C-IgG1	17	18	19	543
k0.R142C-IgG1	17	18	19	544
k0.E143C-IgG1	17	18	19	545
k0.A144C-IgG1	17	18	19	546
k0.K145C-IgG1	17	18	19	547
k0.V146C-IgG1	17	18	19	548
k0.Q147C-IgG1	17	18	19	549
k0.W148C-IgG1	17	18	19	550

k0.K149C-IgG1	17	18	19	551
k0.V150C-IgG1	17	18	19	552
k0.D151C-IgG1	17	18	19	553
k0.N152C-IgG1	17	18	19	554
k0.A153C-IgG1	17	18	19	555
k0.L154C-IgG1	17	18	19	556
k0.Q155C-IgG1	17	18	19	557
k0.S156C-IgG1	17	18	19	558
k0.G157C-IgG1	17	18	19	559
k0.N158C-IgG1	17	18	19	560
k0.S159C-IgG1	17	18	19	561
k0.Q160C-IgG1	17	18	19	562
k0.E161C-IgG1	17	18	19	563
k0.S162C-IgG1	17	18	19	564
k0.V163C-IgG1	17	18	19	565
k0.T164C-IgG1	17	18	19	566
k0.E165C-IgG1	17	18	19	567
k0.Q166C-IgG1	17	18	19	568
k0.D167C-IgG1	17	18	19	569
k0.S168C-IgG1	17	18	19	570
k0.K169C-IgG1	17	18	19	571
k0.D170C-IgG1	17	18	19	572
k0.S171C-IgG1	17	18	19	573
k0.T172C-IgG1	17	18	19	574
k0.Y173C-IgG1	17	18	19	575
k0.S174C-IgG1	17	18	19	576
k0.L175C-IgG1	17	18	19	577
k0.T180C-IgG1	17	18	19	578
k0.L181C-IgG1	17	18	19	579
k0.S182C-IgG1	17	18	19	580
k0.K183C-IgG1	17	18	19	581
k0.A184C-IgG1	17	18	19	582
k0.D185C-IgG1	17	18	19	583

k0.Y186C-IgG1	17	18	19	584
k0.E187C-IgG1	17	18	19	585
k0.K188C-IgG1	17	18	19	586
k0.H189C-IgG1	17	18	19	587
k0.K190C-IgG1	17	18	19	588
k0.V191C-IgG1	17	18	19	589
k0.Y192C-IgG1	17	18	19	590
k0.A193C-IgG1	17	18	19	591
k0.E195C-IgG1	17	18	19	592
k0.V196C-IgG1	17	18	19	593
k0.T197C-IgG1	17	18	19	594
k0.H198C-IgG1	17	18	19	595
k0.Q199C-IgG1	17	18	19	596
k0.G200C-IgG1	17	18	19	597
k0.L201C-IgG1	17	18	19	598
k0.S202C-IgG1	17	18	19	599
k0.S203C-IgG1	17	18	19	600
k0.P204C-IgG1	17	18	19	601
k0.V205C-IgG1	17	18	19	602
k0.T206C-IgG1	17	18	19	603
k0.K207C-IgG1	17	18	19	604
k0.S208C-IgG1	17	18	19	605
k0.F209C-IgG1	17	18	19	606
k0.N210C-IgG1	17	18	19	607
k0.R211C-IgG1	17	18	19	608
k0.G212C-IgG1	17	18	19	609
k0.E213C-IgG1	17	18	19	610

[0318] Reference Example 8-2 Assessment of electrophoretic mobility in polyacrylamide gel of antibodies having cysteine substitution at various positions of IgG1

It was examined with non-reducing SDS-PAGE whether the MRA-IgG1 variants produced in Reference Example 8-1 show a different electrophoretic mobility to MRA-IgG1. Sample Buffer Solution (2ME-) (x4) (Wako; 198-13282) was used for preparing electrophoresis samples, the samples were treated for 10 minutes under the condition of specimen concentration 50 microgram/mL and 70 degrees C, and then subjected to non-reducing SDS-PAGE. In non-reducing SDS-PAGE, electrophoresis was carried out for 90 minutes at 125 V, using 4% SDS-PAGE mini 15well 1.0mm 15well (TEFCO; Cat#01-052-6). Then, the gel was stained with CBB stain, the gel image was captured with ChemiDocTouchMP (BIORAD), and the bands were quantified with

Image Lab (BIORAD).

From the obtained gel image, the variants were classified into 7 groups according to the band pattern of each of the MRA-IgG1 variants: Single (one band at a molecular weight region similar to that of MRA-IgG1), Double (two bands at a molecular weight region similar to that of MRA-IgG1), Triple (three bands at a molecular weight region similar to that of MRA-IgG1), Several (four or more bands at a molecular weight region similar to that of MRA-IgG1), LMW (band(s) at a molecular weight region lower than that of MRA-IgG1), HMW (band(s) at a molecular weight region higher than that of MRA-IgG1), and Faint (band(s) blurry and difficult to determine). Regarding the MRA-IgG1 variants classified as "Double", one of the two bands showed the same electrophoretic mobility as MRA-IgG1 while the other band showed slightly faster or slower mobility. Thus, for the MRA-IgG1 variants classified as "Double", the percentage of the bands showing different mobility to MRA-IgG1 (percentage of new band (%)) was also calculated. Grouping of the band patterns for MRA-IgG1 heavy chain variants and MRA-IgG1 light chain variants, and the calculation results of the band percentage are respectively shown in Tables 34 and 35. From Tables 34 and 35, variants classified into the Double and Triple groups are shown in Table 36. In these variants, it is highly likely that cysteine substitution caused structural changes such as crosslinkage of Fabs, which resulted in the change in electrophoretic mobility. It is noted that while Table 35 indicates "no data" for MRAL.K107C-IgG1, position 107 (Kabat numbering), which is the position of cysteine substitution in this variant, is a position where the residue structurally exposed to the surface is present in the hinge region. Thus, in this variant also, it is highly likely that cysteine substitution causes structural changes such as crosslinkage of Fabs, and results in the change in electrophoretic mobility.

[0319]

[Table 34]

MRA-IgG1 heavy chain variant name	Group	Percentage of new band (%)
MRAH.Q5C-IgG1	Single	-
MRAH.E6C-IgG1	Double	30.7
MRAH.S7C-IgG1	Single	-
MRAH.G8C-IgG1	Single	-
MRAH.P9C-IgG1	Single	-
MRAH.G10C-IgG1	Single	-
MRAH.L11C-IgG1	Single	-
MRAH.V12C-IgG1	Single	-
MRAH.R13C-IgG1	Single	-
MRAH.P14C-IgG1	Single	-
MRAH.S15C-IgG1	Single	-
MRAH.Q16C-IgG1	Single	-
MRAH.T17C-IgG1	Single	-
MRAH.L18C-IgG1	Faint	-
MRAH.S19C-IgG1	Single	-
MRAH.L20C-IgG1	Faint	-
MRAH.T21C-IgG1	Single	-
MRAH.T23C-IgG1	no data	-
MRAH.S25C-IgG1	Double	20.2
MRAH.G26C-IgG1	Double	14.5
MRAH.S28C-IgG1	Single	-
MRAH.T30C-IgG1	Single	-
MRAH.S31C-IgG1	Single	-
MRAH.W35C-IgG1	Faint	-
MRAH.S35aC-IgG1	Faint	-
MRAH.Y50C-IgG1	Single	-
MRAH.I51C-IgG1	Faint	-
MRAH.S52C-IgG1	Single	-
MRAH.S62C-IgG1	Single	-
MRAH.L63C-IgG1	Single	-

MRAH.K64C-IgG1	Single	-
MRAH.S65C-IgG1	Single	-
MRAH.R66C-IgG1	Single	-
MRAH.V67C-IgG1	Single	-
MRAH.T68C-IgG1	Single	-
MRAH.L70C-IgG1	no data	-
MRAH.D72C-IgG1	Single	-
MRAH.T73C-IgG1	Single	-
MRAH.S74C-IgG1	Single	-
MRAH.K75C-IgG1	Single	-
MRAH.N76C-IgG1	Single	-
MRAH.Q77C-IgG1	Single	-
MRAH.S79C-IgG1	Single	-
MRAH.L80C-IgG1	Faint	-
MRAH.R81C-IgG1	Single	-
MRAH.L82C-IgG1	Faint	-
MRAH.S82aC-IgG1	Single	-
MRAH.S82bC-IgG1	Single	-
MRAH.V82cC-IgG1	Faint	-
MRAH.D101C-IgG1	Single	-
MRAH.Y102C-IgG1	Single	-
MRAH.S112C-IgG1	Single	-
MRAH.S113C-IgG1	Single	-
G1T4.A118C-IgG1	Single	-
G1T4.S119C-IgG1	Double	18.4
G1T4.T120C-IgG1	Single	-
G1T4.K121C-IgG1	Single	-
G1T4.G122C-IgG1	Single	-
G1T4.P123C-IgG1	LMW	-
G1T4.S124C-IgG1	Single	-
G1T4.V125C-IgG1	LMW	-
G1T4.F126C-IgG1	Single	-
G1T4.P127C-IgG1	LMW	-

G1T4.S131C-IgG1	Triple	-
G1T4.S132C-IgG1	Triple	-
G1T4.K133C-IgG1	Triple	-
G1T4.S134C-IgG1	Triple	-
G1T4.T135C-IgG1	Triple	-
G1T4.S136C-IgG1	Triple	-
G1T4.G137C-IgG1	Triple	-
G1T4.G138C-IgG1	Double	56.7
G1T4.T139C-IgG1	Single	-
G1T4.A140C-IgG1	Single	-
G1T4.A141C-IgG1	Faint	-
G1T4.D148C-IgG1	Single	-
G1T4.Y149C-IgG1	Faint	-
G1T4.F150C-IgG1	Single	-
G1T4.P151C-IgG1	Faint	-
G1T4.E152C-IgG1	Single	-
G1T4.P153C-IgG1	Single	-
G1T4.V154C-IgG1	LMW	-
G1T4.T155C-IgG1	Single	-
G1T4.V156C-IgG1	LMW	-
G1T4.S157C-IgG1	Single	-
G1T4.W158C-IgG1	LMW	-
G1T4.N159C-IgG1	Double	24
G1T4.S160C-IgG1	Double	35.7
G1T4.G161C-IgG1	Double	27.2
G1T4.A162C-IgG1	Double	27.8
G1T4.L163C-IgG1	Double	16.7
G1T4.T164C-IgG1	Double	13.8
G1T4.S165C-IgG1	Single	-
G1T4.G166C-IgG1	Single	-
G1T4.V167C-IgG1	Single	-
G1T4.V173C-IgG1	Single	-
G1T4.L174C-IgG1	Single	-

G1T4.Q175C-IgG1	Single	-
G1T4.S176C-IgG1	Single	-
G1T4.S177C-IgG1	Single	-
G1T4.G178C-IgG1	Single	-
G1T4.L179C-IgG1	Single	-
G1T4.Y180C-IgG1	LMW	-
G1T4.V186C-IgG1	LMW	-
G1T4.T187C-IgG1	Single	-
G1T4.V188C-IgG1	LMW	-
G1T4.P189C-IgG1	no data	-
G1T4.S190C-IgG1	Double	31.8
G1T4.S191C-IgG1	Double	66.3
G1T4.S192C-IgG1	Double	26.8
G1T4.L193C-IgG1	LMW	-
G1T4.G194C-IgG1	Faint	-
G1T4.T195C-IgG1	Double	78.1
G1T4.Q196C-IgG1	Double	27.4
G1T4.T197C-IgG1	Double	84.4
G1T4.Y198C-IgG1	Faint	-
G1T4.I199C-IgG1	Single	-
G1T4.N201C-IgG1	Double	17.5
G1T4.V202C-IgG1	LMW	-
G1T4.N203C-IgG1	Double	17.2
G1T4.H204C-IgG1	Faint	-
G1T4.K205C-IgG1	Double	18.4
G1T4.P206C-IgG1	Double	14.4
G1T4.S207C-IgG1	Double	21.5
G1T4.N208C-IgG1	Double	16.1
G1T4.T209C-IgG1	Single	-
G1T4.K210C-IgG1	Single	-
G1T4.V211C-IgG1	Double	27.2
G1T4.D212C-IgG1	Double	28.2
G1T4.K213C-IgG1	LMW	-

G1T4.R214C-IgG1	LMW	-
G1T4.V215C-IgG1	LMW	-
G1T4.E216C-IgG1	LMW	-
G1T4.P217C-IgG1	LMW	-
G1T4.K218C-IgG1	Double	39.3
G1T4.S219C-IgG1	Double	68.7

[0320]

[Table 35]

MRA-IgG1 light chain variant name	Group	Percentage of new band (%)
MRAL.T5C-IgG1	Single	-
MRAL.Q6C-IgG1	LMW	-
MRAL.S7C-IgG1	Single	-
MRAL.P8C-IgG1	no data	-
MRAL.S9C-IgG1	Single	-
MRAL.S10C-IgG1	Single	-
MRAL.L11C-IgG1	Single	-
MRAL.S12C-IgG1	Single	-
MRAL.A13C-IgG1	Single	-
MRAL.S14C-IgG1	Single	-
MRAL.V15C-IgG1	Single	-
MRAL.G16C-IgG1	Single	-
MRAL.D17C-IgG1	Single	-
MRAL.R18C-IgG1	Single	-
MRAL.V19C-IgG1	LMW	-
MRAL.T20C-IgG1	Single	-
MRAL.I21C-IgG1	Double	68.9
MRAL.T22C-IgG1	Single	-
MRAL.A25C-IgG1	no data	-
MRAL.S26C-IgG1	no data	-
MRAL.Q27C-IgG1	Triple	-
MRAL.Y32C-IgG1	Single	-
MRAL.L33C-IgG1	LMW	-
MRAL.N34C-IgG1	LMW	-
MRAL.Y50C-IgG1	Single	-
MRAL.T51C-IgG1	Single	-
MRAL.H55C-IgG1	Single	-
MRAL.S56C-IgG1	Single	-
MRAL.G57C-IgG1	Single	-
MRAL.V58C-IgG1	Double	17.4

MRAL.P59C-IgG1	Single	-
MRAL.S60C-IgG1	Single	-
MRAL.R61C-IgG1	Single	-
MRAL.F62C-IgG1	LMW	-
MRAL.S63C-IgG1	Single	-
MRAL.S65C-IgG1	Single	-
MRAL.S67C-IgG1	Single	-
MRAL.G68C-IgG1	Single	-
MRAL.T69C-IgG1	Single	-
MRAL.D70C-IgG1	Single	-
MRAL.T72C-IgG1	Single	-
MRAL.F73C-IgG1	LMW	-
MRAL.T74C-IgG1	Single	-
MRAL.I75C-IgG1	no data	-
MRAL.S76C-IgG1	Single	-
MRAL.S77C-IgG1	Double	18.1
MRAL.L78C-IgG1	LMW	-
MRAL.Q79C-IgG1	Single	-
MRAL.Y96C-IgG1	LMW	-
MRAL.T97C-IgG1	Single	-
MRAL.F98C-IgG1	LMW	-
MRAL.G99C-IgG1	no data	-
MRAL.Q100C-IgG1	Single	-
MRAL.G101C-IgG1	Single	-
MRAL.T102C-IgG1	LMW	-
MRAL.K103C-IgG1	Single	-
MRAL.V104C-IgG1	LMW	-
MRAL.E105C-IgG1	no data	-
MRAL.I106C-IgG1	Single	-
MRAL.K107C-IgG1	no data	-
k0.R108C-IgG1	Single	-
k0.T109C-IgG1	Double	23.1
k0.V110C-IgG1	Single	-

k0.A111C-IgG1	Single	-
k0.A112C-IgG1	Double	21.6
k0.P113C-IgG1	Single	-
k0.S114C-IgG1	Single	-
k0.V115C-IgG1	LMW	-
k0.F116C-IgG1	Single	-
k0.P120C-IgG1	LMW	-
k0.S121C-IgG1	Several	-
k0.D122C-IgG1	LMW	-
k0.E123C-IgG1	Double	18.1
k0.Q124C-IgG1	LMW	-
k0.L125C-IgG1	LMW	-
k0.K126C-IgG1	Triple	-
k0.S127C-IgG1	Single	-
k0.G128C-IgG1	Double	19.4
k0.T129C-IgG1	Single	-
k0.A130C-IgG1	LMW	-
k0.S131C-IgG1	LMW	-
k0.L136C-IgG1	LMW	-
k0.N137C-IgG1	LMW	-
k0.N138C-IgG1	Single	-
k0.F139C-IgG1	LMW	-
k0.Y140C-IgG1	LMW	-
k0.P141C-IgG1	Single	-
k0.R142C-IgG1	Single	-
k0.E143C-IgG1	Single	-
k0.A144C-IgG1	LMW	-
k0.K145C-IgG1	Single	-
k0.V146C-IgG1	LMW	-
k0.Q147C-IgG1	Single	-
k0.W148C-IgG1	LMW	-
k0.K149C-IgG1	Single	-
k0.V150C-IgG1	LMW	-

k0.D151C-IgG1	Single	-
k0.N152C-IgG1	Double	62.4
k0.A153C-IgG1	Single	-
k0.L154C-IgG1	Single	-
k0.Q155C-IgG1	Single	-
k0.S156C-IgG1	HMW	-
k0.G157C-IgG1	Single	-
k0.N158C-IgG1	Single	-
k0.S159C-IgG1	Single	-
k0.Q160C-IgG1	Single	-
k0.E161C-IgG1	Single	-
k0.S162C-IgG1	Single	-
k0.V163C-IgG1	Single	-
k0.T164C-IgG1	Single	-
k0.E165C-IgG1	Single	-
k0.Q166C-IgG1	Single	-
k0.D167C-IgG1	Single	-
k0.S168C-IgG1	Single	-
k0.K169C-IgG1	Single	-
k0.D170C-IgG1	Single	-
k0.S171C-IgG1	Single	-
k0.T172C-IgG1	LMW	-
k0.Y173C-IgG1	LMW	-
k0.S174C-IgG1	Single	-
k0.L175C-IgG1	LMW	-
k0.T180C-IgG1	Single	-
k0.L181C-IgG1	Single	-
k0.S182C-IgG1	Single	-
k0.K183C-IgG1	Single	-
k0.A184C-IgG1	Single	-
k0.D185C-IgG1	Single	-
k0.Y186C-IgG1	Double	26.3
k0.E187C-IgG1	LMW	-

k0.K188C-IgG1	Single	-
k0.H189C-IgG1	Triple	-
k0.K190C-IgG1	LMW	-
k0.V191C-IgG1	LMW	-
k0.Y192C-IgG1	Single	-
k0.A193C-IgG1	Single	-
k0.E195C-IgG1	Single	-
k0.V196C-IgG1	Single	-
k0.T197C-IgG1	Single	-
k0.H198C-IgG1	Faint	-
k0.Q199C-IgG1	Single	-
k0.G200C-IgG1	Double	18.7
k0.L201C-IgG1	Single	-
k0.S202C-IgG1	Double	42.3
k0.S203C-IgG1	Double	45.5
k0.P204C-IgG1	Single	-
k0.V205C-IgG1	Single	-
k0.T206C-IgG1	Single	-
k0.K207C-IgG1	Single	-
k0.S208C-IgG1	Single	-
k0.F209C-IgG1	LMW	-
k0.N210C-IgG1	LMW	-
k0.R211C-IgG1	Single	-
k0.G212C-IgG1	Double	68.5
k0.E213C-IgG1	LMW	-

[0321]

[Table 36]

MRA-lgG1 variant name	Group	Percentage of new band (%)
MRAH.E6C-lgG1	Double	30.7
MRAH.S25C-IgG1	Double	20.2
MRAH.G26C-IgG1	Double	14.5
G1T4.S119C-IgG1	Double	18.4
G1T4.S131C-IgG1	Triple	-
G1T4.S132C-IgG1	Triple	-
G1T4.K133C-IgG1	Triple	-
G1T4.S134C-IgG1	Triple	-
G1T4.T135C-IgG1	Triple	-
G1T4.S136C-IgG1	Triple	-
G1T4.G137C-IgG1	Triple	-
G1T4.G138C-IgG1	Double	56.7
G1T4.N159C-IgG1	Double	24
G1T4.S160C-IgG1	Double	35.7
G1T4.G161C-IgG1	Double	27.2
G1T4.A162C-IgG1	Double	27.8
G1T4.L163C-IgG1	Double	16.7
G1T4.T164C-IgG1	Double	13.8
G1T4.S190C-IgG1	Double	31.8
G1T4.S191C-IgG1	Double	66.3
G1T4.S192C-IgG1	Double	26.8
G1T4.T195C-IgG1	Double	78.1
G1T4.Q196C-IgG1	Double	27.4
G1T4.T197C-IgG1	Double	84.4
G1T4.N201C-IgG1	Double	17.5
G1T4.N203C-IgG1	Double	17.2
G1T4.K205C-IgG1	Double	18.4
G1T4.P206C-IgG1	Double	14.4
G1T4.S207C-IgG1	Double	21.5
G1T4.N208C-IgG1	Double	16.1

G1T4.V211C-IgG1	Double	27.2
G1T4.D212C-IgG1	Double	28.2
G1T4.K218C-IgG1	Double	39.3
G1T4.S219C-IgG1	Double	68.7
MRAL.I21C-IgG1	Double	68.9
MRAL.Q27C-IgG1	Triple	-
MRAL.V58C-IgG1	Double	17.4
MRAL.S77C-IgG1	Double	18.1
k0.T109C-IgG1	Double	23.1
k0.A112C-IgG1	Double	21.6
k0.E123C-IgG1	Double	18.1
k0.K126C-IgG1	Triple	-
k0.G128C-IgG1	Double	19.4
k0.N152C-IgG1	Double	62.4
k0.Y186C-IgG1	Double	26.3
k0.H189C-IgG1	Triple	-
k0.G200C-IgG1	Double	18.7
k0.S202C-IgG1	Double	42.3
k0.S203C-IgG1	Double	45.5
k0.G212C-IgG1	Double	68.5

[0322] [Reference Example 9] Assessment of antibodies having cysteine substitution at various positions of IgG4

Reference Example 9-1 Production of antibodies having cysteine substitution at various positions of IgG4

The heavy chain and light chain of an anti-human IL6R neutralizing antibody, MRA-IgG4 (heavy chain: MRAH-G4T1 (SEQ ID NO: 310), light chain: MRAL-k0 (SEQ ID NO: 16)), were subjected to a study in which an arbitrary amino acid residue structurally exposed to the surface was substituted with cysteine.

Amino acid residues within the MRA-IgG4 heavy chain variable region (MRAH, SEQ ID NO: 17) were substituted with cysteine to produce variants of the MRA-IgG4 heavy chain variable region shown in Table 37. These variants of the MRA-IgG4 heavy chain variable region were each linked with the MRA-IgG4 heavy chain constant region (G4T1, SEQ ID NO: 311) to produce MRA-IgG4 heavy chain variants, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art. In addition, amino acid residues within the MRA-IgG4 heavy chain constant region (G4T1, SEQ ID NO: 311) were substituted with cysteine to produce variants of the MRA-IgG4 heavy chain constant region shown in Table 38. These variants of the MRA-IgG4 heavy chain constant region were each

linked with the MRA-IgG4 heavy chain variable region (MRAH, SEQ ID NO: 17) to produce MRA-IgG4 heavy chain variants, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art.

[0323] [Table 37]

Variant of MRA-IgG4 heavy chain variable region	Position of cysteine substitution (Kabat numbering)	SEQ ID NO:
MRAH.Q5C	5	322
MRAH.E6C	6	323
MRAH.S7C	7	324
MRAH.G8C	8	325
MRAH.P9C	9	326
MRAH.G10C	10	327
MRAH.L11C	11	328
MRAH.V12C	12	329
MRAH.R13C	13	330
MRAH.P14C	14	331
MRAH.S15C	15	332
MRAH.Q16C	16	333
MRAH.T17C	17	334
MRAH.L18C	18	335
MRAH.S19C	19	336
MRAH.L20C	20	337
MRAH.T21C	21	338
MRAH.T23C	23	339
MRAH.S25C	25	340
MRAH.G26C	26	341
MRAH.S28C	28	342
MRAH.T30C	30	343
MRAH.S31C	31	344
MRAH.W35C	35	345
MRAH.S35aC	35a	346
MRAH.Y50C	50	347
MRAH.I51C	51	348
MRAH.S52C	52	349
MRAH.S62C	62	350

MRAH.L63C	63	351
MRAH.K64C	64	352
MRAH.S65C	65	353
MRAH.R66C	66	354
MRAH.V67C	67	355
MRAH.T68C	68	356
MRAH.L70C	70	357
MRAH.D72C	72	358
MRAH.T73C	73	359
MRAH.S74C	74	360
MRAH.K75C	75	361
MRAH.N76C	76	362
MRAH.Q77C	77	363
MRAH.S79C	79	364
MRAH.L80C	80	365
MRAH.R81C	81	366
MRAH.L82C	82	367
MRAH.S82aC	82a	368
MRAH.S82bC	82b	369
MRAH.V82cC	82c	370
MRAH.D101C	101	371
MRAH.Y102C	102	372
MRAH.S112C	112	373
MRAH.S113C	113	374

[0324]

[Table 38]

Variant of MRA-IgG4 heavy chain constant region	Position of cysteine substitution (EU numbering)	SEQ ID NO:
G4T1.A118C	118	611
G4T1.S119C	119	612
G4T1.T120C	120	613
G4T1.K121C	121	614
G4T1.G122C	122	615
G4T1.P123C	123	616
G4T1.S124C	124	617
G4T1.V125C	125	618
G4T1.F126C	126	619
G4T1.P127C	127	620
G4T1.S132C	132	621
G4T1.R133C	133	622
G4T1.S134C	134	623
G4T1.T135C	135	624
G4T1.S136C	136	625
G4T1.E137C	137	626
G4T1.S138C	138	627
G4T1.T139C	139	628
G4T1.A140C	140	629
G4T1.A141C	141	630
G4T1.D148C	148	631
G4T1.Y149C	149	632
G4T1.F150C	150	633
G4T1.P151C	151	634
G4T1.E152C	152	635
G4T1.P153C	153	636
G4T1.V154C	154	637
G4T1.T155C	155	638
G4T1.V156C	156	639

G4T1.S157C	157	640
G4T1.W158C	158	641
G4T1.N159C	159	642
G4T1.S160C	160	643
G4T1.G161C	161	644
G4T1.A162C	162	645
G4T1.L163C	163	646
G4T1.T164C	164	647
G4T1.S165C	165	648
G4T1.G166C	166	649
G4T1.V167C	167	650
G4T1.V173C	173	651
G4T1.L174C	174	652
G4T1.Q175C	175	653
G4T1.S176C	176	654
G4T1.S177C	177	655
G4T1.G178C	178	656
G4T1.L179C	179	657
G4T1.Y180C	180	658
G4T1.V186C	186	659
G4T1.T187C	187	660
G4T1.V188C	188	661
G4T1.P189C	189	662
G4T1.S190C	190	663
G4T1.S191C	191	664
G4T1.S192C	192	665
G4T1.L193C	193	666
G4T1.G194C	194	667
G4T1.T195C	195	668
G4T1.Q196C	196	669
G4T1.T197C	197	670
G4T1.Y198C	198	671
G4T1.T199C	199	672

G4T1.N201C	201	673
G4T1.V202C	202	674
G4T1.D203C	203	675
G4T1.H204C	204	676
G4T1.K205C	205	677
G4T1.P206C	206	678
G4T1.S207C	207	679
G4T1.N208C	208	680
G4T1.T209C	209	681
G4T1.K210C	210	682
G4T1.V211C	211	683
G4T1.D212C	212	684
G4T1.K213C	213	685
G4T1.R214C	214	686
G4T1.V215C	215	687
G4T1.E216C	216	688
G4T1.S217C	217	689
G4T1.K218C	218	690

[0325] The MRA-IgG4 heavy chain variants produced above were combined with the MRA-IgG4 light chain, or the MRA-IgG4 heavy chain was combined with the MRA-IgG4 light chain variants produced in Reference Example 8-1. The resultant MRA-IgG4 heavy chain variants and MRA-IgG4 light chain variants shown in Tables 39 and 40 were expressed by transient expression using FreeStyle293 cells (Invitrogen) or Expi293 cells (Life technologies) by a method known to the person skilled in the art, and purified with Protein A by a method known to the person skilled in the art.

[0326]

[Table 39]

MRA-IgG4 heavy chain variant name	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:	Light chain variable region SEQ ID NO:	Light chain constant region SEQ ID NO:
MRAH.Q5C-IgG4	322	311	19	20
MRAH.E6C-IgG4	323	311	19	20
MRAH.S7C-IgG4	324	311	19	20
MRAH.G8C-IgG4	325	311	19	20
MRAH.P9C-IgG4	326	311	19	20
MRAH.G10C-IgG4	327	311	19	20
MRAH.L11C-IgG4	328	311	19	20
MRAH.V12C-IgG4	329	311	19	20
MRAH.R13C-IgG4	330	311	19	20
MRAH.P14C-IgG4	331	311	19	20
MRAH.S15C-IgG4	332	311	19	20
MRAH.Q16C-IgG4	333	311	19	20
MRAH.T17C-IgG4	334	311	19	20
MRAH.L18C-IgG4	335	311	19	20
MRAH.S19C-IgG4	336	311	19	20
MRAH.L20C-IgG4	337	311	19	20
MRAH.T21C-IgG4	338	311	19	20
MRAH.T23C-IgG4	339	311	19	20
MRAH.S25C-IgG4	340	311	19	20
MRAH.G26C-IgG4	341	311	19	20
MRAH.S28C-IgG4	342	311	19	20
MRAH.T30C-IgG4	343	311	19	20
MRAH.S31C-IgG4	344	311	19	20
MRAH.W35C-IgG4	345	311	19	20
MRAH.S35aC-IgG4	346	311	19	20
MRAH.Y50C-IgG4	347	311	19	20
MRAH.I51C-IgG4	348	311	19	20
MRAH.S52C-IgG4	349	311	19	20

MRAH.S62C-IgG4	350	311	19	20
MRAH.L63C-IgG4	351	311	19	20
MRAH.K64C-IgG4	352	311	19	20
MRAH.S65C-IgG4	353	311	19	20
MRAH.R66C-IgG4	354	311	19	20
MRAH.V67C-IgG4	355	311	19	20
MRAH.T68C-IgG4	356	311	19	20
MRAH.L70C-IgG4	357	311	19	20
MRAH.D72C-IgG4	358	311	19	20
MRAH.T73C-IgG4	359	311	19	20
MRAH.S74C-IgG4	360	311	19	20
MRAH.K75C-IgG4	361	311	19	20
MRAH.N76C-IgG4	362	311	19	20
MRAH.Q77C-IgG4	363	311	19	20
MRAH.S79C-IgG4	364	311	19	20
MRAH.L80C-IgG4	365	311	19	20
MRAH.R81C-IgG4	366	311	19	20
MRAH.L82C-IgG4	367	311	19	20
MRAH.S82aC-IgG4	368	311	19	20
MRAH.S82bC-IgG4	369	311	19	20
MRAH.V82cC-IgG4	370	311	19	20
MRAH.D101C-IgG4	371	311	19	20
MRAH.Y102C-IgG4	372	311	19	20
MRAH.S112C-IgG4	373	311	19	20
MRAH.S113C-IgG4	374	311	19	20
G4T1.A118C-IgG4	17	611	19	20
G4T1.S119C-IgG4	17	612	19	20
G4T1.T120C-IgG4	17	613	19	20
G4T1.K121C-IgG4	17	614	19	20
G4T1.G122C-IgG4	17	615	19	20
G4T1.P123C-IgG4	17	616	19	20
G4T1.S124C-IgG4	17	617	19	20
G4T1.V125C-IgG4	17	618	19	20

G4T1.F126C-IgG4	17	619	19	20
G4T1.P127C-IgG4	17	620	19	20
G4T1.S132C-IgG4	17	621	19	20
G4T1.R133C-IgG4	17	622	19	20
G4T1.S134C-IgG4	17	623	19	20
G4T1.T135C-IgG4	17	624	19	20
G4T1.S136C-IgG4	17	625	19	20
G4T1.E137C-IgG4	17	626	19	20
G4T1.S138C-IgG4	17	627	19	20
G4T1.T139C-IgG4	17	628	19	20
G4T1.A140C-IgG4	17	629	19	20
G4T1.A141C-IgG4	17	630	19	20
G4T1.D148C-IgG4	17	631	19	20
G4T1.Y149C-IgG4	17	632	19	20
G4T1.F150C-IgG4	17	633	19	20
G4T1.P151C-IgG4	17	634	19	20
G4T1.E152C-IgG4	17	635	19	20
G4T1.P153C-IgG4	17	636	19	20
G4T1.V154C-IgG4	17	637	19	20
G4T1.T155C-IgG4	17	638	19	20
G4T1.V156C-IgG4	17	639	19	20
G4T1.S157C-IgG4	17	640	19	20
G4T1.W158C-IgG4	17	641	19	20
G4T1.N159C-IgG4	17	642	19	20
G4T1.S160C-IgG4	17	643	19	20
G4T1.G161C-IgG4	17	644	19	20
G4T1.A162C-IgG4	17	645	19	20
G4T1.L163C-IgG4	17	646	19	20
G4T1.T164C-IgG4	17	647	19	20
G4T1.S165C-IgG4	17	648	19	20
G4T1.G166C-IgG4	17	649	19	20
G4T1.V167C-IgG4	17	650	19	20
G4T1.V173C-IgG4	17	651	19	20

G4T1.L174C-IgG4	17	652	19	20
G4T1.Q175C-IgG4	17	653	19	20
G4T1.S176C-IgG4	17	654	19	20
G4T1.S177C-IgG4	17	655	19	20
G4T1.G178C-IgG4	17	656	19	20
G4T1.L179C-IgG4	17	657	19	20
G4T1.Y180C-IgG4	17	658	19	20
G4T1.V186C-IgG4	17	659	19	20
G4T1.T187C-IgG4	17	660	19	20
G4T1.V188C-IgG4	17	661	19	20
G4T1.P189C-IgG4	17	662	19	20
G4T1.S190C-IgG4	17	663	19	20
G4T1.S191C-IgG4	17	664	19	20
G4T1.S192C-IgG4	17	665	19	20
G4T1.L193C-IgG4	17	666	19	20
G4T1.G194C-IgG4	17	667	19	20
G4T1.T195C-IgG4	17	668	19	20
G4T1.Q196C-IgG4	17	669	19	20
G4T1.T197C-IgG4	17	670	19	20
G4T1.Y198C-IgG4	17	671	19	20
G4T1.T199C-IgG4	17	672	19	20
G4T1.N201C-IgG4	17	673	19	20
G4T1.V202C-IgG4	17	674	19	20
G4T1.D203C-IgG4	17	675	19	20
G4T1.H204C-IgG4	17	676	19	20
G4T1.K205C-IgG4	17	677	19	20
G4T1.P206C-IgG4	17	678	19	20
G4T1.S207C-IgG4	17	679	19	20
G4T1.N208C-IgG4	17	680	19	20
G4T1.T209C-IgG4	17	681	19	20
G4T1.K210C-IgG4	17	682	19	20
G4T1.V211C-IgG4	17	683	19	20
G4T1.D212C-IgG4	17	684	19	20

G4T1.K213C-IgG4	17	685	19	20
G4T1.R214C-IgG4	17	686	19	20
G4T1.V215C-IgG4	17	687	19	20
G4T1.E216C-IgG4	17	688	19	20
G4T1.S217C-IgG4	17	689	19	20
G4T1.K218C-IgG4	17	690	19	20

[0327]

[Table 40]

MRA-IgG4 light chain variant name	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:	Light chain variable region SEQ ID NO:	Light chain constant region SEQ ID NO:
MRAL.T5C-IgG4	17	311	457	20
MRAL.Q6C-IgG4	17	311	458	20
MRAL.S7C-IgG4	17	311	459	20
MRAL.P8C-IgG4	17	311	460	20
MRAL.S9C-IgG4	17	311	461	20
MRAL.S10C-IgG4	17	311	462	20
MRAL.L11C-IgG4	17	311	463	20
MRAL.S12C-IgG4	17	311	464	20
MRAL.A13C-IgG4	17	311	465	20
MRAL.S14C-IgG4	17	311	466	20
MRAL.V15C-IgG4	17	311	467	20
MRAL.G16C-IgG4	17	311	468	20
MRAL.D17C-IgG4	17	311	469	20
MRAL.R18C-IgG4	17	311	470	20
MRAL.V19C-IgG4	17	311	471	20
MRAL.T20C-IgG4	17	311	472	20
MRAL.I21C-IgG4	17	311	473	20
MRAL.T22C-IgG4	17	311	474	20
MRAL.A25C-IgG4	17	311	475	20
MRAL.S26C-IgG4	17	311	476	20
MRAL.Q27C-IgG4	17	311	477	20
MRAL.Y32C-IgG4	17	311	478	20
MRAL.L33C-IgG4	17	311	479	20
MRAL.N34C-IgG4	17	311	480	20
MRAL.Y50C-IgG4	17	311	481	20
MRAL.T51C-IgG4	17	311	482	20
MRAL.H55C-IgG4	17	311	483	20
MRAL.S56C-IgG4	17	311	484	20

MRAL.G57C-IgG4	17	311	485	20
MRAL.V58C-IgG4	17	311	486	20
MRAL.P59C-IgG4	17	311	487	20
MRAL.S60C-IgG4	17	311	488	20
MRAL.R61C-IgG4	17	311	489	20
MRAL.F62C-IgG4	17	311	490	20
MRAL.S63C-IgG4	17	311	491	20
MRAL.S65C-IgG4	17	311	492	20
MRAL.S67C-IgG4	17	311	493	20
MRAL.G68C-IgG4	17	311	494	20
MRAL.T69C-IgG4	17	311	495	20
MRAL.D70C-IgG4	17	311	496	20
MRAL.T72C-IgG4	17	311	497	20
MRAL.F73C-IgG4	17	311	498	20
MRAL.T74C-IgG4	17	311	499	20
MRAL.I75C-IgG4	17	311	500	20
MRAL.S76C-IgG4	17	311	501	20
MRAL.S77C-IgG4	17	311	502	20
MRAL.L78C-IgG4	17	311	503	20
MRAL.Q79C-IgG4	17	311	504	20
MRAL.Y96C-IgG4	17	311	505	20
MRAL.T97C-IgG4	17	311	506	20
MRAL.F98C-IgG4	17	311	507	20
MRAL.G99C-IgG4	17	311	508	20
MRAL.Q100C-IgG4	17	311	509	20
MRAL.G101C-IgG4	17	311	510	20
MRAL.T102C-IgG4	17	311	511	20
MRAL.K103C-IgG4	17	311	512	20
MRAL.V104C-IgG4	17	311	513	20
MRAL.E105C-IgG4	17	311	514	20
MRAL.I106C-IgG4	17	311	515	20
MRAL.K107C-IgG4	17	311	516	20
k0.R108C-IgG4	17	311	19	517

k0.T109C-IgG4	17	311	19	518
k0.V110C-IgG4	17	311	19	519
k0.A111C-IgG4	17	311	19	520
k0.A112C-IgG4	17	311	19	521
k0.P113C-IgG4	17	311	19	522
k0.S114C-IgG4	17	311	19	523
k0.V115C-IgG4	17	311	19	524
k0.F116C-IgG4	17	311	19	525
k0.P120C-IgG4	17	311	19	526
k0.S121C-IgG4	17	311	19	527
k0.D122C-IgG4	17	311	19	528
k0.E123C-IgG4	17	311	19	529
k0.Q124C-IgG4	17	311	19	530
k0.L125C-IgG4	17	311	19	531
k0.K126C-IgG4	17	311	19	532
k0.S127C-IgG4	17	311	19	533
k0.G128C-IgG4	17	311	19	534
k0.T129C-IgG4	17	311	19	535
k0.A130C-IgG4	17	311	19	536
k0.S131C-IgG4	17	311	19	537
k0.L136C-IgG4	17	311	19	538
k0.N137C-IgG4	17	311	19	539
k0.N138C-IgG4	17	311	19	540
k0.F139C-IgG4	17	311	19	541
k0.Y140C-IgG4	17	311	19	542
k0.P141C-IgG4	17	311	19	543
k0.R142C-IgG4	17	311	19	544
k0.E143C-IgG4	17	311	19	545
k0.A144C-IgG4	17	311	19	546
k0.K145C-IgG4	17	311	19	547
k0.V146C-IgG4	17	311	19	548
k0.Q147C-IgG4	17	311	19	549
k0.W148C-IgG4	17	311	19	550

k0.K149C-IgG4	17	311	19	551
k0.V150C-IgG4	17	311	19	552
k0.D151C-IgG4	17	311	19	553
k0.N152C-IgG4	17	311	19	554
k0.A153C-IgG4	17	311	19	555
k0.L154C-IgG4	17	311	19	556
k0.Q155C-IgG4	17	311	19	557
k0.S156C-IgG4	17	311	19	558
k0.G157C-IgG4	17	311	19	559
k0.N158C-IgG4	17	311	19	560
k0.S159C-IgG4	17	311	19	561
k0.Q160C-IgG4	17	311	19	562
k0.E161C-IgG4	17	311	19	563
k0.S162C-IgG4	17	311	19	564
k0.V163C-IgG4	17	311	19	565
k0.T164C-IgG4	17	311	19	566
k0.E165C-IgG4	17	311	19	567
k0.Q166C-IgG4	17	311	19	568
k0.D167C-IgG4	17	311	19	569
k0.S168C-IgG4	17	311	19	570
k0.K169C-IgG4	17	311	19	571
k0.D170C-IgG4	17	311	19	572
k0.S171C-IgG4	17	311	19	573
k0.T172C-IgG4	17	311	19	574
k0.Y173C-IgG4	17	311	19	575
k0.S174C-IgG4	17	311	19	576
k0.L175C-IgG4	17	311	19	577
k0.T180C-IgG4	17	311	19	578
k0.L181C-IgG4	17	311	19	579
k0.S182C-IgG4	17	311	19	580
k0.K183C-IgG4	17	311	19	581
k0.A184C-IgG4	17	311	19	582
k0.D185C-IgG4	17	311	19	583

k0.Y186C-IgG4	17	311	19	584
k0.E187C-IgG4	17	311	19	585
k0.K188C-IgG4	17	311	19	586
k0.H189C-IgG4	17	311	19	587
k0.K190C-IgG4	17	311	19	588
k0.V191C-IgG4	17	311	19	589
k0.Y192C-IgG4	17	311	19	590
k0.A193C-IgG4	17	311	19	591
k0.E195C-IgG4	17	311	19	592
k0.V196C-IgG4	17	311	19	593
k0.T197C-IgG4	17	311	19	594
k0.H198C-IgG4	17	311	19	595
k0.Q199C-IgG4	17	311	19	596
k0.G200C-IgG4	17	311	19	597
k0.L201C-IgG4	17	311	19	598
k0.S202C-IgG4	17	311	19	599
k0.S203C-IgG4	17	311	19	600
k0.P204C-IgG4	17	311	19	601
k0.V205C-IgG4	17	311	19	602
k0.T206C-IgG4	17	311	19	603
k0.K207C-IgG4	17	311	19	604
k0.S208C-IgG4	17	311	19	605
k0.F209C-IgG4	17	311	19	606
k0.N210C-IgG4	17	311	19	607
k0.R211C-IgG4	17	311	19	608
k0.G212C-IgG4	17	311	19	609
k0.E213C-IgG4	17	311	19	610

[0328] Reference Example 9-2 Assessment of electrophoretic mobility in polyacrylamide gel of antibodies having cysteine substitution at various positions of IgG4

Similarly to Reference Example 8-2, non-reducing SDS-PAGE was carried out with the MRA-IgG4 variants produced in Reference Example 9-1, the gel image was captured, and bands were quantified.

From the obtained gel image, the variants were classified into 7 groups according to the band pattern of each of the MRA-IgG4 variants: Single (one band at a molecular weight region similar to that of MRA-IgG4), Double (two bands at a molecular weight region similar to that of MRA-IgG4), Triple (three bands at a molecular weight region similar to that of MRA-IgG4), Several (four or more bands at a molecular weight region similar to that of MRA-IgG4), LMW (band(s) at a molecular weight region

lower than that of MRA-IgG4), HMW (band(s) at a molecular weight region higher than that of MRA-IgG4), and Faint (band(s) blurry and difficult to determine). Regarding the MRA-IgG4 variants classified as "Double", one of the two bands showed the same electrophoretic mobility as MRA-IgG4 while the other band showed slightly faster or slower mobility. Thus, for the MRA-IgG4 variants classified as "Double", the percentage of the bands showing different mobility to MRA-IgG4 (percentage of new band (%)) was also calculated. Grouping of the band patterns for MRA-IgG4 heavy chain variants and MRA-IgG4 light chain variants, and the calculation results of the band percentage are respectively shown in Tables 41 and 42. From Tables 41 and 42, variants classified into the Double and Triple groups are shown in Table 43. In these variants, it is highly likely that cysteine substitution caused structural changes such as crosslinkage of Fabs, which resulted in the change in electrophoretic mobility. It is noted that while Table 26 indicates "no data" for MRAL.K107C-IgG4, position 107 (Kabat numbering), which is the position of cysteine substitution in this variant, is a position where the residue structurally exposed to the surface is present in the hinge region. Thus, in this variant also, it is highly likely that cysteine substitution causes structural changes such as crosslinkage of Fabs, and results in the change in electrophoretic mobility.

[0329]

[Table 41]

MRA-IgG4 heavy chain variant name	Group	Percentage of new band (%)
MRAH.Q5C-IgG4	Single	-
MRAH.E6C-IgG4	Double	5.8
MRAH.S7C-IgG4	Single	-
MRAH.G8C-IgG4	Single	-
MRAH.P9C-IgG4	Single	-
MRAH.G10C-IgG4	Single	-
MRAH.L11C-IgG4	Single	-
MRAH.V12C-IgG4	Faint	-
MRAH.R13C-IgG4	Single	-
MRAH.P14C-IgG4	Single	-
MRAH.S15C-IgG4	Single	-
MRAH.Q16C-IgG4	Single	-
MRAH.T17C-IgG4	Single	-
MRAH.L18C-IgG4	LMW	-
MRAH.S19C-IgG4	Single	-
MRAH.L20C-IgG4	LMW	-
MRAH.T21C-IgG4	Single	-
MRAH.T23C-IgG4	Single	-
MRAH.S25C-IgG4	Double	62.1
MRAH.G26C-IgG4	Double	9.4
MRAH.S28C-IgG4	Single	-
MRAH.T30C-IgG4	Single	-
MRAH.S31C-IgG4	Single	-
MRAH.W35C-IgG4	LMW	-
MRAH.S35aC-IgG4	LMW	-
MRAH.Y50C-IgG4	Single	-
MRAH.I51C-IgG4	LMW	-
MRAH.S52C-IgG4	Single	-
MRAH.S62C-IgG4	Single	-
MRAH.L63C-IgG4	Single	-

MRAH.K64C-IgG4	Single	-
MRAH.S65C-IgG4	Single	-
MRAH.R66C-IgG4	Single	-
MRAH.V67C-IgG4	LMW	-
MRAH.T68C-IgG4	Single	-
MRAH.L70C-IgG4	Single	-
MRAH.D72C-IgG4	Single	-
MRAH.T73C-IgG4	Single	-
MRAH.S74C-IgG4	Double	5.3
MRAH.K75C-IgG4	Single	-
MRAH.N76C-IgG4	Single	-
MRAH.Q77C-IgG4	Single	-
MRAH.S79C-IgG4	Single	-
MRAH.L80C-IgG4	LMW	-
MRAH.R81C-IgG4	Single	-
MRAH.L82C-IgG4	LMW	-
MRAH.S82aC-IgG4	Single	-
MRAH.S82bC-IgG4	Single	-
MRAH.V82cC-IgG4	LMW	-
MRAH.D101C-IgG4	Single	-
MRAH.Y102C-IgG4	Single	-
MRAH.S112C-IgG4	Single	-
MRAH.S113C-IgG4	Single	-
G4T1.A118C-IgG4	Single	-
G4T1.S119C-IgG4	Double	11
G4T1.T120C-IgG4	Single	-
G4T1.K121C-IgG4	Single	-
G4T1.G122C-IgG4	Single	-
G4T1.P123C-IgG4	LMW	-
G4T1.S124C-IgG4	Single	-
G4T1.V125C-IgG4	LMW	-
G4T1.F126C-IgG4	LMW	-
G4T1.P127C-IgG4	LMW	-

G4T1.S132C-IgG4	Triple	-
G4T1.R133C-IgG4	Double	82.9
G4T1.S134C-IgG4	Double	80.4
G4T1.T135C-IgG4	Double	88.6
G4T1.S136C-IgG4	Double	82.4
G4T1.E137C-IgG4	Double	44.7
G4T1.S138C-IgG4	Double	52.6
G4T1.T139C-IgG4	Single	-
G4T1.A140C-IgG4	Triple	-
G4T1.A141C-IgG4	Single	-
G4T1.D148C-IgG4	Single	-
G4T1.Y149C-IgG4	Faint	-
G4T1.F150C-IgG4	Single	-
G4T1.P151C-IgG4	LMW	-
G4T1.E152C-IgG4	Single	-
G4T1.P153C-IgG4	Single	-
G4T1.V154C-IgG4	LMW	-
G4T1.T155C-IgG4	Single	-
G4T1.V156C-IgG4	LMW	-
G4T1.S157C-IgG4	Single	-
G4T1.W158C-IgG4	LMW	-
G4T1.N159C-IgG4	Double	19.9
G4T1.S160C-IgG4	Double	29.5
G4T1.G161C-IgG4	Double	21.4
G4T1.A162C-IgG4	Double	35.6
G4T1.L163C-IgG4	Double	21.1
G4T1.T164C-IgG4	Double	12.8
G4T1.S165C-IgG4	Double	17
G4T1.G166C-IgG4	Double	13
G4T1.V167C-IgG4	Double	20.4
G4T1.V173C-IgG4	Double	15.6
G4T1.L174C-IgG4	Double	18.6
G4T1.Q175C-IgG4	Single	-

G4T1.S176C-IgG4	Double	20.3
G4T1.S177C-IgG4	Single	-
G4T1.G178C-IgG4	Double	22.5
G4T1.L179C-IgG4	Double	26.1
G4T1.Y180C-IgG4	LMW	-
G4T1.V186C-IgG4	LMW	-
G4T1.T187C-IgG4	Double	23.3
G4T1.V188C-IgG4	Double	25.5
G4T1.P189C-IgG4	Double	30.4
G4T1.S190C-IgG4	Double	54.7
G4T1.S191C-IgG4	Double	78.3
G4T1.S192C-IgG4	Double	46.9
G4T1.L193C-IgG4	Double	89.5
G4T1.G194C-IgG4	Double	89.2
G4T1.T195C-IgG4	Double	90.3
G4T1.Q196C-IgG4	Double	63.4
G4T1.T197C-IgG4	Double	79.8
G4T1.Y198C-IgG4	LMW	-
G4T1.T199C-IgG4	LMW	-
G4T1.N201C-IgG4	LMW	-
G4T1.V202C-IgG4	LMW	-
G4T1.D203C-IgG4	LMW	-
G4T1.H204C-IgG4	LMW	-
G4T1.K205C-IgG4	LMW	-
G4T1.P206C-IgG4	LMW	-
G4T1.S207C-IgG4	LMW	-
G4T1.N208C-IgG4	LMW	-
G4T1.T209C-IgG4	LMW	-
G4T1.K210C-IgG4	Single	-
G4T1.V211C-IgG4	Single	-
G4T1.D212C-IgG4	Single	-
G4T1.K213C-IgG4	Triple	-
G4T1.R214C-IgG4	Single	-
G4T1.V215C-IgG4	Double	57.3
G4T1.E216C-IgG4	Single	-
G4T1.S217C-IgG4	Single	-
G4T1.K218C-IgG4	Single	-

[0330]

[Table 42]

MRA-IgG4 light chain variant name	Group	Percentage of new band (%)
MRAL.T5C-IgG4	HMW	-
MRAL.Q6C-IgG4	Faint	-
MRAL.S7C-IgG4	Single	-
MRAL.P8C-IgG4	no data	-
MRAL.S9C-IgG4	Single	-
MRAL.S10C-IgG4	Single	-
MRAL.L11C-IgG4	Single	-
MRAL.S12C-IgG4	Single	-
MRAL.A13C-IgG4	Single	-
MRAL.S14C-IgG4	Single	-
MRAL.V15C-IgG4	Single	-
MRAL.G16C-IgG4	Single	-
MRAL.D17C-IgG4	Single	-
MRAL.R18C-IgG4	Single	-
MRAL.V19C-IgG4	Double	29.2
MRAL.T20C-IgG4	Single	-
MRAL.I21C-IgG4	Faint	-
MRAL.T22C-IgG4	Single	-
MRAL.A25C-IgG4	Faint	-
MRAL.S26C-IgG4	Single	-
MRAL.Q27C-IgG4	Single	-
MRAL.Y32C-IgG4	Single	-
MRAL.L33C-IgG4	Faint	-
MRAL.N34C-IgG4	Faint	-
MRAL.Y50C-IgG4	Single	-
MRAL.T51C-IgG4	Single	-
MRAL.H55C-IgG4	Single	-
MRAL.S56C-IgG4	Double	12.2
MRAL.G57C-IgG4	Double	13.5
MRAL.V58C-IgG4	Double	12.3

MRAL.P59C-IgG4	Double	3.4
MRAL.S60C-IgG4	Double	17.9
MRAL.R61C-IgG4	Single	-
MRAL.F62C-IgG4	Double	39.1
MRAL.S63C-IgG4	Single	-
MRAL.S65C-IgG4	Single	-
MRAL.S67C-IgG4	Single	-
MRAL.G68C-IgG4	Single	-
MRAL.T69C-IgG4	Single	-
MRAL.D70C-IgG4	Single	-
MRAL.T72C-IgG4	Single	-
MRAL.F73C-IgG4	Double	36.9
MRAL.T74C-IgG4	Single	-
MRAL.I75C-IgG4	no data	-
MRAL.S76C-IgG4	Single	-
MRAL.S77C-IgG4	Double	51.2
MRAL.L78C-IgG4	Faint	-
MRAL.Q79C-IgG4	Single	-
MRAL.Y96C-IgG4	Faint	-
MRAL.T97C-IgG4	Single	-
MRAL.F98C-IgG4	Faint	-
MRAL.G99C-IgG4	Double	26.7
MRAL.Q100C-IgG4	Single	-
MRAL.G101C-IgG4	Single	-
MRAL.T102C-IgG4	Faint	-
MRAL.K103C-IgG4	Single	-
MRAL.V104C-IgG4	Faint	-
MRAL.E105C-IgG4	Single	-
MRAL.I106C-IgG4	Faint	-
MRAL.K107C-IgG4	no data	-
k0.R108C-IgG4	Single	-
k0.T109C-IgG4	Double	14.5
k0.V110C-IgG4	Double	13.2

k0.A111C-IgG4	Single	-
k0.A112C-IgG4	Double	12
k0.P113C-IgG4	Single	-
k0.S114C-IgG4	Single	-
k0.V115C-IgG4	Faint	-
k0.F116C-IgG4	Triple	-
k0.P120C-IgG4	Faint	-
k0.S121C-IgG4	Single	-
k0.D122C-IgG4	LMW	-
k0.E123C-IgG4	Single	-
k0.Q124C-IgG4	Faint	-
k0.L125C-IgG4	Single	-
k0.K126C-IgG4	Double	86.3
k0.S127C-IgG4	Single	-
k0.G128C-IgG4	Single	-
k0.T129C-IgG4	Single	-
k0.A130C-IgG4	Faint	-
k0.S131C-IgG4	LMW	-
k0.L136C-IgG4	LMW	-
k0.N137C-IgG4	Triple	-
k0.N138C-IgG4	Single	-
k0.F139C-IgG4	LMW	-
k0.Y140C-IgG4	LMW	-
k0.P141C-IgG4	Single	-
k0.R142C-IgG4	Single	-
k0.E143C-IgG4	Single	-
k0.A144C-IgG4	LMW	-
k0.K145C-IgG4	Single	-
k0.V146C-IgG4	LMW	-
k0.Q147C-IgG4	Single	-
k0.W148C-IgG4	LMW	-
k0.K149C-IgG4	Single	-
k0.V150C-IgG4	LMW	-

k0.D151C-IgG4	Double	21.9
k0.N152C-IgG4	Double	68.7
k0.A153C-IgG4	Single	-
k0.L154C-IgG4	Single	-
k0.Q155C-IgG4	Single	-
k0.S156C-IgG4	HMW	-
k0.G157C-IgG4	Single	-
k0.N158C-IgG4	Single	-
k0.S159C-IgG4	Single	-
k0.Q160C-IgG4	Single	-
k0.E161C-IgG4	Single	-
k0.S162C-IgG4	Single	-
k0.V163C-IgG4	Single	-
k0.T164C-IgG4	Single	-
k0.E165C-IgG4	Single	-
k0.Q166C-IgG4	Single	-
k0.D167C-IgG4	Single	-
k0.S168C-IgG4	Single	-
k0.K169C-IgG4	Single	-
k0.D170C-IgG4	Single	-
k0.S171C-IgG4	Single	-
k0.T172C-IgG4	Faint	-
k0.Y173C-IgG4	Faint	-
k0.S174C-IgG4	Faint	-
k0.L175C-IgG4	Faint	-
k0.T180C-IgG4	Single	-
k0.L181C-IgG4	Faint	-
k0.S182C-IgG4	Single	-
k0.K183C-IgG4	Single	-
k0.A184C-IgG4	Double	11.8
k0.D185C-IgG4	Single	-
k0.Y186C-IgG4	Double	31.7
k0.E187C-IgG4	LMW	-

k0.K188C-IgG4	Single	-
k0.H189C-IgG4	Faint	-
k0.K190C-IgG4	LMW	-
k0.V191C-IgG4	LMW	-
k0.Y192C-IgG4	Faint	-
k0.A193C-IgG4	Single	-
k0.E195C-IgG4	Single	-
k0.V196C-IgG4	Faint	-
k0.T197C-IgG4	Single	-
k0.H198C-IgG4	Faint	-
k0.Q199C-IgG4	Single	-
k0.G200C-IgG4	Double	21.7
k0.L201C-IgG4	Double	3.7
k0.S202C-IgG4	Double	61.5
k0.S203C-IgG4	Double	39
k0.P204C-IgG4	Single	-
k0.V205C-IgG4	Single	-
k0.T206C-IgG4	Single	-
k0.K207C-IgG4	Single	-
k0.S208C-IgG4	Single	-
k0.F209C-IgG4	Double	82.2
k0.N210C-IgG4	LMW	-
k0.R211C-IgG4	Double	12.1
k0.G212C-IgG4	Double	25.6
k0.E213C-IgG4	Double	90.9

[0331]

[Table 43]

MRA-IgG4 variant name	Group	Percentage of new band (%)
MRAH.E6C-IgG4	Double	5.8
MRAH.S25C-IgG4	Double	62.1
MRAH.G26C-IgG4	Double	9.4
MRAH.S74C-IgG4	Double	5.3
G4T1.S119C-IgG4	Double	11
G4T1.S132C-IgG4	Triple	-
G4T1.R133C-IgG4	Double	82.9
G4T1.S134C-IgG4	Double	80.4
G4T1.T135C-IgG4	Double	88.6
G4T1.S136C-IgG4	Double	82.4
G4T1.E137C-IgG4	Double	44.7
G4T1.S138C-IgG4	Double	52.6
G4T1.A140C-IgG4	Triple	-
G4T1.N159C-IgG4	Double	19.9
G4T1.S160C-IgG4	Double	29.5
G4T1.G161C-IgG4	Double	21.4
G4T1.A162C-IgG4	Double	35.6
G4T1.L163C-IgG4	Double	21.1
G4T1.T164C-IgG4	Double	12.8
G4T1.S165C-IgG4	Double	17
G4T1.G166C-IgG4	Double	13
G4T1.V167C-IgG4	Double	20.4
G4T1.V173C-IgG4	Double	15.6
G4T1.L174C-IgG4	Double	18.6
G4T1.S176C-IgG4	Double	20.3
G4T1.G178C-IgG4	Double	22.5
G4T1.L179C-IgG4	Double	26.1
G4T1.T187C-IgG4	Double	23.3
G4T1.V188C-IgG4	Double	25.5
G4T1.P189C-IgG4	Double	30.4

G4T1.S190C-IgG4	Double	54.7
G4T1.S191C-IgG4	Double	78.3
G4T1.S192C-IgG4	Double	46.9
G4T1.L193C-IgG4	Double	89.5
G4T1.G194C-IgG4	Double	89.2
G4T1.T195C-IgG4	Double	90.3
G4T1.Q196C-IgG4	Double	63.4
G4T1.T197C-IgG4	Double	79.8
G4T1.K213C-IgG4	Triple	-
G4T1.V215C-IgG4	Double	57.3
MRAL.V19C-IgG4	Double	29.2
MRAL.S56C-IgG4	Double	12.2
MRAL.G57C-IgG4	Double	13.5
MRAL.V58C-IgG4	Double	12.3
MRAL.P59C-IgG4	Double	3.4
MRAL.S60C-IgG4	Double	17.9
MRAL.F62C-IgG4	Double	39.1
MRAL.F73C-IgG4	Double	36.9
MRAL.S77C-IgG4	Double	51.2
MRAL.G99C-IgG4	Double	26.7
k0.T109C-IgG4	Double	14.5
k0.V110C-IgG4	Double	13.2
k0.A112C-IgG4	Double	12
k0.F116C-IgG4	Triple	-
k0.K126C-IgG4	Double	86.3
k0.N137C-IgG4	Triple	-
k0.D151C-IgG4	Double	21.9
k0.N152C-IgG4	Double	68.7
k0.A184C-IgG4	Double	11.8
k0.Y186C-IgG4	Double	31.7
k0.G200C-IgG4	Double	21.7
k0.L201C-IgG4	Double	3.7
k0.S202C-IgG4	Double	61.5
k0.S203C-IgG4	Double	39
k0.F209C-IgG4	Double	82.2
k0.R211C-IgG4	Double	12.1
k0.G212C-IgG4	Double	25.6
k0.E213C-IgG4	Double	90.9

[0332] [Reference Example 10] Assessment of antibodies having cysteine substitution at various positions of IgG2

Reference Example 10-1 Production of antibodies having cysteine substitution at various positions of IgG2

The heavy chain and light chain of an anti-human IL6R neutralizing antibody, MRA-IgG2 (heavy chain: MRAH-G2d (SEQ ID NO: 312), light chain: MRAL-k0 (SEQ ID NO: 16)), were subjected to a study in which an arbitrary amino acid residue structurally exposed to the surface was substituted with cysteine.

Amino acid residues within the MRA-IgG2 heavy chain variable region (MRAH, SEQ ID NO: 17) were substituted with cysteine to produce variants of the MRA-IgG2 heavy chain variable region shown in Table 44. These variants of the MRA-IgG2 heavy chain variable region were each linked with the MRA-IgG2 heavy chain constant region (G2d, SEQ ID NO: 313) to produce MRA-IgG2 heavy chain variants, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art. In addition, amino acid residues within the MRA-IgG2 heavy chain constant region (G2d, SEQ ID NO: 313) were substituted with cysteine to produce variants of the MRA-IgG2 heavy chain constant region shown in Table 45. These variants of the MRA-IgG2 heavy chain constant region were each linked with the MRA-IgG2 heavy chain variable region (MRAH, SEQ ID NO: 17) to produce MRA-IgG2 heavy chain variants, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art.

[0333]

[Table 44]

Variant of MRA-IgG2 heavy chain variable region	Position of cysteine substitution (Kabat numbering)	SEQ ID NO:
MRAH.Q5C	5	322
MRAH.E6C	6	323
MRAH.S7C	7	324
MRAH.G8C	8	325
MRAH.P9C	9	326
MRAH.G10C	10	327
MRAH.L11C	11	328
MRAH.V12C	12	329
MRAH.R13C	13	330
MRAH.P14C	14	331
MRAH.S15C	15	332
MRAH.Q16C	16	333
MRAH.T17C	17	334
MRAH.L18C	18	335
MRAH.S19C	19	336
MRAH.L20C	20	337
MRAH.T21C	21	338
MRAH.T23C	23	339
MRAH.S25C	25	340
MRAH.G26C	26	341
MRAH.S28C	28	342
MRAH.T30C	30	343
MRAH.S31C	31	344
MRAH.W35C	35	345
MRAH.S35aC	35a	346
MRAH.Y50C	50	347
MRAH.I51C	51	348
MRAH.S52C	52	349
MRAH.S62C	62	350

MRAH.L63C	63	351
MRAH.K64C	64	352
MRAH.S65C	65	353
MRAH.R66C	66	354
MRAH.V67C	67	355
MRAH.T68C	68	356
MRAH.L70C	70	357
MRAH.D72C	72	358
MRAH.T73C	73	359
MRAH.S74C	74	360
MRAH.K75C	75	361
MRAH.N76C	76	362
MRAH.Q77C	77	363
MRAH.S79C	79	364
MRAH.L80C	80	365
MRAH.R81C	81	366
MRAH.L82C	82	367
MRAH.S82aC	82a	368
MRAH.S82bC	82b	369
MRAH.V82cC	82c	370
MRAH.D101C	101	371
MRAH.Y102C	102	372
MRAH.S112C	112	373
MRAH.S113C	113	374

[0334]

[Table 45]

Variant of MRA-IgG2 heavy chain constant region	Position of cysteine substitution (EU numbering)	SEQ ID NO:
G2d.A118C	118	691
G2d.S119C	119	692
G2d.T120C	120	693
G2d.K121C	121	694
G2d.G122C	122	695
G2d.P123C	123	696
G2d.S124C	124	697
G2d.V125C	125	698
G2d.F126C	126	699
G2d.P127C	127	700
G2d.S132C	132	701
G2d.R133C	133	702
G2d.S134C	134	703
G2d.T135C	135	704
G2d.S136C	136	705
G2d.E137C	137	706
G2d.S138C	138	707
G2d.T139C	139	708
G2d.A140C	140	709
G2d.A141C	141	710
G2d.D148C	148	711
G2d.Y149C	149	712
G2d.F150C	150	713
G2d.P151C	151	714
G2d.E152C	152	715
G2d.P153C	153	716
G2d.V154C	154	717
G2d.T155C	155	718
G2d.V156C	156	719

G2d.S157C	157	720
G2d.W158C	158	721
G2d.N159C	159	722
G2d.S160C	160	723
G2d.G161C	161	724
G2d.A162C	162	725
G2d.L163C	163	726
G2d.T164C	164	727
G2d.S165C	165	728
G2d.G166C	166	729
G2d.V167C	167	730
G2d.V173C	173	731
G2d.L174C	174	732
G2d.Q175C	175	733
G2d.S176C	176	734
G2d.S177C	177	735
G2d.G178C	178	736
G2d.L179C	179	737
G2d.Y180C	180	738
G2d.V186C	186	739
G2d.T187C	187	740
G2d.V188C	188	741
G2d.P189C	189	742
G2d.S190C	190	743
G2d.S191C	191	744
G2d.N192C	192	745
G2d.F193C	193	746
G2d.G194C	194	747
G2d.T195C	195	748
G2d.Q196C	196	749
G2d.T197C	197	750
G2d.Y198C	198	751
G2d.T199C	199	752

G2d.N201C	201	753
G2d.V202C	202	754
G2d.D203C	203	755
G2d.H204C	204	756
G2d.K205C	205	757
G2d.P206C	206	758
G2d.S207C	207	759
G2d.N208C	208	760
G2d.T209C	209	761
G2d.K210C	210	762
G2d.V211C	211	763
G2d.D212C	212	764
G2d.K213C	213	765
G2d.T214C	214	766
G2d.V215C	215	767
G2d.E216C	216	768
G2d.R217C	217	769
G2d.K218C	218	770

[0335] The MRA-IgG2 heavy chain variants produced above were combined with the MRA-IgG2 light chain, or the MRA-IgG2 heavy chain was combined with the MRA-IgG2 light chain variants produced in Reference Example 8-1. The resultant MRA-IgG2 heavy chain variants and MRA-IgG2 light chain variants shown in Tables 46 and 47 were expressed by transient expression using FreeStyle293 cells (Invitrogen) or Expi293 cells (Life technologies) by a method known to the person skilled in the art, and purified with Protein A by a method known to the person skilled in the art.

[0336]

[Table 46]

MRA-IgG2 heavy chain variant name	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:	Light chain variable region SEQ ID NO:	Light chain constant region SEQ ID NO:
MRAH.Q5C-IgG2	322	313	19	20
MRAH.E6C-IgG2	323	313	19	20
MRAH.S7C-IgG2	324	313	19	20
MRAH.G8C-IgG2	325	313	19	20
MRAH.P9C-IgG2	326	313	19	20
MRAH.G10C-IgG2	327	313	19	20
MRAH.L11C-IgG2	328	313	19	20
MRAH.V12C-IgG2	329	313	19	20
MRAH.R13C-IgG2	330	313	19	20
MRAH.P14C-IgG2	331	313	19	20
MRAH.S15C-IgG2	332	313	19	20
MRAH.Q16C-IgG2	333	313	19	20
MRAH.T17C-IgG2	334	313	19	20
MRAH.L18C-IgG2	335	313	19	20
MRAH.S19C-IgG2	336	313	19	20
MRAH.L20C-IgG2	337	313	19	20
MRAH.T21C-IgG2	338	313	19	20
MRAH.T23C-IgG2	339	313	19	20
MRAH.S25C-IgG2	340	313	19	20
MRAH.G26C-IgG2	341	313	19	20
MRAH.S28C-IgG2	342	313	19	20
MRAH.T30C-IgG2	343	313	19	20
MRAH.S31C-IgG2	344	313	19	20
MRAH.W35C-IgG2	345	313	19	20
MRAH.S35aC-IgG2	346	313	19	20
MRAH.Y50C-IgG2	347	313	19	20
MRAH.I51C-IgG2	348	313	19	20
MRAH.S52C-IgG2	349	313	19	20

MRAH.S62C-IgG2	350	313	19	20
MRAH.L63C-IgG2	351	313	19	20
MRAH.K64C-IgG2	352	313	19	20
MRAH.S65C-IgG2	353	313	19	20
MRAH.R66C-IgG2	354	313	19	20
MRAH.V67C-IgG2	355	313	19	20
MRAH.T68C-IgG2	356	313	19	20
MRAH.L70C-IgG2	357	313	19	20
MRAH.D72C-IgG2	358	313	19	20
MRAH.T73C-IgG2	359	313	19	20
MRAH.S74C-IgG2	360	313	19	20
MRAH.K75C-IgG2	361	313	19	20
MRAH.N76C-IgG2	362	313	19	20
MRAH.Q77C-IgG2	363	313	19	20
MRAH.S79C-IgG2	364	313	19	20
MRAH.L80C-IgG2	365	313	19	20
MRAH.R81C-IgG2	366	313	19	20
MRAH.L82C-IgG2	367	313	19	20
MRAH.S82aC-IgG2	368	313	19	20
MRAH.S82bC-IgG2	369	313	19	20
MRAH.V82cC-IgG2	370	313	19	20
MRAH.D101C-IgG2	371	313	19	20
MRAH.Y102C-IgG2	372	313	19	20
MRAH.S112C-IgG2	373	313	19	20
MRAH.S113C-IgG2	374	313	19	20
G2d.A118C-IgG2	17	691	19	20
G2d.S119C-IgG2	17	692	19	20
G2d.T120C-IgG2	17	693	19	20
G2d.K121C-IgG2	17	694	19	20
G2d.G122C-IgG2	17	695	19	20
G2d.P123C-IgG2	17	696	19	20
G2d.S124C-IgG2	17	697	19	20
G2d.V125C-IgG2	17	698	19	20

G2d.F126C-IgG2	17	699	19	20
G2d.P127C-IgG2	17	700	19	20
G2d.S132C-IgG2	17	701	19	20
G2d.R133C-IgG2	17	702	19	20
G2d.S134C-IgG2	17	703	19	20
G2d.T135C-IgG2	17	704	19	20
G2d.S136C-IgG2	17	705	19	20
G2d.E137C-IgG2	17	706	19	20
G2d.S138C-IgG2	17	707	19	20
G2d.T139C-IgG2	17	708	19	20
G2d.A140C-IgG2	17	709	19	20
G2d.A141C-IgG2	17	710	19	20
G2d.D148C-IgG2	17	711	19	20
G2d.Y149C-IgG2	17	712	19	20
G2d.F150C-IgG2	17	713	19	20
G2d.P151C-IgG2	17	714	19	20
G2d.E152C-IgG2	17	715	19	20
G2d.P153C-IgG2	17	716	19	20
G2d.V154C-IgG2	17	717	19	20
G2d.T155C-IgG2	17	718	19	20
G2d.V156C-IgG2	17	719	19	20
G2d.S157C-IgG2	17	720	19	20
G2d.W158C-IgG2	17	721	19	20
G2d.N159C-IgG2	17	722	19	20
G2d.S160C-IgG2	17	723	19	20
G2d.G161C-IgG2	17	724	19	20
G2d.A162C-IgG2	17	725	19	20
G2d.L163C-IgG2	17	726	19	20
G2d.T164C-IgG2	17	727	19	20
G2d.S165C-IgG2	17	728	19	20
G2d.G166C-IgG2	17	729	19	20
G2d.V167C-IgG2	17	730	19	20
G2d.V173C-IgG2	17	731	19	20

G2d.L174C-IgG2	17	732	19	20
G2d.Q175C-IgG2	17	733	19	20
G2d.S176C-IgG2	17	734	19	20
G2d.S177C-IgG2	17	735	19	20
G2d.G178C-IgG2	17	736	19	20
G2d.L179C-IgG2	17	737	19	20
G2d.Y180C-IgG2	17	738	19	20
G2d.V186C-IgG2	17	739	19	20
G2d.T187C-IgG2	17	740	19	20
G2d.V188C-IgG2	17	741	19	20
G2d.P189C-IgG2	17	742	19	20
G2d.S190C-IgG2	17	743	19	20
G2d.S191C-IgG2	17	744	19	20
G2d.N192C-IgG2	17	745	19	20
G2d.F193C-IgG2	17	746	19	20
G2d.G194C-IgG2	17	747	19	20
G2d.T195C-IgG2	17	748	19	20
G2d.Q196C-IgG2	17	749	19	20
G2d.T197C-IgG2	17	750	19	20
G2d.Y198C-IgG2	17	751	19	20
G2d.T199C-IgG2	17	752	19	20
G2d.N201C-IgG2	17	753	19	20
G2d.V202C-IgG2	17	754	19	20
G2d.D203C-IgG2	17	755	19	20
G2d.H204C-IgG2	17	756	19	20
G2d.K205C-IgG2	17	757	19	20
G2d.P206C-IgG2	17	758	19	20
G2d.S207C-IgG2	17	759	19	20
G2d.N208C-IgG2	17	760	19	20
G2d.T209C-IgG2	17	761	19	20
G2d.K210C-IgG2	17	762	19	20
G2d.V211C-IgG2	17	763	19	20
G2d.D212C-IgG2	17	764	19	20

G2d.K213C-IgG2	17	765	19	20
G2d.T214C-IgG2	17	766	19	20
G2d.V215C-IgG2	17	767	19	20
G2d.E216C-IgG2	17	768	19	20
G2d.R217C-IgG2	17	769	19	20
G2d.K218C-IgG2	17	770	19	20

[0337]

[Table 47]

MRA-IgG2 light chain variant name	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:	Light chain variable region SEQ ID NO:	Light chain constant region SEQ ID NO:
MRAL.T5C-IgG2	17	313	457	20
MRAL.Q6C-IgG2	17	313	458	20
MRAL.S7C-IgG2	17	313	459	20
MRAL.P8C-IgG2	17	313	460	20
MRAL.S9C-IgG2	17	313	461	20
MRAL.S10C-IgG2	17	313	462	20
MRAL.L11C-IgG2	17	313	463	20
MRAL.S12C-IgG2	17	313	464	20
MRAL.A13C-IgG2	17	313	465	20
MRAL.S14C-IgG2	17	313	466	20
MRAL.V15C-IgG2	17	313	467	20
MRAL.G16C-IgG2	17	313	468	20
MRAL.D17C-IgG2	17	313	469	20
MRAL.R18C-IgG2	17	313	470	20
MRAL.V19C-IgG2	17	313	471	20
MRAL.T20C-IgG2	17	313	472	20
MRAL.I21C-IgG2	17	313	473	20
MRAL.T22C-IgG2	17	313	474	20
MRAL.A25C-IgG2	17	313	475	20
MRAL.S26C-IgG2	17	313	476	20
MRAL.Q27C-IgG2	17	313	477	20
MRAL.Y32C-IgG2	17	313	478	20
MRAL.L33C-IgG2	17	313	479	20
MRAL.N34C-IgG2	17	313	480	20
MRAL.Y50C-IgG2	17	313	481	20
MRAL.T51C-IgG2	17	313	482	20
MRAL.H55C-IgG2	17	313	483	20
MRAL.S56C-IgG2	17	313	484	20

MRAL.G57C-IgG2	17	313	485	20
MRAL.V58C-IgG2	17	313	486	20
MRAL.P59C-IgG2	17	313	487	20
MRAL.S60C-IgG2	17	313	488	20
MRAL.R61C-IgG2	17	313	489	20
MRAL.F62C-IgG2	17	313	490	20
MRAL.S63C-IgG2	17	313	491	20
MRAL.S65C-IgG2	17	313	492	20
MRAL.S67C-IgG2	17	313	493	20
MRAL.G68C-IgG2	17	313	494	20
MRAL.T69C-IgG2	17	313	495	20
MRAL.D70C-IgG2	17	313	496	20
MRAL.T72C-IgG2	17	313	497	20
MRAL.F73C-IgG2	17	313	498	20
MRAL.T74C-IgG2	17	313	499	20
MRAL.I75C-IgG2	17	313	500	20
MRAL.S76C-IgG2	17	313	501	20
MRAL.S77C-IgG2	17	313	502	20
MRAL.L78C-IgG2	17	313	503	20
MRAL.Q79C-IgG2	17	313	504	20
MRAL.Y96C-IgG2	17	313	505	20
MRAL.T97C-IgG2	17	313	506	20
MRAL.F98C-IgG2	17	313	507	20
MRAL.G99C-IgG2	17	313	508	20
MRAL.Q100C-IgG2	17	313	509	20
MRAL.G101C-IgG2	17	313	510	20
MRAL.T102C-IgG2	17	313	511	20
MRAL.K103C-IgG2	17	313	512	20
MRAL.V104C-IgG2	17	313	513	20
MRAL.E105C-IgG2	17	313	514	20
MRAL.I106C-IgG2	17	313	515	20
MRAL.K107C-IgG2	17	313	516	20
k0.R108C-IgG2	17	313	19	517

k0.T109C-IgG2	17	313	19	518
k0.V110C-IgG2	17	313	19	519
k0.A111C-IgG2	17	313	19	520
k0.A112C-IgG2	17	313	19	521
k0.P113C-IgG2	17	313	19	522
k0.S114C-IgG2	17	313	19	523
k0.V115C-IgG2	17	313	19	524
k0.F116C-IgG2	17	313	19	525
k0.P120C-IgG2	17	313	19	526
k0.S121C-IgG2	17	313	19	527
k0.D122C-IgG2	17	313	19	528
k0.E123C-IgG2	17	313	19	529
k0.Q124C-IgG2	17	313	19	530
k0.L125C-IgG2	17	313	19	531
k0.K126C-IgG2	17	313	19	532
k0.S127C-IgG2	17	313	19	533
k0.G128C-IgG2	17	313	19	534
k0.T129C-IgG2	17	313	19	535
k0.A130C-IgG2	17	313	19	536
k0.S131C-IgG2	17	313	19	537
k0.L136C-IgG2	17	313	19	538
k0.N137C-IgG2	17	313	19	539
k0.N138C-IgG2	17	313	19	540
k0.F139C-IgG2	17	313	19	541
k0.Y140C-IgG2	17	313	19	542
k0.P141C-IgG2	17	313	19	543
k0.R142C-IgG2	17	313	19	544
k0.E143C-IgG2	17	313	19	545
k0.A144C-IgG2	17	313	19	546
k0.K145C-IgG2	17	313	19	547
k0.V146C-IgG2	17	313	19	548
k0.Q147C-IgG2	17	313	19	549
k0.W148C-IgG2	17	313	19	550

k0.K149C-IgG2	17	313	19	551
k0.V150C-IgG2	17	313	19	552
k0.D151C-IgG2	17	313	19	553
k0.N152C-IgG2	17	313	19	554
k0.A153C-IgG2	17	313	19	555
k0.L154C-IgG2	17	313	19	556
k0.Q155C-IgG2	17	313	19	557
k0.S156C-IgG2	17	313	19	558
k0.G157C-IgG2	17	313	19	559
k0.N158C-IgG2	17	313	19	560
k0.S159C-IgG2	17	313	19	561
k0.Q160C-IgG2	17	313	19	562
k0.E161C-IgG2	17	313	19	563
k0.S162C-IgG2	17	313	19	564
k0.V163C-IgG2	17	313	19	565
k0.T164C-IgG2	17	313	19	566
k0.E165C-IgG2	17	313	19	567
k0.Q166C-IgG2	17	313	19	568
k0.D167C-IgG2	17	313	19	569
k0.S168C-IgG2	17	313	19	570
k0.K169C-IgG2	17	313	19	571
k0.D170C-IgG2	17	313	19	572
k0.S171C-IgG2	17	313	19	573
k0.T172C-IgG2	17	313	19	574
k0.Y173C-IgG2	17	313	19	575
k0.S174C-IgG2	17	313	19	576
k0.L175C-IgG2	17	313	19	577
k0.T180C-IgG2	17	313	19	578
k0.L181C-IgG2	17	313	19	579
k0.S182C-IgG2	17	313	19	580
k0.K183C-IgG2	17	313	19	581
k0.A184C-IgG2	17	313	19	582
k0.D185C-IgG2	17	313	19	583

k0.Y186C-IgG2	17	313	19	584
k0.E187C-IgG2	17	313	19	585
k0.K188C-IgG2	17	313	19	586
k0.H189C-IgG2	17	313	19	587
k0.K190C-IgG2	17	313	19	588
k0.V191C-IgG2	17	313	19	589
k0.Y192C-IgG2	17	313	19	590
k0.A193C-IgG2	17	313	19	591
k0.E195C-IgG2	17	313	19	592
k0.V196C-IgG2	17	313	19	593
k0.T197C-IgG2	17	313	19	594
k0.H198C-IgG2	17	313	19	595
k0.Q199C-IgG2	17	313	19	596
k0.G200C-IgG2	17	313	19	597
k0.L201C-IgG2	17	313	19	598
k0.S202C-IgG2	17	313	19	599
k0.S203C-IgG2	17	313	19	600
k0.P204C-IgG2	17	313	19	601
k0.V205C-IgG2	17	313	19	602
k0.T206C-IgG2	17	313	19	603
k0.K207C-IgG2	17	313	19	604
k0.S208C-IgG2	17	313	19	605
k0.F209C-IgG2	17	313	19	606
k0.N210C-IgG2	17	313	19	607
k0.R211C-IgG2	17	313	19	608
k0.G212C-IgG2	17	313	19	609
k0.E213C-IgG2	17	313	19	610

[0338] Reference Example 10-2 Assessment of electrophoretic mobility in polyacrylamide gel of antibodies having cysteine substitution at various positions of IgG2

Similarly to Reference Example 8-2, non-reducing SDS-PAGE was carried out with the MRA-IgG2 variants produced in Reference Example 10-1, the gel image was captured, and bands were analyzed.

From the obtained gel image, the variants were classified into 7 groups according to the band pattern of each of the MRA-IgG2 variants: Single (one band at a molecular weight region near 140 kDa), Double (two bands at a molecular weight region near 140 kDa), Triple (three bands at a molecular weight region near 140 kDa), Several (four or more bands at a molecular weight region near 140 kDa), LMW (band(s) at a molecular weight region lower than near 140 kDa), HMW (band(s) at a molecular

weight region higher than near 140 kDa), and Faint (band(s) blurry and difficult to determine). Grouping results of the band patterns for MRA-IgG2 heavy chain variants and MRA-IgG2 light chain variants are respectively shown in Tables 48 and 49. From Tables 48 and 49, variants classified into the Double and Triple groups are shown in Table 50. It is noted that while Table 33 indicates "no data" for MRAL.K107C-IgG2, position 107 (Kabat numbering), which is the position of cysteine substitution in this variant, is a position where the residue structurally exposed to the surface is present in the hinge region. Accordingly, this variant may also be classified as "Double".

[0339]

[Table 48]

MRA-IgG2 heavy chain variant name	Group
MRAH.Q5C-IgG2	Double
MRAH.E6C-IgG2	Double
MRAH.S7C-IgG2	Faint
MRAH.G8C-IgG2	Double
MRAH.P9C-IgG2	Double
MRAH.G10C-IgG2	Double
MRAH.L11C-IgG2	Double
MRAH.V12C-IgG2	Double
MRAH.R13C-IgG2	Double
MRAH.P14C-IgG2	Double
MRAH.S15C-IgG2	Double
MRAH.Q16C-IgG2	Double
MRAH.T17C-IgG2	Double
MRAH.L18C-IgG2	Faint
MRAH.S19C-IgG2	Double
MRAH.L20C-IgG2	Faint
MRAH.T21C-IgG2	Double
MRAH.T23C-IgG2	Double
MRAH.S25C-IgG2	Double
MRAH.G26C-IgG2	Double
MRAH.S28C-IgG2	Double
MRAH.T30C-IgG2	Double
MRAH.S31C-IgG2	Double
MRAH.W35C-IgG2	Double
MRAH.S35aC-IgG2	Faint
MRAH.Y50C-IgG2	Faint
MRAH.I51C-IgG2	Double
MRAH.S52C-IgG2	Double
MRAH.S62C-IgG2	Double
MRAH.L63C-IgG2	Double

MRAH.K64C-IgG2	Double
MRAH.S65C-IgG2	Double
MRAH.R66C-IgG2	Double
MRAH.V67C-IgG2	Faint
MRAH.T68C-IgG2	Double
MRAH.L70C-IgG2	Double
MRAH.D72C-IgG2	Double
MRAH.T73C-IgG2	Double
MRAH.S74C-IgG2	HMW
MRAH.K75C-IgG2	Double
MRAH.N76C-IgG2	no data
MRAH.Q77C-IgG2	Double
MRAH.S79C-IgG2	Double
MRAH.L80C-IgG2	Faint
MRAH.R81C-IgG2	Double
MRAH.L82C-IgG2	Faint
MRAH.S82aC-IgG2	Double
MRAH.S82bC-IgG2	Double
MRAH.V82cC-IgG2	Faint
MRAH.D101C-IgG2	Double
MRAH.Y102C-IgG2	Double
MRAH.S112C-IgG2	Double
MRAH.S113C-IgG2	Double
G2d.A118C-IgG2	LMW
G2d.S119C-IgG2	Double
G2d.T120C-IgG2	Double
G2d.K121C-IgG2	Double
G2d.G122C-IgG2	Double
G2d.P123C-IgG2	LMW
G2d.S124C-IgG2	Double
G2d.V125C-IgG2	LMW
G2d.F126C-IgG2	Double
G2d.P127C-IgG2	Faint

G2d.S132C-IgG2	Double
G2d.R133C-IgG2	Double
G2d.S134C-IgG2	Double
G2d.T135C-IgG2	Double
G2d.S136C-IgG2	Double
G2d.E137C-IgG2	Double
G2d.S138C-IgG2	Double
G2d.T139C-IgG2	Faint
G2d.A140C-IgG2	Double
G2d.A141C-IgG2	Faint
G2d.D148C-IgG2	Double
G2d.Y149C-IgG2	Double
G2d.F150C-IgG2	LMW
G2d.P151C-IgG2	no data
G2d.E152C-IgG2	LMW
G2d.P153C-IgG2	HMW
G2d.V154C-IgG2	Faint
G2d.T155C-IgG2	Double
G2d.V156C-IgG2	Double
G2d.S157C-IgG2	no data
G2d.W158C-IgG2	no data
G2d.N159C-IgG2	Double
G2d.S160C-IgG2	Double
G2d.G161C-IgG2	Double
G2d.A162C-IgG2	Double
G2d.L163C-IgG2	Double
G2d.T164C-IgG2	Double
G2d.S165C-IgG2	LMW
G2d.G166C-IgG2	Faint
G2d.V167C-IgG2	Double
G2d.V173C-IgG2	Double
G2d.L174C-IgG2	LMW
G2d.Q175C-IgG2	Double

G2d.S176C-IgG2	Double
G2d.S177C-IgG2	Double
G2d.G178C-IgG2	Double
G2d.L179C-IgG2	Double
G2d.Y180C-IgG2	LMW
G2d.V186C-IgG2	LMW
G2d.T187C-IgG2	Double
G2d.V188C-IgG2	Double
G2d.P189C-IgG2	Double
G2d.S190C-IgG2	Double
G2d.S191C-IgG2	Double
G2d.N192C-IgG2	Double
G2d.F193C-IgG2	Double
G2d.G194C-IgG2	Double
G2d.T195C-IgG2	Double
G2d.Q196C-IgG2	Double
G2d.T197C-IgG2	Double
G2d.Y198C-IgG2	LMW
G2d.T199C-IgG2	LMW
G2d.N201C-IgG2	LMW
G2d.V202C-IgG2	LMW
G2d.D203C-IgG2	LMW
G2d.H204C-IgG2	LMW
G2d.K205C-IgG2	LMW
G2d.P206C-IgG2	LMW
G2d.S207C-IgG2	LMW
G2d.N208C-IgG2	Double
G2d.T209C-IgG2	Double
G2d.K210C-IgG2	Double
G2d.V211C-IgG2	Double
G2d.D212C-IgG2	Double
G2d.K213C-IgG2	Double
G2d.T214C-IgG2	Single
G2d.V215C-IgG2	Single
G2d.E216C-IgG2	Single
G2d.R217C-IgG2	Double
G2d.K218C-IgG2	Double

[0340]

[Table 49]

MRA-IgG2 light chain variant name	Group
MRAL.T5C-IgG2	Double
MRAL.Q6C-IgG2	Faint
MRAL.S7C-IgG2	Double
MRAL.P8C-IgG2	no data
MRAL.S9C-IgG2	Double
MRAL.S10C-IgG2	Double
MRAL.L11C-IgG2	Double
MRAL.S12C-IgG2	Double
MRAL.A13C-IgG2	Double
MRAL.S14C-IgG2	Double
MRAL.V15C-IgG2	Double
MRAL.G16C-IgG2	Double
MRAL.D17C-IgG2	Double
MRAL.R18C-IgG2	Double
MRAL.V19C-IgG2	Double
MRAL.T20C-IgG2	Double
MRAL.I21C-IgG2	Double
MRAL.T22C-IgG2	Double
MRAL.A25C-IgG2	Faint
MRAL.S26C-IgG2	Double
MRAL.Q27C-IgG2	Double
MRAL.Y32C-IgG2	Double
MRAL.L33C-IgG2	Faint
MRAL.N34C-IgG2	Faint
MRAL.Y50C-IgG2	Double
MRAL.T51C-IgG2	Double
MRAL.H55C-IgG2	Double
MRAL.S56C-IgG2	Double
MRAL.G57C-IgG2	Double
MRAL.V58C-IgG2	Double

MRAL.P59C-IgG2	Double
MRAL.S60C-IgG2	Double
MRAL.R61C-IgG2	Double
MRAL.F62C-IgG2	Faint
MRAL.S63C-IgG2	Double
MRAL.S65C-IgG2	Double
MRAL.S67C-IgG2	Double
MRAL.G68C-IgG2	Double
MRAL.T69C-IgG2	Double
MRAL.D70C-IgG2	Double
MRAL.T72C-IgG2	Double
MRAL.F73C-IgG2	Faint
MRAL.T74C-IgG2	Double
MRAL.I75C-IgG2	no data
MRAL.S76C-IgG2	Double
MRAL.S77C-IgG2	Double
MRAL.L78C-IgG2	Faint
MRAL.Q79C-IgG2	Double
MRAL.Y96C-IgG2	Faint
MRAL.T97C-IgG2	Double
MRAL.F98C-IgG2	Faint
MRAL.G99C-IgG2	Double
MRAL.Q100C-IgG2	Double
MRAL.G101C-IgG2	Double
MRAL.T102C-IgG2	Faint
MRAL.K103C-IgG2	Double
MRAL.V104C-IgG2	Faint
MRAL.E105C-IgG2	Double
MRAL.I106C-IgG2	Faint
MRAL.K107C-IgG2	no data
k0.R108C-IgG2	Double
k0.T109C-IgG2	Double
k0.V110C-IgG2	Double

k0.A111C-IgG2	Double
k0.A112C-IgG2	Double
k0.P113C-IgG2	Double
k0.S114C-IgG2	Double
k0.V115C-IgG2	Faint
k0.F116C-IgG2	Double
k0.P120C-IgG2	Faint
k0.S121C-IgG2	Faint
k0.D122C-IgG2	LMW
k0.E123C-IgG2	Double
k0.Q124C-IgG2	Faint
k0.L125C-IgG2	Double
k0.K126C-IgG2	Triple
k0.S127C-IgG2	Double
k0.G128C-IgG2	Double
k0.T129C-IgG2	Double
k0.A130C-IgG2	Faint
k0.S131C-IgG2	Faint
k0.L136C-IgG2	Faint
k0.N137C-IgG2	no data
k0.N138C-IgG2	Double
k0.F139C-IgG2	Faint
k0.Y140C-IgG2	Faint
k0.P141C-IgG2	Double
k0.R142C-IgG2	Double
k0.E143C-IgG2	Double
k0.A144C-IgG2	Double
k0.K145C-IgG2	Double
k0.V146C-IgG2	Faint
k0.Q147C-IgG2	Double
k0.W148C-IgG2	no data
k0.K149C-IgG2	Double
k0.V150C-IgG2	Faint

k0.D151C-IgG2	Double
k0.N152C-IgG2	Double
k0.A153C-IgG2	Double
k0.L154C-IgG2	Double
k0.Q155C-IgG2	Double
k0.S156C-IgG2	Double
k0.G157C-IgG2	Double
k0.N158C-IgG2	Double
k0.S159C-IgG2	Double
k0.Q160C-IgG2	Double
k0.E161C-IgG2	Double
k0.S162C-IgG2	Double
k0.V163C-IgG2	Double
k0.T164C-IgG2	Double
k0.E165C-IgG2	Double
k0.Q166C-IgG2	Double
k0.D167C-IgG2	Double
k0.S168C-IgG2	Double
k0.K169C-IgG2	Double
k0.D170C-IgG2	Double
k0.S171C-IgG2	Double
k0.T172C-IgG2	Double
k0.Y173C-IgG2	Faint
k0.S174C-IgG2	Faint
k0.L175C-IgG2	Faint
k0.T180C-IgG2	Double
k0.L181C-IgG2	Double
k0.S182C-IgG2	Double
k0.K183C-IgG2	Double
k0.A184C-IgG2	Double
k0.D185C-IgG2	Double
k0.Y186C-IgG2	Double
k0.E187C-IgG2	LMW

k0.K188C-IgG2	Double
k0.H189C-IgG2	Faint
k0.K190C-IgG2	LMW
k0.V191C-IgG2	Double
k0.Y192C-IgG2	Double
k0.A193C-IgG2	Double
k0.E195C-IgG2	Double
k0.V196C-IgG2	Double
k0.T197C-IgG2	Double
k0.H198C-IgG2	Faint
k0.Q199C-IgG2	Double
k0.G200C-IgG2	Triple
k0.L201C-IgG2	Triple
k0.S202C-IgG2	Double
k0.S203C-IgG2	Triple
k0.P204C-IgG2	Double
k0.V205C-IgG2	Triple
k0.T206C-IgG2	Double
k0.K207C-IgG2	Triple
k0.S208C-IgG2	Double
k0.F209C-IgG2	LMW
k0.N210C-IgG2	LMW
k0.R211C-IgG2	LMW
k0.G212C-IgG2	Double
k0.E213C-IgG2	Double

[0341]

[Table 50]

MRA-IgG2 variant name	Group
MRAH.Q5C-IgG2	Double
MRAH.E6C-IgG2	Double
MRAH.G8C-IgG2	Double
MRAH.P9C-IgG2	Double
MRAH.G10C-IgG2	Double
MRAH.L11C-IgG2	Double
MRAH.V12C-IgG2	Double
MRAH.R13C-IgG2	Double
MRAH.P14C-IgG2	Double
MRAH.S15C-IgG2	Double
MRAH.Q16C-IgG2	Double
MRAH.T17C-IgG2	Double
MRAH.S19C-IgG2	Double
MRAH.T21C-IgG2	Double
MRAH.T23C-IgG2	Double
MRAH.S25C-IgG2	Double
MRAH.G26C-IgG2	Double
MRAH.S28C-IgG2	Double
MRAH.T30C-IgG2	Double
MRAH.S31C-IgG2	Double
MRAH.W35C-IgG2	Double
MRAH.I51C-IgG2	Double
MRAH.S52C-IgG2	Double
MRAH.S62C-IgG2	Double
MRAH.L63C-IgG2	Double
MRAH.K64C-IgG2	Double
MRAH.S65C-IgG2	Double
MRAH.R66C-IgG2	Double
MRAH.T68C-IgG2	Double
MRAH.L70C-IgG2	Double

MRAH.D72C-IgG2	Double
MRAH.T73C-IgG2	Double
MRAH.K75C-IgG2	Double
MRAH.Q77C-IgG2	Double
MRAH.S79C-IgG2	Double
MRAH.R81C-IgG2	Double
MRAH.S82aC-IgG2	Double
MRAH.S82bC-IgG2	Double
MRAH.D101C-IgG2	Double
MRAH.Y102C-IgG2	Double
MRAH.S112C-IgG2	Double
MRAH.S113C-IgG2	Double
G2d.S119C-IgG2	Double
G2d.T120C-IgG2	Double
G2d.K121C-IgG2	Double
G2d.G122C-IgG2	Double
G2d.S124C-IgG2	Double
G2d.F126C-IgG2	Double
G2d.S132C-IgG2	Double
G2d.R133C-IgG2	Double
G2d.S134C-IgG2	Double
G2d.T135C-IgG2	Double
G2d.S136C-IgG2	Double
G2d.E137C-IgG2	Double
G2d.S138C-IgG2	Double
G2d.A140C-IgG2	Double
G2d.D148C-IgG2	Double
G2d.Y149C-IgG2	Double
G2d.T155C-IgG2	Double
G2d.V156C-IgG2	Double
G2d.N159C-IgG2	Double
G2d.S160C-IgG2	Double
G2d.G161C-IgG2	Double

G2d.A162C-IgG2	Double
G2d.L163C-IgG2	Double
G2d.T164C-IgG2	Double
G2d.V167C-IgG2	Double
G2d.V173C-IgG2	Double
G2d.Q175C-IgG2	Double
G2d.S176C-IgG2	Double
G2d.S177C-IgG2	Double
G2d.G178C-IgG2	Double
G2d.L179C-IgG2	Double
G2d.T187C-IgG2	Double
G2d.V188C-IgG2	Double
G2d.P189C-IgG2	Double
G2d.S190C-IgG2	Double
G2d.S191C-IgG2	Double
G2d.N192C-IgG2	Double
G2d.F193C-IgG2	Double
G2d.G194C-IgG2	Double
G2d.T195C-IgG2	Double
G2d.Q196C-IgG2	Double
G2d.T197C-IgG2	Double
G2d.N208C-IgG2	Double
G2d.T209C-IgG2	Double
G2d.K210C-IgG2	Double
G2d.V211C-IgG2	Double
G2d.D212C-IgG2	Double
G2d.K213C-IgG2	Double
G2d.R217C-IgG2	Double
G2d.K218C-IgG2	Double
MRAL.T5C-IgG2	Double
MRAL.S7C-IgG2	Double
MRAL.S9C-IgG2	Double
MRAL.S10C-IgG2	Double

MRAL.L11C-IgG2	Double
MRAL.S12C-IgG2	Double
MRAL.A13C-IgG2	Double
MRAL.S14C-IgG2	Double
MRAL.V15C-IgG2	Double
MRAL.G16C-IgG2	Double
MRAL.D17C-IgG2	Double
MRAL.R18C-IgG2	Double
MRAL.V19C-IgG2	Double
MRAL.T20C-IgG2	Double
MRAL.I21C-IgG2	Double
MRAL.T22C-IgG2	Double
MRAL.S26C-IgG2	Double
MRAL.Q27C-IgG2	Double
MRAL.Y32C-IgG2	Double
MRAL.Y50C-IgG2	Double
MRAL.T51C-IgG2	Double
MRAL.H55C-IgG2	Double
MRAL.S56C-IgG2	Double
MRAL.G57C-IgG2	Double
MRAL.V58C-IgG2	Double
MRAL.P59C-IgG2	Double
MRAL.S60C-IgG2	Double
MRAL.R61C-IgG2	Double
MRAL.S63C-IgG2	Double
MRAL.S65C-IgG2	Double
MRAL.S67C-IgG2	Double
MRAL.G68C-IgG2	Double
MRAL.T69C-IgG2	Double
MRAL.D70C-IgG2	Double
MRAL.T72C-IgG2	Double
MRAL.T74C-IgG2	Double
MRAL.S76C-IgG2	Double

MRAL.S77C-IgG2	Double
MRAL.Q79C-IgG2	Double
MRAL.T97C-IgG2	Double
MRAL.G99C-IgG2	Double
MRAL.Q100C-IgG2	Double
MRAL.G101C-IgG2	Double
MRAL.K103C-IgG2	Double
MRAL.E105C-IgG2	Double
k0.R108C-IgG2	Double
k0.T109C-IgG2	Double
k0.V110C-IgG2	Double
k0.A111C-IgG2	Double
k0.A112C-IgG2	Double
k0.P113C-IgG2	Double
k0.S114C-IgG2	Double
k0.F116C-IgG2	Double
k0.E123C-IgG2	Double
k0.L125C-IgG2	Double
k0.K126C-IgG2	Triple
k0.S127C-IgG2	Double
k0.G128C-IgG2	Double
k0.T129C-IgG2	Double
k0.N138C-IgG2	Double
k0.P141C-IgG2	Double
k0.R142C-IgG2	Double
k0.E143C-IgG2	Double
k0.A144C-IgG2	Double
k0.K145C-IgG2	Double
k0.Q147C-IgG2	Double
k0.K149C-IgG2	Double
k0.D151C-IgG2	Double
k0.N152C-IgG2	Double
k0.A153C-IgG2	Double

k0.L154C-IgG2	Double
k0.Q155C-IgG2	Double
k0.S156C-IgG2	Double
k0.G157C-IgG2	Double
k0.N158C-IgG2	Double
k0.S159C-IgG2	Double
k0.Q160C-IgG2	Double
k0.E161C-IgG2	Double
k0.S162C-IgG2	Double
k0.V163C-IgG2	Double
k0.T164C-IgG2	Double
k0.E165C-IgG2	Double
k0.Q166C-IgG2	Double
k0.D167C-IgG2	Double
k0.S168C-IgG2	Double
k0.K169C-IgG2	Double
k0.D170C-IgG2	Double
k0.S171C-IgG2	Double
k0.T172C-IgG2	Double
k0.T180C-IgG2	Double
k0.L181C-IgG2	Double
k0.S182C-IgG2	Double
k0.K183C-IgG2	Double
k0.A184C-IgG2	Double
k0.D185C-IgG2	Double
k0.Y186C-IgG2	Double
k0.K188C-IgG2	Double
k0.V191C-IgG2	Double
k0.Y192C-IgG2	Double
k0.A193C-IgG2	Double
k0.E195C-IgG2	Double
k0.V196C-IgG2	Double
k0.T197C-IgG2	Double

k0.Q199C-IgG2	Double
k0.G200C-IgG2	Triple
k0.L201C-IgG2	Triple
k0.S202C-IgG2	Double
k0.S203C-IgG2	Triple
k0.P204C-IgG2	Double
k0.V205C-IgG2	Triple
k0.T206C-IgG2	Double
k0.K207C-IgG2	Triple
k0.S208C-IgG2	Double
k0.G212C-IgG2	Double
k0.E213C-IgG2	Double

[0342] [Reference Example 11] Assessment of antibodies having cysteine substitution at various positions of the Lambda chain

Reference Example 11-1 Production of antibodies having cysteine substitution at various positions of the Lambda chain

The light chain (Lambda chain) of an anti-human CXCL10 neutralizing antibody, G7-IgG1 (heavy chain: G7H-G1T4 (SEQ ID NO: 314), light chain: G7L-LT0 (SEQ ID NO: 316)), was subjected to a study in which an arbitrary amino acid residue structurally exposed to the surface was substituted with cysteine.

Amino acid residues within the G7-IgG1 light chain variable region (G7L, SEQ ID NO: 317) were substituted with cysteine to produce variants of the G7-IgG1 light chain variable region shown in Table 51. These variants of the G7-IgG1 light chain variable region were each linked with the G7-IgG1 light chain constant region (LT0, SEQ ID NO: 318) to produce G7-IgG1 light chain variants, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art. In addition, amino acid residues within the G7-IgG1 light chain constant region (LT0, SEQ ID NO: 318) were substituted with cysteine to produce variants of the G7-IgG1 light chain constant region shown in Table 52. These variants of the G7-IgG1 heavy chain constant region were each linked with the G7-IgG1 light chain variable region (G7L, SEQ ID NO: 317) to produce G7-IgG1 light chain variants, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art.

[0343]

[Table 51]

Variant of G7-IgG1 light chain variable region	Position of cysteine substitution (Kabat numbering)	SEQ ID NO:
G7L.T5C	5	771
G7L.Q6C	6	772
G7L.P7C	7	773
G7L.P8C	8	774
G7L.S9C	9	775
G7L.A11C	11	776
G7L.S12C	12	777
G7L.G13C	13	778
G7L.T14C	14	779
G7L.P15C	15	780
G7L.G16C	16	781
G7L.Q17C	17	782
G7L.R18C	18	783
G7L.V19C	19	784
G7L.T20C	20	785
G7L.I21C	21	786
G7L.S22C	22	787
G7L.G25C	25	788
G7L.S26C	26	789
G7L.S27C	27	790
G7L.S27aC	27a	791
G7L.T32C	32	792
G7L.V33C	33	793
G7L.N34C	34	794
G7L.N50C	50	795
G7L.N51C	51	796
G7L.P55C	55	797
G7L.S56C	56	798
G7L.G57C	57	799

G7L.I58C	58	800
G7L.P59C	59	801
G7L.D60C	60	802
G7L.R61C	61	803
G7L.F62C	62	804
G7L.S63C	63	805
G7L.S65C	65	806
G7L.S67C	67	807
G7L.G68C	68	808
G7L.T69C	69	809
G7L.S70C	70	810
G7L.S72C	72	811
G7L.L73C	73	812
G7L.V74C	74	813
G7L.I75C	75	814
G7L.S76C	76	815
G7L.G77C	77	816
G7L.L78C	78	817
G7L.Q79C	79	818
G7L.R96C	96	819
G7L.V97C	97	820
G7L.F98C	98	821
G7L.G99C	99	822
G7L.G100C	100	823
G7L.G101C	101	824
G7L.T102C	102	825
G7L.K103C	103	826
G7L.L104C	104	827
G7L.T105C	105	828
G7L.V106C	106	829
G7L.L106aC	106a	830

[0344]

[Table 52]

Variant of G7-IgG1 light chain constant region	Position of cysteine substitution (Kabat numbering)	SEQ ID NO:
LT0.Q108C	108	831
LT0.P109C	109	832
LT0.K110C	110	833
LT0.A111C	111	834
LT0.A112C	112	835
LT0.P113C	113	836
LT0.S114C	114	837
LT0.V115C	115	838
LT0.T116C	116	839
LT0.P120C	120	840
LT0.S121C	121	841
LT0.S122C	122	842
LT0.E123C	123	843
LT0.E124C	124	844
LT0.L125C	125	845
LT0.Q126C	126	846
LT0.A127C	127	847
LT0.N128C	128	848
LT0.K129C	129	849
LT0.A130C	130	850
LT0.T131C	131	851
LT0.I136C	136	852
LT0.S137C	137	853
LT0.D138C	138	854
LT0.F139C	139	855
LT0.Y140C	140	856
LT0.P141C	141	857
LT0.G142C	142	858
LT0.A143C	143	859

LT0.V144C	144	860
LT0.T145C	145	861
LT0.V146C	146	862
LT0.A147C	147	863
LT0.W148C	148	864
LT0.K149C	149	865
LT0.A150C	150	866
LT0.D151C	151	867
LT0.S152C	152	868
LT0.S153C	153	869
LT0.P154C	154	870
LT0.V155C	155	871
LT0.K156C	156	872
LT0.A157C	157	873
LT0.G158C	158	874
LT0.V159C	159	875
LT0.E160C	160	876
LT0.T161C	161	877
LT0.T162C	162	878
LT0.T163C	163	879
LT0.P164C	164	880
LT0.S165C	165	881
LT0.K166C	166	882
LT0.Q167C	167	883
LT0.S168C	168	884
LT0.N170C	170	885
LT0.N171C	171	886
LT0.K172C	172	887
LT0.Y173C	173	888
LT0.A174C	174	889
LT0.A175C	175	890
LT0.S180C	180	891
LT0.L181C	181	892

LT0.T182C	182	893
LT0.P183C	183	894
LT0.E184C	184	895
LT0.Q185C	185	896
LT0.W186C	186	897
LT0.K187C	187	898
LT0.S188C	188	899
LT0.H189C	189	900
LT0.R190C	190	901
LT0.S191C	191	902
LT0.Y192C	192	903
LT0.S193C	193	904
LT0.Q195C	195	905
LT0.V196C	196	906
LT0.T197C	197	907
LT0.H198C	198	908
LT0.E199C	199	909
LT0.G200C	200	910
LT0.S203C	203	911
LT0.T204C	204	912
LT0.V205C	205	913
LT0.E206C	206	914
LT0.K207C	207	915
LT0.T208C	208	916
LT0.V209C	209	917
LT0.A210C	210	918
LT0.P211C	211	919
LT0.T212C	212	920
LT0.E213C	213	921

[0345] The G7-IgG1 light chain variants produced above were combined with the G7-IgG1 heavy chain and the resultant G7-IgG1 light chain variants shown in Table 53 were expressed by transient expression using FreeStyle293 cells (Invitrogen) or Expi293 cells (Life technologies) by a method known to the person skilled in the art, and purified with Protein A by a method known to the person skilled in the art.

[0346]

[Table 53]

G7-IgG1 light chain variant name	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:	Light chain variable region SEQ ID NO:	Light chain constant region SEQ ID NO:
G7L.T5C-IgG1	315	18	771	318
G7L.Q6C-IgG1	315	18	772	318
G7L.P7C-IgG1	315	18	773	318
G7L.P8C-IgG1	315	18	774	318
G7L.S9C-IgG1	315	18	775	318
G7L.A11C-IgG1	315	18	776	318
G7L.S12C-IgG1	315	18	777	318
G7L.G13C-IgG1	315	18	778	318
G7L.T14C-IgG1	315	18	779	318
G7L.P15C-IgG1	315	18	780	318
G7L.G16C-IgG1	315	18	781	318
G7L.Q17C-IgG1	315	18	782	318
G7L.R18C-IgG1	315	18	783	318
G7L.V19C-IgG1	315	18	784	318
G7L.T20C-IgG1	315	18	785	318
G7L.I21C-IgG1	315	18	786	318
G7L.S22C-IgG1	315	18	787	318
G7L.G25C-IgG1	315	18	788	318
G7L.S26C-IgG1	315	18	789	318
G7L.S27C-IgG1	315	18	790	318
G7L.S27aC-IgG1	315	18	791	318
G7L.T32C-IgG1	315	18	792	318
G7L.V33C-IgG1	315	18	793	318
G7L.N34C-IgG1	315	18	794	318
G7L.N50C-IgG1	315	18	795	318
G7L.N51C-IgG1	315	18	796	318
G7L.P55C-IgG1	315	18	797	318
G7L.S56C-IgG1	315	18	798	318

G7L.G57C-IgG1	315	18	799	318
G7L.I58C-IgG1	315	18	800	318
G7L.P59C-IgG1	315	18	801	318
G7L.D60C-IgG1	315	18	802	318
G7L.R61C-IgG1	315	18	803	318
G7L.F62C-IgG1	315	18	804	318
G7L.S63C-IgG1	315	18	805	318
G7L.S65C-IgG1	315	18	806	318
G7L.S67C-IgG1	315	18	807	318
G7L.G68C-IgG1	315	18	808	318
G7L.T69C-IgG1	315	18	809	318
G7L.S70C-IgG1	315	18	810	318
G7L.S72C-IgG1	315	18	811	318
G7L.L73C-IgG1	315	18	812	318
G7L.V74C-IgG1	315	18	813	318
G7L.I75C-IgG1	315	18	814	318
G7L.S76C-IgG1	315	18	815	318
G7L.G77C-IgG1	315	18	816	318
G7L.L78C-IgG1	315	18	817	318
G7L.Q79C-IgG1	315	18	818	318
G7L.R96C-IgG1	315	18	819	318
G7L.V97C-IgG1	315	18	820	318
G7L.F98C-IgG1	315	18	821	318
G7L.G99C-IgG1	315	18	822	318
G7L.G100C-IgG1	315	18	823	318
G7L.G101C-IgG1	315	18	824	318
G7L.T102C-IgG1	315	18	825	318
G7L.K103C-IgG1	315	18	826	318
G7L.L104C-IgG1	315	18	827	318
G7L.T105C-IgG1	315	18	828	318
G7L.V106C-IgG1	315	18	829	318
G7L.L106aC-IgG1	315	18	830	318
LT0.Q108C-IgG1	315	18	317	831

LT0.P109C-IgG1	315	18	317	832
LT0.K110C-IgG1	315	18	317	833
LT0.A111C-IgG1	315	18	317	834
LT0.A112C-IgG1	315	18	317	835
LT0.P113C-IgG1	315	18	317	836
LT0.S114C-IgG1	315	18	317	837
LT0.V115C-IgG1	315	18	317	838
LT0.T116C-IgG1	315	18	317	839
LT0.P120C-IgG1	315	18	317	840
LT0.S121C-IgG1	315	18	317	841
LT0.S122C-IgG1	315	18	317	842
LT0.E123C-IgG1	315	18	317	843
LT0.E124C-IgG1	315	18	317	844
LT0.L125C-IgG1	315	18	317	845
LT0.Q126C-IgG1	315	18	317	846
LT0.A127C-IgG1	315	18	317	847
LT0.N128C-IgG1	315	18	317	848
LT0.K129C-IgG1	315	18	317	849
LT0.A130C-IgG1	315	18	317	850
LT0.T131C-IgG1	315	18	317	851
LT0.I136C-IgG1	315	18	317	852
LT0.S137C-IgG1	315	18	317	853
LT0.D138C-IgG1	315	18	317	854
LT0.F139C-IgG1	315	18	317	855
LT0.Y140C-IgG1	315	18	317	856
LT0.P141C-IgG1	315	18	317	857
LT0.G142C-IgG1	315	18	317	858
LT0.A143C-IgG1	315	18	317	859
LT0.V144C-IgG1	315	18	317	860
LT0.T145C-IgG1	315	18	317	861
LT0.V146C-IgG1	315	18	317	862
LT0.A147C-IgG1	315	18	317	863
LT0.W148C-IgG1	315	18	317	864

LT0.K149C-IgG1	315	18	317	865
LT0.A150C-IgG1	315	18	317	866
LT0.D151C-IgG1	315	18	317	867
LT0.S152C-IgG1	315	18	317	868
LT0.S153C-IgG1	315	18	317	869
LT0.P154C-IgG1	315	18	317	870
LT0.V155C-IgG1	315	18	317	871
LT0.K156C-IgG1	315	18	317	872
LT0.A157C-IgG1	315	18	317	873
LT0.G158C-IgG1	315	18	317	874
LT0.V159C-IgG1	315	18	317	875
LT0.E160C-IgG1	315	18	317	876
LT0.T161C-IgG1	315	18	317	877
LT0.T162C-IgG1	315	18	317	878
LT0.T163C-IgG1	315	18	317	879
LT0.P164C-IgG1	315	18	317	880
LT0.S165C-IgG1	315	18	317	881
LT0.K166C-IgG1	315	18	317	882
LT0.Q167C-IgG1	315	18	317	883
LT0.S168C-IgG1	315	18	317	884
LT0.N170C-IgG1	315	18	317	885
LT0.N171C-IgG1	315	18	317	886
LT0.K172C-IgG1	315	18	317	887
LT0.Y173C-IgG1	315	18	317	888
LT0.A174C-IgG1	315	18	317	889
LT0.A175C-IgG1	315	18	317	890
LT0.S180C-IgG1	315	18	317	891
LT0.L181C-IgG1	315	18	317	892
LT0.T182C-IgG1	315	18	317	893
LT0.P183C-IgG1	315	18	317	894
LT0.E184C-IgG1	315	18	317	895
LT0.Q185C-IgG1	315	18	317	896
LT0.W186C-IgG1	315	18	317	897

LT0.K187C-IgG1	315	18	317	898
LT0.S188C-IgG1	315	18	317	899
LT0.H189C-IgG1	315	18	317	900
LT0.R190C-IgG1	315	18	317	901
LT0.S191C-IgG1	315	18	317	902
LT0.Y192C-IgG1	315	18	317	903
LT0.S193C-IgG1	315	18	317	904
LT0.Q195C-IgG1	315	18	317	905
LT0.V196C-IgG1	315	18	317	906
LT0.T197C-IgG1	315	18	317	907
LT0.H198C-IgG1	315	18	317	908
LT0.E199C-IgG1	315	18	317	909
LT0.G200C-IgG1	315	18	317	910
LT0.S203C-IgG1	315	18	317	911
LT0.T204C-IgG1	315	18	317	912
LT0.V205C-IgG1	315	18	317	913
LT0.E206C-IgG1	315	18	317	914
LT0.K207C-IgG1	315	18	317	915
LT0.T208C-IgG1	315	18	317	916
LT0.V209C-IgG1	315	18	317	917
LT0.A210C-IgG1	315	18	317	918
LT0.P211C-IgG1	315	18	317	919
LT0.T212C-IgG1	315	18	317	920
LT0.E213C-IgG1	315	18	317	921

[0347] Reference Example 11-2 Assessment of electrophoretic mobility in polyacrylamide gel of antibodies having cysteine substitution at various positions of the Lambda chain

Similarly to Reference Example 8-2, non-reducing SDS-PAGE was carried out with the G7-IgG1 variants produced in Reference Example 11-1, the gel image was captured, and bands were quantified.

From the obtained gel image, the variants were classified into 7 groups according to the band pattern of each of the G7-IgG1 variants: Single (one band at a molecular weight region similar to that of G7-IgG1), Double (two bands at a molecular weight region similar to that of G7-IgG1), Triple (three bands at a molecular weight region similar to that of G7-IgG1), Several (four or more bands at a molecular weight region similar to that of G7-IgG1), LMW (band(s) at a molecular weight region lower than that of G7-IgG1), HMW (band(s) at a molecular weight region higher than that of G7-IgG1), and Faint (band(s) blurry and difficult to determine). Regarding the G7-IgG1 variants classified as "Double", one of the two bands showed the same elec-

trophoretic mobility as G7-IgG1 while the other band showed slightly faster or slower mobility. Thus, for the G7-IgG1 variants classified as "Double", the percentage of the bands showing different mobility to G7-IgG1 (percentage of new band (%)) was also calculated. Grouping of the band patterns for G7-IgG1 light chain variants and the calculation results of the band percentage are shown in Table 54. From Table 54, variants classified into the Double and Triple groups are shown in Table 55. In these variants, it is highly likely that cysteine substitution caused structural changes such as crosslinkage of Fabs, which resulted in the change in electrophoretic mobility. In this Reference Example, the variant in which the amino acid residue at position 107a (Kabat numbering) was substituted with cysteine was not assessed. However, position 107a (Kabat numbering) is a position where the residue structurally exposed to the surface is present in the hinge region. Thus, in this variant also, it is highly likely that cysteine substitution causes structural changes such as crosslinkage of Fabs, and results in the change in electrophoretic mobility.

[0348]

[Table 54]

G7-IgG1 light chain variant name	Group	Percentage of new band (%)
G7L.T5C-IgG1	Single	-
G7L.Q6C-IgG1	Triple	-
G7L.P7C-IgG1	Single	-
G7L.P8C-IgG1	Single	-
G7L.S9C-IgG1	Single	-
G7L.A11C-IgG1	Single	-
G7L.S12C-IgG1	Single	-
G7L.G13C-IgG1	Single	-
G7L.T14C-IgG1	Single	-
G7L.P15C-IgG1	Single	-
G7L.G16C-IgG1	Faint	-
G7L.Q17C-IgG1	Single	-
G7L.R18C-IgG1	Single	-
G7L.V19C-IgG1	Double	32.3
G7L.T20C-IgG1	Single	-
G7L.I21C-IgG1	Faint	-
G7L.S22C-IgG1	Single	-
G7L.G25C-IgG1	Single	-
G7L.S26C-IgG1	Single	-
G7L.S27C-IgG1	Single	-
G7L.S27aC-IgG1	Single	-
G7L.T32C-IgG1	Single	-
G7L.V33C-IgG1	Triple	-
G7L.N34C-IgG1	Double	43.8
G7L.N50C-IgG1	Single	-
G7L.N51C-IgG1	Single	-
G7L.P55C-IgG1	Single	-
G7L.S56C-IgG1	Single	-
G7L.G57C-IgG1	Single	-
G7L.I58C-IgG1	Single	-

G7L.P59C-IgG1	Single	-
G7L.D60C-IgG1	Single	-
G7L.R61C-IgG1	Single	-
G7L.F62C-IgG1	Faint	-
G7L.S63C-IgG1	Single	-
G7L.S65C-IgG1	Single	-
G7L.S67C-IgG1	Single	-
G7L.G68C-IgG1	Single	-
G7L.T69C-IgG1	Single	-
G7L.S70C-IgG1	Single	-
G7L.S72C-IgG1	Single	-
G7L.L73C-IgG1	Faint	-
G7L.V74C-IgG1	Single	-
G7L.I75C-IgG1	Faint	-
G7L.S76C-IgG1	Single	-
G7L.G77C-IgG1	Single	-
G7L.L78C-IgG1	Faint	-
G7L.Q79C-IgG1	Single	-
G7L.R96C-IgG1	Single	-
G7L.V97C-IgG1	Faint	-
G7L.F98C-IgG1	Single	-
G7L.G99C-IgG1	Faint	-
G7L.G100C-IgG1	Single	-
G7L.G101C-IgG1	Single	-
G7L.T102C-IgG1	Faint	-
G7L.K103C-IgG1	Single	-
G7L.L104C-IgG1	Faint	-
G7L.T105C-IgG1	Single	-
G7L.V106C-IgG1	Faint	-
G7L.L106aC-IgG1	Single	-
LT0.Q108C-IgG1	Double	10.6
LT0.P109C-IgG1	Double	42.9
LT0.K110C-IgG1	Single	-

LT0.A111C-IgG1	Single	-
LT0.A112C-IgG1	Single	-
LT0.P113C-IgG1	LMW	-
LT0.S114C-IgG1	Single	-
LT0.V115C-IgG1	LMW	-
LT0.T116C-IgG1	Single	-
LT0.P120C-IgG1	LMW	-
LT0.S121C-IgG1	LMW	-
LT0.S122C-IgG1	no data	-
LT0.E123C-IgG1	Double	57.5
LT0.E124C-IgG1	LMW	-
LT0.L125C-IgG1	LMW	-
LT0.Q126C-IgG1	Triple	-
LT0.A127C-IgG1	Single	-
LT0.N128C-IgG1	Single	-
LT0.K129C-IgG1	Single	-
LT0.A130C-IgG1	LMW	-
LT0.T131C-IgG1	LMW	-
LT0.I136C-IgG1	LMW	-
LT0.S137C-IgG1	Single	-
LT0.D138C-IgG1	Single	-
LT0.F139C-IgG1	LMW	-
LT0.Y140C-IgG1	Single	-
LT0.P141C-IgG1	Single	-
LT0.G142C-IgG1	Single	-
LT0.A143C-IgG1	Single	-
LT0.V144C-IgG1	LMW	-
LT0.T145C-IgG1	Single	-
LT0.V146C-IgG1	LMW	-
LT0.A147C-IgG1	Single	-
LT0.W148C-IgG1	no data	-
LT0.K149C-IgG1	Single	-
LT0.A150C-IgG1	Single	-

LT0.D151C-IgG1	Single	-
LT0.S152C-IgG1	Single	-
LT0.S153C-IgG1	Single	-
LT0.P154C-IgG1	Single	-
LT0.V155C-IgG1	Single	-
LT0.K156C-IgG1	Single	-
LT0.A157C-IgG1	Single	-
LT0.G158C-IgG1	no data	-
LT0.V159C-IgG1	Single	-
LT0.E160C-IgG1	Single	-
LT0.T161C-IgG1	Single	-
LT0.T162C-IgG1	Single	-
LT0.T163C-IgG1	Single	-
LT0.P164C-IgG1	Single	-
LT0.S165C-IgG1	Single	-
LT0.K166C-IgG1	Single	-
LT0.Q167C-IgG1	Single	-
LT0.S168C-IgG1	Single	-
LT0.N170C-IgG1	Single	-
LT0.N171C-IgG1	Single	-
LT0.K172C-IgG1	Single	-
LT0.Y173C-IgG1	Single	-
LT0.A174C-IgG1	LMW	-
LT0.A175C-IgG1	LMW	-
LT0.S180C-IgG1	Single	-
LT0.L181C-IgG1	Single	-
LT0.T182C-IgG1	Single	-
LT0.P183C-IgG1	LMW	-
LT0.E184C-IgG1	Single	-
LT0.Q185C-IgG1	Single	-
LT0.W186C-IgG1	LMW	-
LT0.K187C-IgG1	LMW	-
LT0.S188C-IgG1	LMW	-

LT0.H189C-IgG1	LMW	-
LT0.R190C-IgG1	LMW	-
LT0.S191C-IgG1	Single	-
LT0.Y192C-IgG1	Single	-
LT0.S193C-IgG1	Single	-
LT0.Q195C-IgG1	Double	30.1
LT0.V196C-IgG1	Double	82.9
LT0.T197C-IgG1	Single	-
LT0.H198C-IgG1	Faint	-
LT0.E199C-IgG1	Single	-
LT0.G200C-IgG1	Double	15.5
LT0.S203C-IgG1	Double	32.4
LT0.T204C-IgG1	Single	-
LT0.V205C-IgG1	Single	-
LT0.E206C-IgG1	Single	-
LT0.K207C-IgG1	Single	-
LT0.T208C-IgG1	Single	-
LT0.V209C-IgG1	LMW	-
LT0.A210C-IgG1	LMW	-
LT0.P211C-IgG1	Faint	-
LT0.T212C-IgG1	LMW	-
LT0.E213C-IgG1	Single	-

[0349] [Table 55]

G7-IgG1 light chain variant name	Group	Percentage of new band (%)
G7L.Q6C-IgG1	Triple	-
G7L.V19C-IgG1	Double	32.3
G7L.V33C-IgG1	Triple	-
G7L.N34C-IgG1	Double	43.8
LT0.Q108C-IgG1	Double	10.6
LT0.P109C-IgG1	Double	42.9
LT0.E123C-IgG1	Double	57.5
LT0.Q126C-IgG1	Triple	-
LT0.Q195C-IgG1	Double	30.1
LT0.V196C-IgG1	Double	82.9
LT0.G200C-IgG1	Double	15.5
LT0.S203C-IgG1	Double	32.4

[0350] [Reference Example 12] Assessment of antibodies having cysteine substitution at

various positions of VHH

Reference Example 12-1 Production of antibodies having cysteine substitution at various positions of VHH

An anti-human IL6R neutralizing VHH, IL6R90 (SEQ ID NO: 319) was fused with a human IgG1 Fc region (G1T3dCH1dC, SEQ ID NO: 320) to produce IL6R90-Fc (IL6R90-G1T3dCH1dC, SEQ ID NO: 321), and this was subjected to a study in which an arbitrary amino acid residue among the IL6R90 region structurally exposed to the surface was substituted with cysteine.

Amino acid residues within the IL6R90 region were substituted with cysteine, and expression vectors encoding the genes of IL6R90-Fc VHH region variants shown in Table 56 were produced by a method known to the person skilled in the art. These variants of the IL6R90-Fc VHH region were each linked with the Fc region of human IgG1 (G1T3dCH1dC, SEQ ID NO: 320) to produce IL6R90-Fc variants, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art.

[0351]

[Table 56]

Variant of IL6R90-Fc VHH region	Position of cysteine substitution (Kabat numbering)	SEQ ID NO:
IL6R90.E1C	1	922
IL6R90.V2C	2	923
IL6R90.Q3C	3	924
IL6R90.L4C	4	925
IL6R90.V5C	5	926
IL6R90.E6C	6	927
IL6R90.S7C	7	928
IL6R90.G8C	8	929
IL6R90.G9C	9	930
IL6R90.G10C	10	931
IL6R90.L11C	11	932
IL6R90.V12C	12	933
IL6R90.Q13C	13	934
IL6R90.P14C	14	935
IL6R90.G15C	15	936
IL6R90.G16C	16	937
IL6R90.S17C	17	938
IL6R90.L18C	18	939
IL6R90.R19C	19	940
IL6R90.L20C	20	941
IL6R90.S21C	21	942
IL6R90.A23C	23	943
IL6R90.A24C	24	944
IL6R90.S25C	25	945
IL6R90.G26C	26	946
IL6R90.F27C	27	947
IL6R90.T28C	28	948
IL6R90.F29C	29	949
IL6R90.D30C	30	950

IL6R90.W36C	36	951
IL6R90.V37C	37	952
IL6R90.R38C	38	953
IL6R90.Q39C	39	954
IL6R90.A40C	40	955
IL6R90.P41C	41	956
IL6R90.G42C	42	957
IL6R90.K43C	43	958
IL6R90.A44C	44	959
IL6R90.L45C	45	960
IL6R90.E46C	46	961
IL6R90.W47C	47	962
IL6R90.V48C	48	963
IL6R90.S49C	49	964
IL6R90.R66C	66	965
IL6R90.F67C	67	966
IL6R90.T68C	68	967
IL6R90.I69C	69	968
IL6R90.S70C	70	969
IL6R90.R71C	71	970
IL6R90.D72C	72	971
IL6R90.N73C	73	972
IL6R90.A74C	74	973
IL6R90.K75C	75	974
IL6R90.N76C	76	975
IL6R90.T77C	77	976
IL6R90.L78C	78	977
IL6R90.Y79C	79	978
IL6R90.L80C	80	979
IL6R90.Q81C	81	980
IL6R90.M82C	82	981
IL6R90.N82aC	82a	982
IL6R90.S82bC	82b	983

IL6R90.L82cC	82c	984
IL6R90.R83C	83	985
IL6R90.P84C	84	986
IL6R90.E85C	85	987
IL6R90.D86C	86	988
IL6R90.T87C	87	989
IL6R90.A88C	88	990
IL6R90.V89C	89	991
IL6R90.Y90C	90	992
IL6R90.Y91C	91	993
IL6R90.V93C	93	994
IL6R90.K94C	94	995
IL6R90.W103C	103	996
IL6R90.G104C	104	997
IL6R90.Q105C	105	998
IL6R90.G106C	106	999
IL6R90.T107C	107	1000
IL6R90.L108C	108	1001
IL6R90.V109C	109	1002
IL6R90.T110C	110	1003
IL6R90.V111C	111	1004
IL6R90.S112C	112	1005
IL6R90.S113C	113	1006

IL6R90-Fc variants produced above and shown in Table 57 were expressed by transient expression using FreeStyle293 cells (Invitrogen) or Expi293 cells (Life technologies) by a method known to the person skilled in the art, and purified with Protein A by a method known to the person skilled in the art.

[0352]

[Table 57]

IL6R90-Fc variant name	VHH region SEQ ID NO:	Fc region SEQ ID NO:
IL6R90.E1C-Fc	922	320
IL6R90.V2C-Fc	923	320
IL6R90.Q3C-Fc	924	320
IL6R90.L4C-Fc	925	320
IL6R90.V5C-Fc	926	320
IL6R90.E6C-Fc	927	320
IL6R90.S7C-Fc	928	320
IL6R90.G8C-Fc	929	320
IL6R90.G9C-Fc	930	320
IL6R90.G10C-Fc	931	320
IL6R90.L11C-Fc	932	320
IL6R90.V12C-Fc	933	320
IL6R90.Q13C-Fc	934	320
IL6R90.P14C-Fc	935	320
IL6R90.G15C-Fc	936	320
IL6R90.G16C-Fc	937	320
IL6R90.S17C-Fc	938	320
IL6R90.L18C-Fc	939	320
IL6R90.R19C-Fc	940	320
IL6R90.L20C-Fc	941	320
IL6R90.S21C-Fc	942	320
IL6R90.A23C-Fc	943	320
IL6R90.A24C-Fc	944	320
IL6R90.S25C-Fc	945	320
IL6R90.G26C-Fc	946	320
IL6R90.F27C-Fc	947	320
IL6R90.T28C-Fc	948	320
IL6R90.F29C-Fc	949	320

IL6R90.D30C-Fc	950	320
IL6R90.W36C-Fc	951	320
IL6R90.V37C-Fc	952	320
IL6R90.R38C-Fc	953	320
IL6R90.Q39C-Fc	954	320
IL6R90.A40C-Fc	955	320
IL6R90.P41C-Fc	956	320
IL6R90.G42C-Fc	957	320
IL6R90.K43C-Fc	958	320
IL6R90.A44C-Fc	959	320
IL6R90.L45C-Fc	960	320
IL6R90.E46C-Fc	961	320
IL6R90.W47C-Fc	962	320
IL6R90.V48C-Fc	963	320
IL6R90.S49C-Fc	964	320
IL6R90.R66C-Fc	965	320
IL6R90.F67C-Fc	966	320
IL6R90.T68C-Fc	967	320
IL6R90.I69C-Fc	968	320
IL6R90.S70C-Fc	969	320
IL6R90.R71C-Fc	970	320
IL6R90.D72C-Fc	971	320
IL6R90.N73C-Fc	972	320
IL6R90.A74C-Fc	973	320
IL6R90.K75C-Fc	974	320
IL6R90.N76C-Fc	975	320
IL6R90.T77C-Fc	976	320
IL6R90.L78C-Fc	977	320
IL6R90.Y79C-Fc	978	320
IL6R90.L80C-Fc	979	320
IL6R90.Q81C-Fc	980	320
IL6R90.M82C-Fc	981	320
IL6R90.N82aC-Fc	982	320

IL6R90.S82bC-Fc	983	320
IL6R90.L82cC-Fc	984	320
IL6R90.R83C-Fc	985	320
IL6R90.P84C-Fc	986	320
IL6R90.E85C-Fc	987	320
IL6R90.D86C-Fc	988	320
IL6R90.T87C-Fc	989	320
IL6R90.A88C-Fc	990	320
IL6R90.V89C-Fc	991	320
IL6R90.Y90C-Fc	992	320
IL6R90.Y91C-Fc	993	320
IL6R90.V93C-Fc	994	320
IL6R90.K94C-Fc	995	320
IL6R90.W103C-Fc	996	320
IL6R90.G104C-Fc	997	320
IL6R90.Q105C-Fc	998	320
IL6R90.G106C-Fc	999	320
IL6R90.T107C-Fc	1000	320
IL6R90.L108C-Fc	1001	320
IL6R90.V109C-Fc	1002	320
IL6R90.T110C-Fc	1003	320
IL6R90.V111C-Fc	1004	320
IL6R90.S112C-Fc	1005	320
IL6R90.S113C-Fc	1006	320

[0353] Reference Example 12-2 Assessment of electrophoretic mobility in polyacrylamide gel of antibodies having cysteine substitution at various positions of VHH

It was examined with non-reducing SDS-PAGE whether the IL6R90-Fc variants produced in Reference Example 12-1 show a different electrophoretic mobility to IL6R90-Fc. Sample Buffer Solution (2ME-) (x4) (Wako; 198-13282) was used for preparing electrophoresis samples, the samples were treated for 10 minutes under the condition of specimen concentration 50 microgram/mL and 70 degrees C, and then subjected to non-reducing SDS-PAGE. Mini-PROTEAN TGX Precast gel 4-20% 15well (BIORAD; 456-1096) was used for non-reducing SDS-PAGE and electrophoresis was carried out at 200 V for 2.5 hours. Then, the gel was stained with CBB stain, the gel image was captured with ChemiDocTouchMP (BIORAD), and the bands were quantified with Image Lab (BIORAD).

From the obtained gel image, the variants were classified into 7 groups according to the band pattern of each of the IL6R90-Fc variants: Single (one band at a molecular

weight region similar to that of IL6R90-Fc), Double (two bands at a molecular weight region similar to that of IL6R90-Fc), Triple (three bands at a molecular weight region similar to that of IL6R90-Fc), Several (four or more bands at a molecular weight region similar to that of IL6R90-Fc), LMW (band(s) at a molecular weight region lower than that of IL6R90-Fc), HMW (band(s) at a molecular weight region higher than that of IL6R90-Fc), and Faint (band(s) blurry and difficult to determine). Regarding the IL6R90-Fc variants classified as "Double", one of the two bands showed the same electrophoretic mobility as IL6R90-Fc while the other band showed slightly faster or slower mobility. Thus, for the IL6R90-Fc variants classified as "Double", the percentage of the bands showing different electrophoretic mobility to IL6R90-Fc (percentage of new band (%)) was also calculated. Grouping of the band patterns for IL6R90-Fc variants and the calculation results of the band percentage are shown in Table 58. From Table 58, variants classified into the Double and Triple groups are shown in Table 59. In these variants, it is highly likely that cysteine substitution caused structural changes such as crosslinkage of VHHs, which resulted in the change in electrophoretic mobility.

[0354]

[Table 58]

IL6R90-Fc variant name	Group	Percentage of new band (%)
IL6R90.E1C-Fc	Single	-
IL6R90.V2C-Fc	Single	-
IL6R90.Q3C-Fc	Single	-
IL6R90.L4C-Fc	Triple	-
IL6R90.V5C-Fc	Single	-
IL6R90.E6C-Fc	Double	65.2
IL6R90.S7C-Fc	Double	16.4
IL6R90.G8C-Fc	Double	38.4
IL6R90.G9C-Fc	Double	71.8
IL6R90.G10C-Fc	Double	9.7
IL6R90.L11C-Fc	Double	59.8
IL6R90.V12C-Fc	Double	24.8
IL6R90.Q13C-Fc	no data	-
IL6R90.P14C-Fc	Double	16.8
IL6R90.G15C-Fc	Double	18.6
IL6R90.G16C-Fc	Single	-
IL6R90.S17C-Fc	Double	16.6
IL6R90.L18C-Fc	Single	-
IL6R90.R19C-Fc	Single	-
IL6R90.L20C-Fc	Double	57.4
IL6R90.S21C-Fc	Single	-
IL6R90.A23C-Fc	Single	-
IL6R90.A24C-Fc	Double	59.3
IL6R90.S25C-Fc	Single	-
IL6R90.G26C-Fc	Single	-
IL6R90.F27C-Fc	Double	61.5
IL6R90.T28C-Fc	Single	-
IL6R90.F29C-Fc	Double	56.7
IL6R90.D30C-Fc	Single	-
IL6R90.W36C-Fc	no data	-

IL6R90.V37C-Fc	Single	-
IL6R90.R38C-Fc	Double	64.5
IL6R90.Q39C-Fc	Double	12.9
IL6R90.A40C-Fc	Double	3.2
IL6R90.P41C-Fc	Double	15.9
IL6R90.G42C-Fc	HMW	-
IL6R90.K43C-Fc	Double	9.2
IL6R90.A44C-Fc	Double	17.9
IL6R90.L45C-Fc	Double	15.4
IL6R90.E46C-Fc	Double	16.4
IL6R90.W47C-Fc	Double	12.6
IL6R90.V48C-Fc	Double	14.7
IL6R90.S49C-Fc	Double	54.1
IL6R90.R66C-Fc	Single	-
IL6R90.F67C-Fc	Double	34.8
IL6R90.T68C-Fc	Single	-
IL6R90.I69C-Fc	Double	57.5
IL6R90.S70C-Fc	Single	-
IL6R90.R71C-Fc	Double	34.3
IL6R90.D72C-Fc	Single	-
IL6R90.N73C-Fc	Single	-
IL6R90.A74C-Fc	Single	-
IL6R90.K75C-Fc	Single	-
IL6R90.N76C-Fc	Single	-
IL6R90.T77C-Fc	Single	-
IL6R90.L78C-Fc	Double	40.6
IL6R90.Y79C-Fc	Single	-
IL6R90.L80C-Fc	Double	54.7
IL6R90.Q81C-Fc	Single	-
IL6R90.M82C-Fc	Double	47.7
IL6R90.N82aC-Fc	Single	-
IL6R90.S82bC-Fc	HMW	-
IL6R90.L82cC-Fc	Double	73.2

IL6R90.R83C-Fc	Single	-
IL6R90.P84C-Fc	Single	-
IL6R90.E85C-Fc	Double	9.4
IL6R90.D86C-Fc	no data	-
IL6R90.T87C-Fc	Single	-
IL6R90.A88C-Fc	Double	66.5
IL6R90.V89C-Fc	LMW	-
IL6R90.Y90C-Fc	no data	-
IL6R90.Y91C-Fc	Triple	-
IL6R90.V93C-Fc	Triple	-
IL6R90.K94C-Fc	Double	37.7
IL6R90.W103C-Fc	Single	-
IL6R90.G104C-Fc	no data	-
IL6R90.Q105C-Fc	Single	-
IL6R90.G106C-Fc	no data	-
IL6R90.T107C-Fc	Double	53.6
IL6R90.L108C-Fc	Single	-
IL6R90.V109C-Fc	Faint	-
IL6R90.T110C-Fc	Single	-
IL6R90.V111C-Fc	Single	-
IL6R90.S112C-Fc	Single	-
IL6R90.S113C-Fc	Single	-

[0355]

[Table 59]

IL6R90-Fc variant name	Group	Percentage of new band (%)
IL6R90.L4C-Fc	Triple	-
IL6R90.E6C-Fc	Double	65.2
IL6R90.S7C-Fc	Double	16.4
IL6R90.G8C-Fc	Double	38.4
IL6R90.G9C-Fc	Double	71.8
IL6R90.G10C-Fc	Double	9.7
IL6R90.L11C-Fc	Double	59.8
IL6R90.V12C-Fc	Double	24.8
IL6R90.P14C-Fc	Double	16.8
IL6R90.G15C-Fc	Double	18.6
IL6R90.S17C-Fc	Double	16.6
IL6R90.L20C-Fc	Double	57.4
IL6R90.A24C-Fc	Double	59.3
IL6R90.F27C-Fc	Double	61.5
IL6R90.F29C-Fc	Double	56.7
IL6R90.R38C-Fc	Double	64.5
IL6R90.Q39C-Fc	Double	12.9
IL6R90.A40C-Fc	Double	3.2
IL6R90.P41C-Fc	Double	15.9
IL6R90.K43C-Fc	Double	9.2
IL6R90.A44C-Fc	Double	17.9
IL6R90.L45C-Fc	Double	15.4
IL6R90.E46C-Fc	Double	16.4
IL6R90.W47C-Fc	Double	12.6
IL6R90.V48C-Fc	Double	14.7
IL6R90.S49C-Fc	Double	54.1
IL6R90.F67C-Fc	Double	34.8
IL6R90.I69C-Fc	Double	57.5
IL6R90.R71C-Fc	Double	34.3
IL6R90.L78C-Fc	Double	40.6

IL6R90.L80C-Fc	Double	54.7
IL6R90.M82C-Fc	Double	47.7
IL6R90.L82cC-Fc	Double	73.2
IL6R90.E85C-Fc	Double	9.4
IL6R90.A88C-Fc	Double	66.5
IL6R90.Y91C-Fc	Triple	-
IL6R90.V93C-Fc	Triple	-
IL6R90.K94C-Fc	Double	37.7
IL6R90.T107C-Fc	Double	53.6

[0356] [Reference Example 13] Assessment of CD3 agonist activity of antibodies having cysteine substitution within the Fab

Reference Example 13-1 Production of antibodies having cysteine substitution at the constant region

An anti-human CD3 agonist antibody, OKT3 (heavy chain: OKT3VH0000-G1T4 (SEQ ID NO: 1007), light chain: OKT3VL0000-KT0 (SEQ ID NO: 1008)), was subjected to a study in which an arbitrary amino acid residue structurally exposed to the surface was substituted with cysteine.

Amino acid residues within the OKT3 heavy chain constant region (G1T4, SEQ ID NO: 1009) were substituted with cysteine to produce variants of the OKT3 heavy chain constant region shown in Table 60. These variants of the OKT3 heavy chain constant region were each linked with the OKT3 heavy chain variable region (OKT3VH0000, SEQ ID NO: 1010) to produce OKT3 heavy chain variants, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art.

[0357] [Table 60]

Variant of OKT3 heavy chain constant region	Position of cysteine substitution (EU numbering)	SEQ ID NO:
G1T4.T135C	135	1017
G1T4.S136C	136	1018
G1T4.S191C	191	1019

[0358] Similarly, an amino acid residue within the OKT3 light chain constant region (KT0, SEQ ID NO: 1011) was substituted with cysteine to produce a variant of the OKT3 light chain constant region shown in Table 61. This variant of the OKT3 light chain constant region was linked with the OKT3 light chain variable region (OKT3VL0000, SEQ ID NO: 1012) to produce an OKT3 light chain variant, and an expression vector encoding the corresponding gene was produced by a method known to the person

skilled in the art.

[0359] [Table 61]

Variant of OKT3 light chain constant region	Position of cysteine substitution (Kabat numbering)	SEQ ID NO:
KT0.K126C	126	1020

[0360] The above-produced OKT3 heavy chain variants and OKT3 light chain variant were each combined with the OKT3 light chain and OKT3 heavy chain, and the OKT3 variants shown in Table 62 were expressed by transient expression using FreeStyle293 cells (Invitrogen) or Expi293 cells (Life technologies) by a method known to the person skilled in the art, and purified with Protein A by a method known to the person skilled in the art. Further, an anti-KLH antibody, IC17 ((heavy chain: IC17HdK-G1T4 (SEQ ID NO: 1013), light chain: IC17L-k0 (SEQ ID NO: 1014)) was similarly prepared as a negative control.

[0361] [Table 62]

Antibody name	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:	Light chain variable region SEQ ID NO:	Light chain constant region SEQ ID NO:
H_T135C	1010	1017	1012	1011
H_S136C	1010	1018	1012	1011
H_S191C	1010	1019	1012	1011
L_K126C	1010	1009	1012	1020

[0362] Reference Example 13-2 Preparation of Jurkat cell suspension

Jurkat cells (TCR/CD3 Effector Cells (NFAT), Promega) were collected from flasks. The cells were washed with Assay Buffer (RPMI 1640 medium (Gibco), 10% FBS (HyClone), 1% MEM Non Essential Amino Acids (Invitrogen), and 1 mM Sodium Pyruvate (Invitrogen)), and then suspended at 3 x 10^6 cells/mL in Assay Buffer. This suspension of Jurkat cells was subjected to subsequent experiments.

[0363] Reference Example 13-3 Preparation of luminescence reagent solution

100 mL of Bio-Glo Luciferase Assay Buffer (Promega) was added to the bottle of Bio-Glo Luciferase Assay Substrate (Promega), and mixed by inversion. The bottle was protected from light and frozen at -20 degrees C. This luminescence reagent solution was subjected to subsequent experiments.

[0364] Reference Example 13-4 Assessment of T cell activation of antibodies having cysteine substitution at the constant region

T cell activation by agonist signaling was assessed based on the fold change of lu-

ciferase luminescence. The aforementioned Jurkat cells are cells transformed with a luciferase reporter gene having an NFAT responsive sequence. When the cells are stimulated by an anti-TCR/CD3 antibody, the NFAT pathway is activated via intracellular signaling, thereby inducing luciferase expression. The Jurkat cell suspension prepared as described above was added to a 384-well flat-bottomed white plate at 10 microliter per well (3 x 10^4 cells/well). Next, the antibody solution prepared at each concentration (10,000, 1,000, 100, 10, 1, and 0.1 ng/mL) was added at 20 microliter per well. This plate was allowed to stand in a 5% CO_2 incubator at 37 degrees C for 24 hours. After the incubation, the luminescence reagent solution was thawed, and 30 microliter of the solution was added to each well. The plate was then allowed to stand at room temperature for 10 minutes. Luciferase luminescence in each well of the plate was measured using a luminometer. The amount of luminescence (fold) was determined by dividing the amount of luminescence in the wells added with the antibody with the amount of luminescence in the wells lacking the antibody.

As a result, among the OKT3 variants having cysteine substitution at the constant region, multiple variants greatly increased the T cell activated state as compared to OKT3 as shown in Fig. 46. This result shows that there are multiple cysteine modifications that can crosslink Fabs and enhance CD3 agonist activities.

[0365] [Reference Example 14] Assessment of CD3 agonist activity of antibodies having different cysteine substitutions in the two Fabs

Reference Example 14-1 Production of antibodies having heterologous cysteine substitution at the constant region

An anti-human CD3 agonist antibody, OKT3 (heavy chain: OKT3VH0000-G1T4 (SEQ ID NO: 1007), light chain: OKT3VL0000-KT0 (SEQ ID NO: 1008)), was subjected to a study in which an arbitrary amino acid residue structurally exposed to the surface was substituted with cysteine.

An amino acid residue within the OKT3 heavy chain constant region 1 (G1T4k, SEQ ID NO: 1015) was substituted with cysteine to produce a variant of the OKT3 heavy chain constant region shown in Table 63. This variant of the OKT3 heavy chain constant region was linked with the OKT3 heavy chain variable region (OKT3VH0000, SEQ ID NO: 1010) to produce OKT3 heavy chain variant 1, and an expression vector encoding the corresponding gene was produced by a method known to the person skilled in the art. Similarly, amino acid residues within the OKT3 heavy chain constant region 2 (G1T4h, SEQ ID NO: 1016) were substituted with cysteine to produce variants of the OKT3 heavy chain constant region shown in Table 64. These variants of the OKT3 heavy chain constant region were each linked with the OKT3 heavy chain variable region (OKT3VH0000, SEQ ID NO: 1010) to produce OKT3 heavy chain variant 2, and expression vectors encoding the corresponding genes were

produced by a method known to the person skilled in the art. It is noted that heavy-chain constant regions 1 and 2 in this Reference Example are introduced with the Knobs-into-Holes (KiH) modification at the CH3 region for promoting heterodimerization.

[0366] [Table 63]

Variant of OKT3 heavy chain constant region 1	Position of cysteine substitution (EU numbering)	SEQ ID NO:
G1T4k.S191C	191	1022

[0367] [Table 64]

Variant of OKT3 heavy chain constant region 2	Position of cysteine substitution (EU numbering)	SEQ ID NO:
G1T4h.V188C	188	1023
G1T4h.P189C	189	1024
G1T4h.S190C	190	1025
G1T4h.S191C	191	1026
G1T4h.S192C	192	1027
G1T4h.L193C	193	1028
G1T4h.G194C	194	1029

[0368] The above-produced OKT3 heavy chain variant 1 and OKT3 heavy chain variant 2 were combined with the OKT3 light chain, and the OKT3 variants shown in Table 65 were expressed by transient expression using FreeStyle293 cells (Invitrogen) or Expi293 cells (Life technologies) by a method known to the person skilled in the art, and purified with Protein A by a method known to the person skilled in the art. Further, an anti-KLH antibody, IC17 (heavy chain: IC17HdK-G1T4 (SEQ ID NO: 1013), light chain: IC17L-k0 (SEQ ID NO: 1014)) was similarly prepared as a negative control.

[0369]

[Table 65]

Antibody name	Heavy chain variant 1		Heavy chain variant 2		Light chain variable region SEQ ID NO:	Light chain constant region SEQ ID NO:
	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:		
OKT3_KiH	1010	1015	1010	1016	1012	1011
H_S191C_KiH	1010	1022	1010	1026	1012	1011
H_S191C/V188C_KiH	1010	1022	1010	1023	1012	1011
H_S191C/P189C_KiH	1010	1022	1010	1024	1012	1011
H_S191C/S190C_KiH	1010	1022	1010	1025	1012	1011
H_S191C/S192C_KiH	1010	1022	1010	1027	1012	1011
H_S191C/L193C_KiH	1010	1022	1010	1028	1012	1011
H_S191C/G194C_KiH	1010	1022	1010	1029	1012	1011

[0370] Reference Example 14-2 Preparation of Jurkat cell suspension

Jurkat cell suspension was prepared as in Reference Example 13-2.

[0371] Reference Example 14-3 Preparation of luminescence reagent solution

Luminescence reagent solution was prepared as in Reference Example 13-3.

[0372] Reference Example 14-4 Assessment of T cell activation of antibodies having heterologous cysteine substitution at the constant region

T cell activation was assessed as in Reference Example 13-4.

As a result, OKT3 variants having different cysteine substitutions at the two constant regions of the antibody greatly increased the T cell activated state as compared to OKT3, as shown in Fig. 47. This result shows that even different cysteine substitutions between the Fabs can crosslink Fabs and enhance CD3 agonist activities.

[0373] [Reference Example 15] Assessment of CD3 agonist activity of antibodies having charge modification within the Fab

Reference Example 15-1 Production of antibodies having charged amino acid substitution at the constant region

The heavy chain of an anti-human CD3 agonist antibody, OKT3 (heavy chain: OKT3VH0000-G1T4 (SEQ ID NO: 1007), light chain: OKT3VL0000-KT0 (SEQ ID NO: 1008)), was subjected to a study in which an arbitrary amino acid residue structurally exposed to the surface was substituted with charged amino acid. Amino acid residues within the OKT3 heavy chain constant region 1 (G1T4k, SEQ ID NO: 1015) were substituted with arginine (R) or lysine (K) to produce a variant of the OKT3 heavy chain constant region shown in Table 66. This variant of the OKT3 heavy chain constant region was linked with the OKT3 heavy chain variable region (OKT3VH0000, SEQ ID NO: 1010) to produce OKT3 heavy chain variant 1, and an expression vector encoding the corresponding gene was produced by a method known to the person skilled in the art. Similarly, amino acid residues within the OKT3 heavy chain constant region 2 (G1T4h, SEQ ID NO: 1016) were substituted with aspartic acid (D) or glutamic acid (E) to produce variants of the OKT3 heavy chain constant region shown in Table 67. These variants of the OKT3 heavy chain constant region were each linked with the OKT3 heavy chain variable region (OKT3VH0000, SEQ ID NO: 1010) to produce OKT3 heavy chain variant 2, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art. It is noted that the CH3 regions of heavy chain constant regions 1 and 2 in this Reference Example are introduced with the Knobs-into-Holes (KiH) modification for promoting heterodimerization.

[0374] [Table 66]

Variant of OKT3 heavy chain constant region 1	Amino acid modification (EU numbering)	SEQ ID NO:
G1T4k0004	S134R/T135R/S136R/G137R/S191R/ S192R/L193R/G194R/T195R/Q196R	1030

[0375] [Table 67]

Variant of OKT3 heavy chain constant region 2	Amino acid modification (EU numbering)	SEQ ID NO:
G1T4h0004	S134D/T135D/S136D/G137D/S191D/ S192D/L193D/G194D/T195D/Q196D	1031
G1T4h0006	S134E/T135E/S136E/G137E/S191E/ S192E/L193E/G194E/T195E/Q196E	1032

[0376] The above-produced OKT3 heavy chain variant 1 and OKT3 heavy chain variant 2 were combined with the OKT3 light chain, and the OKT3 variants shown in Table 68 were expressed by transient expression using FreeStyle293 cells (Invitrogen) or

Expi293 cells (Life technologies) by a method known to the person skilled in the art, and purified with Protein A by a method known to the person skilled in the art. Further, an anti-KLH antibody, IC17 (heavy chain: IC17HdK-G1T4 (SEQ ID NO: 1013), light chain: IC17L-k0 (SEQ ID NO: 1014)) was similarly prepared as a negative control.

[0377] [Table 68]

Antibody name	Heavy chain variant 1		Heavy chain variant 2		Light chain variable region SEQ ID NO:	Light chain constant region SEQ ID NO:
	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:		
OKT3_KiH	1010	1015	1010	1016	1012	1011
0004//0004	1010	1030	1010	1031	1012	1011
0004//0006	1010	1030	1010	1032	1012	1011
0004//OKT3	1010	1030	1010	1016	1012	1011
OKT3//0004	1010	1015	1010	1031	1012	1011
OKT3//0006	1010	1015	1010	1032	1012	1011

[0378] Reference Example 15-2 Preparation of Jurkat cell suspension

Jurkat cell suspension was prepared as in Reference Example 13-2.

[0379] Reference Example 15-3 Preparation of luminescence reagent solution

Luminescence reagent solution was prepared as in Reference Example 13-3.

[0380] Reference Example 15-4 Assessment of T cell activation of antibodies having substitution with amino acids other than cysteine at the constant region

T cell activation was assessed as in Reference Example 13-4.

As a result, OKT3 variants introduced with positively charged amino acid substitution at one constant region and with negatively charged amino acid substitution at the other constant region greatly increased the T cell activated state as compared to OKT3 as shown in Fig. 48. Meanwhile, OKT3 variants introduced with positively or negatively charged amino acid substitution at one constant region and with no modification at the other constant region hardly changed the T cell activated state as compared to OKT3. This result shows that not only cysteine substitution but also charged amino acid substitution can crosslink Fabs by noncovalent bond and enhance CD3 agonist activities.

[0381] [Reference Example 16] Assessment of CD3 agonist activity of antibodies having cysteine substitution within the Fab and lacking disulfide bonds in the hinge region

Reference Example 16-1 Production of antibodies having cysteine substitution within

the Fab and lacking disulfide bonds in the hinge region

The heavy chain of an anti-human CD3 agonist antibody, OKT3 (heavy chain: OKT3VH0000-G1T4 (SEQ ID NO: 1007), light chain: OKT3VL0000-KT0 (SEQ ID NO: 1008)), was subjected to a study in which the disulfide bonds in the hinge region were removed and an amino acid residue structurally exposed to the surface was substituted with cysteine.

Cysteine in the hinge region of OKT3 heavy chain constant region (G1T4, SEQ ID NO: 1009) was substituted with serine to produce variants of the OKT3 heavy chain constant region shown in Table 69. The amino acid residue at position 191 (EU numbering) of these variants of OKT3 heavy chain constant region was substituted with cysteine to produce variants of the OKT3 heavy chain constant region shown in Table 70. These variants of the OKT3 heavy chain constant region were each linked with the OKT3 heavy chain variable region (OKT3VH0000, SEQ ID NO: 1010) to produce OKT3 heavy chain variants, and expression vectors encoding the corresponding genes were produced by a method known to the person skilled in the art.

[0382] [Table 69]

Variant of OKT3 heavy chain constant region	Amino acid modification (EU numbering)	SEQ ID NO:
G1T4.dh1	C226S	1033
G1T4.dh2	C229S	1034
G1T4.dh3	C226S/C229S	1035

[0383] [Table 70]

Variant of OKT3 heavy chain constant region	Amino acid modification (EU numbering)	SEQ ID NO:
G1T4.S191C.dh1	S191C/C226S	1036
G1T4.S191C.dh2	S191C/C229S	1037
G1T4.S191C.dh3	S191C/C226S/C229S	1038

[0384] The above-produced OKT3 heavy chain variants were combined with the OKT3 light chain, and the OKT3 variants shown in Table 71 were expressed by transient expression using FreeStyle293 cells (Invitrogen) or Expi293 cells (Life technologies) by a method known to the person skilled in the art, and purified with Protein A by a method known to the person skilled in the art. Further, an anti-KLH antibody, IC17 (heavy chain: IC17HdK-G1T4 (SEQ ID NO: 1013), light chain: IC17L-k0 (SEQ ID NO: 1014)) was similarly prepared as a negative control.

[0385]

[Table 71]

Antibody name	Heavy chain variable region SEQ ID NO:	Heavy chain constant region SEQ ID NO:	Light chain variable region SEQ ID NO:	Light chain constant region SEQ ID NO:
dh1	1010	1033	1012	1011
dh2	1010	1034	1012	1011
dh3	1010	1035	1012	1011
H_S191C_dh1	1010	1036	1012	1011
H_S191C_dh2	1010	1037	1012	1011
H_S191C_dh3	1010	1038	1012	1011

[0386] Reference Example 16-2 Preparation of Jurkat cell suspension

Jurkat cell suspension was prepared as in Reference Example 13-2.

[0387] Reference Example 16-3 Preparation of luminescence reagent solution

Luminescence reagent solution was prepared as in Reference Example 13-3.

[0388] Reference Example 16-4 Assessment of T cell activation of antibodies having cysteine substitution within the Fab and lacking disulfide bonds in the hinge region

T cell activation was assessed as in Reference Example 13-4.

As a result, OKT3 variants with only the disulfide bonds in the hinge region removed reduced or hardly changed the T cell activated state as compared to OKT3 as shown in Fig. 49. On the other hand, OKT3 variants with the disulfide bonds in the hinge region removed and introduced with cysteine substitution at the constant region greatly increased the T cell activated state as compared to OKT3. This result shows that even when there is no disulfide bond in the hinge region, cysteine substitution within the Fab can crosslink Fabs and enhance CD3 agonist activities.

[0389] [Reference Example 17] Production of expression vectors for modified antibodies, and expression and purification of modified antibodies

An antibody gene inserted in an expression vector for animal cells was subjected to amino acid residue sequence substitution by a method known to the person skilled in the art using PCR, the In-Fusion Advantage PCR cloning kit (TAKARA), or such, to construct an expression vector for a modified antibody. The nucleotide sequence of the resulting expression vector was determined by a method known to the person skilled in the art. The produced expression vector was transiently introduced into FreeStyle293 (registered trademark) or Expi293 (registered trademark) cells (Invitrogen) and the cells were allowed to express the modified antibody into culture supernatant. The modified antibody was purified from the obtained culture supernatant by a method known to the person skilled in the art using Protein A and such. Absorbance at 280 nm was measured using a spectrophotometer. An absorption coefficient was calculated

from the measured value using the PACE method and used to calculate the antibody concentration (Protein Science 1995;4:2411-2423).

[0390] [Reference Example 18] Preparation of bispecific antibodies

The purified antibody was dialyzed into TBS or PBS buffer and its concentration was adjusted to 1 mg /mL. As a 10x reaction buffer, 250 mM 2-MEA (SIGMA) was prepared. Two different homodimeric antibodies prepared in Reference Example 17 were mixed in equal amount. To this mixture, a 1/10 volume of the 10x reaction buffer was added and mixed. The mixture was allowed to stand at 37 degrees C for 90 minutes. After the reaction, the mixture was dialyzed into TBS or PBS to obtain a solution of a bispecific antibody in which the above two different antibodies were heterodimerized. The antibody concentration was measured by the above-mentioned method, and the antibody was subjected to subsequent experiments.

[0391] [Reference Example 19] Assessment of agonist activity

Reference Example 19-1 Preparation of Jurkat cell suspension

Jurkat cells (TCR/CD3 Effector Cells (NFAT), Promega) were collected from flasks. The cells were washed with Assay Buffer (RPMI 1640 medium (Gibco), 10% FBS (HyClone), 1% MEM Non Essential Amino Acids (Invitrogen), and 1 mM Sodium Pyruvate (Invitrogen)), and then suspended at 3 x 10^6 cells/mL in Assay Buffer. This suspension of Jurkat cells was subjected to subsequent experiments.

[0392] Reference Example 19-2 Preparation of luminescence reagent solution

100 mL of Bio-Glo Luciferase Assay Buffer (Promega) was added to the bottle of Bio-Glo Luciferase Assay Substrate (Promega), and mixed by inversion. The bottle was protected from light and frozen at -20 degrees C. This luminescence reagent solution was subjected to subsequent experiments.

[0393] Reference Example 19-3 T cell activation assay

T cell activation by agonist signaling was assessed based on the fold change of luciferase luminescence. The aforementioned Jurkat cells are cells transformed with a luciferase reporter gene having an NFAT responsive sequence. When the cells are stimulated by an anti-TCR/CD3 antibody, the NFAT pathway is activated via intracellular signaling, thereby inducing luciferase expression. The Jurkat cell suspension prepared as described above was added to a 384-well flat-bottomed white plate at 10 microliter per well (3 x 10^4 cells/well). Next, the antibody solution prepared at each concentration (150, 15, 1.5, 0.15, 0.015, 0.0015, 0.00015, and 0.000015 nM) was added at 20 microliter per well. This plate was allowed to stand in a 5% CO_2 incubator at 37 degrees C for 24 hours. After the incubation, the luminescence reagent solution was thawed, and 30 microliter of the solution was added to each well. The plate was then allowed to stand at room temperature for 10 minutes. Luciferase luminescence in each well of the plate was measured using a luminometer.

[0394] [Reference Example 20] Assessment of agonist activity of CD3 biparatopic antibodies using Jurkat cells

Antibodies were prepared and their activities were assessed according to Reference Examples 17, 18, and 19. The antibodies used in this Example are shown in Table 72.

[0395] [Table 72]

Antibody name	SEQ ID NO (Antibody 1):		SEQ ID NO (Antibody 2):		Molecular form
	Heavy chain	Light chain	Heavy chain	Light chain	
CD3-G1sLL	1039	1040	-	-	Monospecific antibody
CD3//OKT3-G1s	1041	1042	1043	1044	Bispecific antibody
CD3//OKT3-G1sHH	1045	1046	1047	1048	Bispecific antibody
CD3//OKT3-G1sLH	1049	1050	1051	1052	Bispecific antibody
OKT3-G1s	1053	1054	-	-	Monospecific antibody
OKT3-G1sHH	1055	1056	-	-	Monospecific antibody
CD3-G1sLL + OKT3-G1s	1057	1058	1059	1060	Monospecific antibody

[0396] As a result, modified molecules with an additional disulfide bond linking the Fab-Fab of two types of anti-CD3 bispecific antibodies showed varied CD3-mediated signaling compared to bispecific antibodies lacking the additional disulfide bond as shown in Fig. 50.

This result suggests that introducing modifications of the present invention can enhance or diminish agonist activity possessed by bispecific antigen-binding molecules having different epitopes for the same target.

[0397] [Reference Example 21] Assessment of CD137 agonist activity using Jurkat cells

Antibodies were prepared and their activities were assessed according to Reference Examples 17, 18, and 19. The antibodies used in this Reference Example were as follows: an ordinary anti-CD137 antibody, an antibody introduced with a mutation that promotes association of antibodies (hexamerization) in its heavy-chain constant region, and modified antibodies produced by linking the Fab-Fab of each of the above antibodies with an additional disulfide bond.

[0398] T cell activation by agonist signaling was assessed based on the fold change of luciferase luminescence. The cells of GloResponseTM NF-kappa B-Luc2/4-1BB Jurkat cell line (Promega) are cells transformed with a luciferase reporter gene having an NFAT responsive sequence. When the cells are stimulated by an anti-CD137 antibody, the NFAT pathway is activated via intracellular signaling, thereby inducing luciferase expression. The Jurkat cell suspension prepared at 2 x 10^6 cells/mL with Assay medium (99% RPMI, 1% FBS) was added to a 96-well flat-bottomed white plate at 25

microliter per well (5 x 10^4 cells/well). Next, the antibody solution containing ATP or the antibody solution without ATP prepared at each antibody concentration (final concentration: 45, 15, 5, 1.667, 0.556, 0.185, 0.062, and 0.021 microgram/mL) was added at 25 microliter per well. The final concentration of ATP was 250 nM. This plate was allowed to stand in a 5% CO_2 incubator at 37 degrees C for 6 hours. After the incubation, the luminescence reagent solution was thawed, and 75 microliter of the solution was added to each well. The plate was then allowed to stand at room temperature for 10 minutes. Luciferase luminescence in each well of the plate was measured using a luminometer. The value of the luminescence of each well divided by the value of the luminescence of the well without antibody addition was defined as Luminescence fold, and it served as an indicator for assessing the activity of each antibody.

[0399] As a result, antibodies introduced with the hexamerization modification showed increased agonist activity as compared to an ordinary anti-CD137 antibody. Further, in modified antibodies where each of the antibodies was introduced with additional disulfide bonds, synergistic increase in agonist activity was observed.

This result suggests that introducing modifications of the present invention can enhance the activity of an anti-CD137 agonist antibody.

[0400] [Reference Example 22] Assessment of agonist activity of CD3//PD1 bispecific antibodies using Jurkat cells

Reference Example 22-1

Antibodies were prepared and their activities were assessed according to Reference Examples 17, 18, and 19. The antibodies used in this Example are shown in Table 74.

[0401]

[Table 74]

Antibody name	SEQ ID NO (Antibody 1):		SEQ ID NO (Antibody 2):		Molecular form
	Heavy chain	Light chain	Heavy chain	Light chain	
OKT3//117-G1silent	1113	1114	1115	1116	Bispecific antibody
OKT3//117-G1silentLL	1117	1118	1119	1120	Bispecific antibody
OKT3//117-G1silentHH	1121	1122	1123	1124	Bispecific antibody
OKT3//117-G1silentHL	1125	1126	1127	1128	Bispecific antibody
OKT3//10-G1silent	1129	1130	1131	1132	Bispecific antibody
OKT3//10-G1silentHH	1133	1134	1135	1136	Bispecific antibody
OKT3//10-G1silentHL	1137	1138	1139	1140	Bispecific antibody
CD3//949-G1silent	1141	1142	1143	1144	Bispecific antibody
CD3//949-G1silentLL	1145	1146	1147	1148	Bispecific antibody
CD3//949-G1silentHH	1149	1150	1151	1152	Bispecific antibody
CD3//949-G1silentLH	1153	1154	1155	1156	Bispecific antibody
CD3//949-G1silentHL	1157	1158	1159	1160	Bispecific antibody
OKT3//949-G1silent	1161	1162	1163	1164	Bispecific antibody
OKT3//949-G1silentLL	1165	1166	1167	1168	Bispecific antibody
OKT3//949-G1silentHH	1169	1170	1171	1172	Bispecific antibody
OKT3//949-G1silentHL	1173	1174	1175	1176	Bispecific antibody

[0402] As a result, in multiple bispecific antibodies consisting of a combination of an anti-CD3 antibody and an anti-PD1 antibody, modified molecules with an additional disulfide bond linking the Fab-Fab showed greatly varied CD3- and/or PD1-mediated signaling compared to bispecific antibodies lacking the additional disulfide bond as shown in Fig. 51.

This result suggests that introducing modifications of the present invention can enhance or diminish agonist activity possessed by antigen-binding molecules such as antibodies.

[0403] Reference Example 22-2

Antibodies were prepared and their activities were assessed according to Reference Examples 2, 3, and 4. The antibodies used in this Reference Example are shown in Table 75.

[0404]

[Table 75]

Antibody name	SEQ ID NO (Antibody 1):		SEQ ID NO (Antibody 2):		Molecular form
	Heavy chain	Light chain	Heavy chain	Light chain	
OKT3//949-G1silent	1161	1162	1163	1164	Bispecific antibody
OKT3//949-G1silentHH	1169	1170	1171	1172	Bispecific antibody
OKT3//949-G1silentHL	1173	1174	1175	1176	Bispecific antibody
OKT3//949-G1silentLL	1165	1166	1167	1168	Bispecific antibody
OKT3//949-G1silentLH	1177	1178	1179	1180	Bispecific antibody

[0405] The presence or absence of PD-1 agonist signaling was assessed by the ratio of the fluorescent signal from BRET when PD-1 is in the vicinity of SHP2 (618 nm) and the luminescence originating from SHP2, which is the donor (460 nm). One day before the assay, antigen presenting cells expressing PD-L1 (Promega, #J109A) were seeded into F-12 medium containing 10% FBS (Gibco, 11765-054) in a 96-well plate (Costar, #3917) at 4.0 x 10^4 cells/100 microliter/well, and the cells were cultured in a CO_2 incubator for 16-24 hours at 37 degrees C. On the day of the assay, HaloTag nanoBRET 618 Ligand (Promega, #G980A) was diluted 250-fold with Opti-MEM (Gibco, #31985-062). The medium for culturing PD-L1-expressing antigen presenting cells were removed, and the diluted HaloTag nanoBRET 618 Ligand was added at 25 microliter/well. The specimen for assessment diluted with Opti-MEM containing 10 microgram/mL of PD-L1-inhibiting antibodies (40, 8, and 1.6 microgram/mL) was added at 25 microliter/well. PD-1/SHP2 Jurkat cells (Promega, #CS2009A01) were added to the above-noted 96-well plate at 5 x 10^4 cells/50 microliter/well, thoroughly suspended, and then incubated in a CO_2 incubator for 2.5 hours at 37 degrees C. nanoBRET Nano-Glo substrate (Promega, #N157A) was diluted 100-fold with Opti-MEM, and this was added at 25 microliter/well to the 96-well plate after incubation. The plate was allowed to stand at room temperature for 30 minutes, and then the Em460mM and Em618nm were measured using Envision (PerkinElmer, 2104 EnVision). The obtained values were applied to the following equation to calculate the BRET Ratio (mBU).

618nm / 460nm = BU

BU x 1000 = mBU

Mean $mBU_{experimental}$ - Mean $mBU_{no\ PD\text{-}L1\ block\ control}$ = BRET Ratio (mBU)

As a result, in the bispecific antibodies consisting of an anti-CD3 antibody and an anti-PD1 antibody, modified molecules with an additional disulfide bond linking the Fab-Fab showed greatly varied CD3- and/or PD1-mediated signaling compared to bispecific antibodies lacking the additional disulfide bond as shown in Fig. 52.

[0406] [Reference Example 23] Assessment of agonist activity of CD28/CD3 clamping bispecific antibodies

Reference Example 23-1 Real-time cell growth inhibition assay (xCELLigence assay)

Antibodies were prepared according to Reference Examples 17 and 18. The antibodies used in this Example are shown in Table 76.

[0407] [Table 76]

Antibody name	SEQ ID NO (Antibody 1):		SEQ ID NO (Antibody 2):		Molecular form
	Heavy chain	Light chain	Heavy chain	Light chain	
GPC3/attCE115	1181	1182	1183	1184	Bispecific antibody
GPC3/attCE115_LL	1185	1186	1187	1188	Bispecific antibody
KLH/clamp CD3	1189	1190	1191	1192	Bispecific antibody
GPC3/clamp CD3	1193	1194	1195	1196	Bispecific antibody
CD28/clamp CD3	1197	1198	1199	1200	Bispecific antibody
CD28/clamp CD3_HH	1201	1202	1203	1204	Bispecific antibody

[0408] T cell-dependent cancer cell growth inhibitory effect of the antibodies was assessed using xCELLigence RTCA MP instrument (ACEA Biosciences). Cells of the human liver cancer cell line SK-Hep-1 forced to express Glypican-3 (GPC3) (SEQ ID NO: 1241) (SK-pca31a) were used as target cells, and human peripheral blood mononuclear cells (PBMC: Cellular Technology Limited (CTL)) were used as effector cells. 1 x 10^4 cells of SK-pca31a were seeded onto E-Plate 96 (ACEA Biosciences). On the next day were added 2 x 10^5 cells of PBMC and antibodies to make a final concentration of 0.001, 0.01, 0.1, 1, or 10 microgram/mL. Cell growth was monitored every 15 minutes with xCELLigence, and culturing was continued for 72 hours. Cell growth inhibitory effect (CGI: %) was calculated by the following equation.

$CGI\ (\%) = 100 - (CI_{Ab}\ X\ 100 / CI_{NoAb})$

In the above equation, "CI_{Ab}" is the Cell index for a well at 72 hours after addition of an antibody (cell growth index measured with xCELLigence). Further, "CI_{NoAb}" is the Cell index for a well after 72 hours without antibody addition.

[0409] Reference Example 23-2 Cytokine production assay

Cytokine production from T cells by antibodies was assessed as discussed below.

SK-pca31a was used as the target cell and PBMC (Cellular Technology Limited (CTL)) was used as the effector cell. 1 x 10^4 cells of SK-pca31a were seeded onto a 96-well plate. On the next day were added 2 x 10^5 cells of PBMC and antibodies to make a final concentration of 0.01, 0.1, 1, or 10 microgram/mL. The culture su-

pernatant was collected after 72 hours, and human IL-6 was measured using AlphaLISA (PerkinElmer).

[0410] Results

Combined use of CD28/CD3 clamping bispecific antibody and GPC3/binding-attenuated CD3 bispecific antibody did not result in cell growth inhibitory effects. However, inhibitory effects on cancer cell growth were observed by applying modifications for introducing an additional disulfide bond between the Fab-Fab of the CD28/CD3 clamping bispecific antibody (Figs. 53 and 55). Further, cytokine production was observed when a CD28/CD3 clamping bispecific antibody introduced with the above-noted modification and a GPC3/binding-attenuated CD3 bispecific antibody were cocultured with GPC3 expressing strain and PBMC; however, mere addition of a CD28/CD3 clamping bispecific antibody introduced with the above-noted modification and a GPC3/binding-attenuated CD3 bispecific antibody to PBMC did not result in cytokine production (Figs. 54 and 56). Accordingly, it was suggested that the effect of the CD28/CD3 clamping bispecific antibody introduced with the above-noted modification and GPC3/binding-attenuated CD3 bispecific antibody on inhibiting cancer cell growth and inducing cytokine production in T cells depends on the expression of cancer antigen.

[0411] [Reference Example 24] Assessment of agonist activity of CD8/CD28 bispecific antibodies

Antibodies were prepared according to Reference Examples 17 and 18. The antibodies used in this Reference Example are shown in Table 77.

[0412] [Table 77]

Antibody name	SEQ ID NO (Antibody 1):		SEQ ID NO (Antibody 2):		Molecular form
	Heavy chain	Light chain	Heavy chain	Light chain	
KLH-P587	1205	1206	-	-	Monospecific antibody
CD8/CD28-P587	1207	1208	1209	1210	Bispecific antibody
CD8/CD28-P587(HH)	1211	1212	1213	1214	Bispecific antibody
CD8/CD28-P587(LL)	1219	1220	1221	1222	Bispecific antibody
CD8/CD28-P587(HL)	1223	1224	1225	1226	Bispecific antibody
CD8/CD28-P587(LH)	1227	1228	1229	1230	Bispecific antibody

[0413] Human peripheral blood mononuclear cells (PBMCs) isolated from healthy volunteer blood samples were used for assessing the prepared specimen. Heparin (0.5 mL) was mixed with 50 mL of blood and was further diluted with 50 mL PBS. Human PBMCs were isolated by the following two steps. In step 1, Leucosep (greiner bio-one) added with Ficoll-Paque PLUS (GE Healthcare) was centrifuged at 1000 x g for 1 minute

under room temperature, then blood diluted with PBS was added thereto and the mixture was centrifuged at 400 x g for 30 minutes under room temperature. In step 2, the buffy coat was collected from the tube after centrifugation and then washed with 60 mL PBS (Wako). The isolated human PBMCs were adjusted to a cell density of 1 x 10^7 /mL with a medium (5% human serum (SIGMA), 95% AIM-V (Thermo Fischer Scientific)). The resulting cell suspension was seeded onto the wells of a 24-well plate at 1 mL/well and the plate was incubated in a 5% CO_2 incubator at 37 degrees C. Two days later, the medium was removed from the seeded cells and the cells were washed with 500 microliter PBS, and then collected using accutase (nacalai tesque). Next, the cells were adjusted to make a cell density of 1 x 10^6/mL with ViaFluor 405 (Biotium) solution diluted with PBS to make a final concentration of 2 micromolar, and then allowed to stand at 37 degrees C for 15 minutes. Subsequently, the cells were suspended again with a medium and then seeded onto the wells of a 96-well plate at 2 x 10^5 cells per well. Antibody solution was added thereto to make a final concentration of 0.1, 1, and 10 microgram/mL, and the cells were cultured in a 5% CO_2 incubator for 4 days at 37 degrees C.

After the end of culturing, the percentage of grown cells was investigated using a flow cytometer (BD LSRFortessa (TM) X-20 (BD Biosciences)) (FCM). The percentage of grown cells was calculated from the percentage of reduced ViaFluor 405 fluorescence intensity. Fluorescently-labeled anti-CD8 alpha antibody, anti-CD4 antibody, anti-Foxp3 antibody, and such were used for performing an analysis with CD8 alpha positive T cells and regulatory T (Treg) cells. As a result, increase in activity was observed in some specimens as shown in Fig. 57.

[0414] [Reference Example 25] Assessment of disulfide bond formation between the introduced cysteines

Modified antibodies were produced by introducing cysteine into the light and heavy chains of a humanized model antibody, and the formation of disulfide bond between the newly introduced cysteines was assessed. Assessment was carried out by incubating sample antibodies in 20 mM phosphate buffer (pH 7.0) with chymotrypsin and detecting the mass of peptides presumed to be produced from the amino acid sequence of each antibody, using LC/MS. Each antibody was prepared according to Reference Examples 17 and 18. The antibodies used in this Example are shown in Table 78.

[0415]

[Table 78]

Antibody name	SEQ ID NO (Antibody 1):		SEQ ID NO (Antibody 2):		Molecular form
	Heavy chain	Light chain	Heavy chain	Light chain	
MRA-G1_LL	1231	1232	-	-	Monospecific antibody
MRA-G2_LL	1233	1234	-	-	Monospecific antibody
MRA-G4_LL	1235	1236	-	-	Monospecific antibody
MRA-G1T4.S191C	1237	1238	-	-	Monospecific antibody
MRA-G1T4.A162C	1239	1240	-	-	Monospecific antibody

[0416] First, modified antibodies of different subclass (IgG1, IgG2, and IgG4) in which lysine at position 126 (Kabat numbering) of the light chain was substituted with cysteine were analyzed. As a result, in all of the antibodies analyzed, components that correspond to the theoretical mass of a peptide having a disulfide bond between the cysteines at position 126 were detected, as shown in Table 79. Further, this component disappeared when tris(2-carboxyethyl)phosphine, which has the reducing effect of disulfide bonds, was added to the IgG1 sample, suggesting that a disulfide bond is formed between the cysteines at position 126 in this peptide. At the same time, it was suggested that the difference in subclass does not affect this disulfide bond formation.

[0417] [Table 79]

Peptide	Ion	Theoretical mass (Da)	Measured value (Da)			
			IgG1		IgG2	IgG4
			unreduced	reduced	unreduced	unreduced
((IFPPSDEQLC^{126}SGTASVVCL)-(ACEVTHQGL))2	$[M+4H]^{4+}$	1460.2	1460.2	n.d.	1460.2	1460.2
	$[M+5H]^{5+}$	1168.3	1168.4	n.d.	1168.4	1168.3
	$[M+6H]^{6+}$	973.8	973.8	n.d.	973.8	973.8

n.d. : not detected

[0418] Next, analysis was performed on modified antibodies in which alanine at position 162 (EU numbering), or serine at position 191 (EU numbering) of IgG1 heavy chain was substituted with cysteine. As a result, components that correspond to the theoretical mass of a peptide having a disulfide bond between the introduced cysteines were detected, as shown respectively in Tables 80 and 81. Further, this component disappeared when tris(2-carboxyethyl)phosphine was added to the sample of a modified antibody introduced with position 191 cysteine (Table 81). From the above, it was suggested that a disulfide bond is formed between cysteines also introduced into the heavy chain.

[0419]

[Table 80]

Peptide	Ion	Theoretical mass (Da)	Measured value (Da)
$(NSGC^{162}L)_2$	$[M+H]^+$	983.4	983.4
	$[M+2H]^{2+}$	492.2	492.2

[0420] [Table 81]

Peptide	Ion	Theoretical mass (Da)	Measured value (Da)	
			unreduced	reduced
$(SLSSVVTVPSC^{191}SLGTQTY)_2$	$[M+2H]^{2+}$	1827.9	1827.9	n.d.
	$[M+3H]^{3+}$	1218.9	1218.9	n.d.

n.d. : not detected

[0421] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention. The disclosures of all patent and scientific literature cited herein are expressly incorporated in their entirety by reference.

Industrial Applicability

[0422] In a non-limiting embodiment, the antigen-binding molecule of the present disclosure is useful in that it can hold multiple antigen molecules at spatially close positions, regulate interaction between multiple antigen molecules, and/or regulate activation of multiple antigen molecules which are activated by association with each other. In other embodiments, the antigen-binding molecule of the present disclosure is useful in that it has increased resistance to protease cleavage as compared to conventional antigen-binding molecules.

Claims

[Claim 1] A method for producing an antibody preparation, said method comprising contacting an antibody preparation with a reducing reagent, wherein the antibody comprises a first antigen-binding domain and a second antigen-binding domain which are capable of being linked with each other via at least one disulfide bond, wherein said at least one disulfide bond is capable of being formed between amino acid residues which are not in a hinge region.

[Claim 2] The method of claim 1, wherein said antibody preparation comprises two structural isoforms which differ by at least one disulfide bond formed between amino acid residues which are not in a hinge region.

[Claim 3] The method of claim 1 or 2, wherein said method preferentially enriches or increases the population of an antibody structural isoform having at least one disulfide bond formed between amino acid residues which is not in a hinge region.

[Claim 4] The method of any one of claims 1 to 3, wherein said at least one disulfide bond is an interchain disulfide bond.

[Claim 5] The method of any one of claims 1 to 4, wherein said at least one disulfide bond is formed between a CH1 region, a CL region, a VL region, a VH region and/or a VHH region of the first antigen-binding domain and the second antigen-binding domain.

[Claim 6] The method of any one of claims 1 to 5, wherein said at least one disulfide bond is formed between a CH1 region of the first antigen-binding domain and a CH1 region of the second antigen-binding domain.

[Claim 7] The method of claim 6, wherein said at least one disulfide bond is formed between the amino acid residues at position 191 according to EU numbering in the respective CH1 regions of the first antigen-binding domain and the second antigen-binding domain.

[Claim 8] The method of any one of claims 1 to 7, wherein said antibody is an IgG antibody, preferably an IgG1, IgG2, IgG3 or IgG4 antibody.

[Claim 9] The method of any one of claims 1 to 8, wherein the pH of said reducing reagent contacting with the antibody is from about 3 to about 10.

[Claim 10] The method of any one of claims 1 to 9, wherein the reducing agent is selected from the group consisting of TCEP, 2-MEA, DTT, Cysteine, GSH and Na_2SO_3.

[Claim 11] The method of any one of claims 1 to 10, wherein the contacting step is performed for at least 30 minutes.

[Claim 12] The method of any one of claims 1 to 11, wherein the contacting step is performed at a temperature of about 20 degrees Celsius to 37 degrees Celsius, preferably at 23 degrees Celsius, 25 degrees Celsius or 37 degrees Celsius, more preferably at 23 degrees Celsius.

[Claim 13] The method of any one of claims 1 to 12, wherein the concentration of the antibody is from about 1 mg/ml and about 50 mg/ml.

[Claim 14] The method of any one of claims 1 to 13, wherein said antibody is partially purified by affinity chromatography prior to said contacting with a reducing agent.

[Claim 15] The method of any one of claims 1 to 14, further comprising a step of removing the reducing agent, preferably by dialysis, more preferably by a chromatography method.