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Summary

Technical Problem

[0011] An objective of the present disclosure 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 disclosure 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 disclosure 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.

[0012a] In one aspect, the present disclosure relates to a method for producing an antibody preparation, said method comprising contacting an antibody solution with a reducing agent, and then re-oxidizing the antibody, wherein the antibody comprises a first antigen-binding domain and a second antigen-binding domain which are linked with each other via at least one disulfide bond, 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, and wherein said antibody solution comprises two structural isoforms which differ by said at least one disulfide bond.

[0013] In one non-limiting example, 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 example, 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 example, 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 example of the present disclosure 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 disclosure 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 disclosure 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 examples of the disclosure, 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 disclosure 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.

[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 example, the present disclosure 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

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 example, the present disclosure 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 example, the present disclosure 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

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 example, 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 example, 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 example, 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,

- (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 example, 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

CLAIMS

[Claim 1]

A method for producing an antibody preparation, said method comprising contacting an antibody solution with a reducing agent, and then re-oxidizing the antibody, wherein the antibody comprises a first antigen-binding domain and a second antigen-binding domain which are linked with each other via at least one disulfide bond, 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, and wherein said antibody solution comprises two structural isoforms which differ by said at least one disulfide bond.

[Claim 2]

The method of claim 1, wherein said method enriches or increases the population of an antibody structural isoform having said at least one disulfide bond.

[Claim 3]

The method of claim 1 or 2, wherein said antibody is an IgG antibody.

[Claim 4]

The method of claim 3, wherein said IgG antibody is an IgG1, IgG2, IgG3 or IgG4 antibody.

[Claim 5]

The method of any one of claims 1 to 4, wherein the pH of said reducing agent contacting with the antibody is from 3 to 10.

[Claim 6]

The method of any one of claims 1 to 5, wherein the reducing agent is selected from the group consisting of tris(2-carboxyethyl)phosphine (TCEP), 2-aminoethanethiol (2-MEA), dithiothreitol (DTT), Cysteine, glutathione (GSH) and Na₂SO₃.

[Claim 7]

The method of any one of claims 1 to 6, wherein the contacting step is performed for at least 30 minutes.

[Claim 8]

The method of any one of claims 1 to 7, wherein the contacting step is performed at a temperature of 20 degrees Celsius to 37 degrees Celsius.

[Claim 9]

The method of claim 8, wherein the contacting step is performed at 23 degrees Celsius, 25 degrees Celsius or 37 degrees Celsius.

[Claim 10]

The method of claim 9, wherein the contacting step is performed at 23 degrees Celsius.

[Claim 11]

The method of any one of claims 1 to 10, wherein the concentration of the antibody is from 1 mg/ml and 50 mg/ml.

[Claim 12]

The method of any one of claims 1 to 11, wherein said antibody is partially purified by affinity chromatography prior to said contacting with a reducing agent.

[Claim 13]

The method of any one of claims 1 to 12, further comprising a step of removing the reducing agent by dialysis or by a chromatography method, prior to said step of re-oxidizing the antibody.