(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 December 2024 (12.12.2024)

WIPO | PCT

(10) International Publication Number
WO 2024/251196 A1

(51) International Patent Classification:
A61K 47/68 (2017.01) *A61K 39/395* (2006.01)
C07K 16/00 (2006.01) *A61P 35/00* (2006.01)
C07K 16/28 (2006.01) *A61P 31/00* (2006.01)

(21) International Application Number:
PCT/CN2024/097745

(22) International Filing Date:
06 June 2024 (06.06.2024)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PCT/CN2023/098791
07 June 2023 (07.06.2023) CN

(71) Applicant *(for CN only)*: **WUXI XDC (SHANGHAI) CO., LTD.** [CN/CN]; Room 368, Section 302, 211 Fute North Road, China (Shanghai) Pilot Free Trade Zone, Pudong New Area, Shanghai 200131 (CN).

(71) Applicant *(for all designated States except CN)*: **WUXI XDC SINGAPORE PRIVATE LIMITED** [SG/SG]; 100 Tras Street #16-01 100 AM, 079027 (SG).

(72) Inventors: **FAN, Qirui**; Room 368, Section 302, 211 Fute North Road, China (Shanghai) Pilot Free Trade Zone, Pudong New Area, Shanghai 200131 (CN). **ZHANG, Lin**; Room 368, Section 302, 211 Fute North Road, China (Shanghai) Pilot Free Trade Zone, Pudong New Area, Shanghai 200131 (CN). **WANG, Laisen**; Room 368, Section 302, 211 Fute North Road, China (Shanghai) Pilot Free Trade Zone, Pudong New Area, Shanghai 200131 (CN).

(74) Agent: **CCPIT PATENT AND TRADEMARK LAW OFFICE**; 10/F, Ocean Plaza, 158 Fuxingmennei Street, Xicheng District, Beijing 100031 (CN).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY,

(54) Title: PROCESS FOR PREPARING ANTIBODY-DRUG CONJUGATES WITH IMPROVED HOMOGENEITY

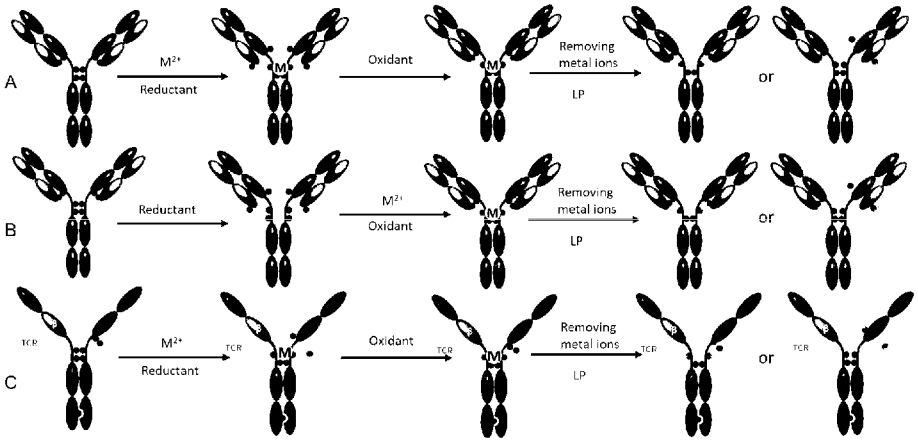


Fig. 22

(57) Abstract: Provided herein is a bio-conjugation process for preparing a composition of antibody-drug conjugates (ADCs) with improved homogeneity, wherein the antibody-drug conjugates (ADCs) with drug to antibody molar ratio of 2 (D2) are present in a high level in the composition. In the composition of ADCs prepared by the process of the present disclosure, the content of D2 is higher than 64 mol%, and the content of D0+D4+D6+D8 is less than 36 mol%, on the basis of total molar of D0, D2, D4, D6 and D8.

[Continued on next page]

WO 2024/251196 A1

MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

Process for Preparing Antibody-Drug Conjugates with Improved Homogeneity

INFORMATION OF PRIORITY

[0000] The present application claims the benefit of the priority application PCT/CN2023/098791 filed on June 7, 2023.

FIELD OF INVENTION

[0001] The present disclosure relates to a process for preparing a composition of antibody-drug conjugates (ADCs). Specifically, the present disclosure relates to a process for preparing a composition of antibody-drug conjugates (ADCs), wherein the antibody-drug conjugate (ADC) with drug to antibody molar ratio of 2 (i.e., DAR2) is present in a high level, and wherein the composition of antibody-drug conjugates have improved homogeneity.

BACKGROUND OF INVENTION

[0002] Antibody-drug conjugate refers to a group of therapeutic molecules in which a therapeutic agent is linked to a target-specific antibody which directs the agent to the target and releases it for therapeutic effects. The specificity of antibodies for specific antigens on the surface of target cells and molecules has led to their extensive use as carriers of a variety of diagnostic and therapeutic agents. For example, antibodies conjugated to labels and reporter groups such as fluorophores, radioisotopes and enzymes find use in labelling and imaging applications, while conjugation antibodies to cytotoxic agents and chemotherapy drugs allows targeted delivery of such agents to specific tissues or structures, for example particular cell types or growth factors, minimizing the impact on normal, healthy tissue and significantly reducing the side effects associated with chemotherapy treatments.

[0003] In developing antibody-drug conjugates, a therapeutic agent could be coupled to an antibody that specifically targets a certain tumor marker (e.g., a protein that, ideally, is only to be found in or on tumor cells). Antibodies track these proteins down in the body and attach themselves to the surface of cancer cells. The biochemical reaction between the antibody and the target protein (i.e., antigen) triggers a signal in the tumor cell, which then absorbs or internalizes the antibody together with the therapeutic agent (e.g., a cytotoxic drug). After the ADC is internalized, the cytotoxic drug is released and kills the tumor cells (Chari, Ravi V. J.; Martell, Bridget A.; Gross, Jonathan L.; Cook, Sherrilyn B.; Shah, Sudhir A.; Blättler, Walter A.; McKenzie, Sara J.;

Goldmacher, Victor S. (1992). "Immunoconjugates containing novel maytansinoids: promising anticancer drugs". Cancer Research. **52** (1): 127–31.). Due to this targeting, ideally the ADC has lower side effects and gives a wider therapeutic window than other chemotherapeutic agents.

[0004] For drug attachment, functional groups with high reactivity and stability on both antibody and linker-payload (i.e., linker-drug) were used for the coupling, to form stable covalent bonds. Conventional means of attaching, i.e., covalent bonding of a drug moiety to an antibody via a linker, generally leads to a heterogeneous mixture of molecules where the drug moieties are attached at several sites on the antibody. For example, cytotoxic drugs have typically been conjugated to antibodies through the often-numerous lysine residues of an antibody, generating a heterogeneous antibody-drug conjugate mixture.

[0005] For example, antibody-drug conjugates are usually produced by two conventional chemical strategies, Lysine-based conjugation and conjugation based on Cysteine from the reduction of interchain sulfide bond. For the reaction of primary amine group on Lysine residue, the most widely used connector on linker-payload is the NHS ester (i.e., N-hydroxysuccinimide). But the application of NHS ester in antibody-drug conjugate production is limited by its inherent properties, for instance, the reaction between NHS ester and primary amine is very slow under acidic conditions, so the conjugation needs to be performed in the buffer with high pH value (i.e., > 7.0), which is not friendly to antibody sometimes, and the NHS is prone to hydrolysis under basic conditions, which makes the purification and identification of free drug after conjugation more complicated. Also, due to the low reactivity of NHS ester to primary amine on antibody, the reaction needs to be carried out with high temperature (i.e., 22°C). Even more, due to the low solubility, more organic solvent is required for linker-payload prepared by NHS ester (i.e., SMCC-DM1) to be fully dissolved in the reaction systems, which increases the risk of aggregation of antibody. For conjugation based on Cysteine from the reduction of interchain sulfide bond, it comprises a step of opening inter-chain disulfide bonds in the presence of various reductants, such TCEP, DTT and so on, followed by nucleophilic reaction of thiol groups. In this conjugation process, antibody-drug conjugates are typically formed by conjugating one or more antibody cysteine thiol groups to one or more linker moieties bound to a drug thereby forming an antibody-linker-drug complex. Cysteine thiols are reactive at neutral pH, unlike most amines which are protonated and less nucleophilic near pH 7. Since free thiol (RSH, sulfhydryl) groups are relatively

reactive, proteins with cysteine residues often exist in their oxidized form as disulfide-linked oligomers or have internally bridged disulfide groups. Antibody cysteine thiol groups are generally more reactive, i.e., more nucleophilic, towards electrophilic conjugation reagents than antibody amine or hydroxyl groups. Engineering in cysteine thiol groups by the mutation of various amino acid residues of a protein to cysteine amino acids is potentially problematic, particularly in the case of unpaired (free Cys) residues or those which are relatively accessible for reaction or oxidation. In concentrated solutions of the protein, whether in the periplasm of E. coli, culture supernatants, or partially or completely purified protein, unpaired Cys residues on the surface of the protein can pair and oxidize to form intermolecular disulfides, and hence protein dimers or multimers form. Disulfide dimer formation renders the new Cys unreactive for conjugation to a drug, ligand, or other label. Furthermore, if the protein oxidatively forms an intramolecular disulfide bond between the newly engineered Cys and an existing Cys residue, both Cys groups are unavailable for active site participation and interactions. Furthermore, the protein may be rendered inactive or non-specific, by misfolding or loss of tertiary structure (Zhang et al. (2002) Anal. Biochem. 311: 1-9).

[0006] It is of great importance to develop new ADCs as therapeutic agents. However, the conventional conjugation processes always result in a heterogeneous mixture of molecules where the drug moieties are attached at several sites on the antibody. Depending on reaction conditions, the heterogeneous mixture typically contains a distribution of antibodies with from 0 to about 8, or more, attached drug moieties. In addition, within each subgroup of conjugates with a particular integer ratio of drug moieties to a single antibody, there is a potentially heterogeneous mixture where the drug moiety is attached at various sites on the antibody. Analytical and preparative methods are inadequate to separate and characterize the antibody-drug conjugate species molecules within the heterogeneous mixture resulting from a conjugation reaction. The heterogeneous mixture is so complex that it is difficult and expensive to characterize and purify. Each conjugation product in such a mixture potentially has different pharmacokinetic, distribution, toxicity and efficacy profiles, and non-specific conjugation also frequently results in impaired antibody function. Antibodies are large, complex, and structurally diverse biomolecules, often with many reactive functional groups. Antibody reactivity with linker reagents and drug-linker intermediates are dependent on factors such as pH, concentration, salt concentration, and co-solvents.

[0007] Furthermore, the number of drugs coupling to a single antibody molecule is an important factor for the efficacy and safety of the resultant ADC. For example, in the conjugation process based on native inter-chain disulfide bond reduction, the inter-chain S-S bonds are more accessible to solvents than other disulfide bonds. Therefore, the inter-chain disulfide bonds can be used as the binding sites for coupling a drug (or a drug-linker) to an antibody. In general, one therapeutic antibody molecule belonging to IgG1 or IgG4 subclass has 4 inter-chain S-S bonds, each of which is formed with two -SH groups, and thus, the number of drugs coupling to a single antibody molecule is 2, 4, 6 or 8. If the number of drugs coupling to a single antibody molecule is 0 (i.e., drug to antibody molar ratio is 0 (i.e., DAR0)), the product is referred to as D0 (i.e., actually representing one single antibody molecule which is not conjugated with any drug). Accordingly, D2 refers to an ADC molecule in which two drug molecules are coupled to one single antibody molecule (i.e., drug to antibody molar ratio is 2 (i.e., DAR2)), where two drug molecules may be coupled to -SH groups generated by reduction of S-S bonds between heavy and light chains, or may be coupled to -SH groups generated by reduction of S-S bonds between heavy and heavy chains. D4 refers to an ADC molecule in which four drug molecules are coupled to one single antibody molecule (i.e., drug to antibody molar ratio is 4 (i.e., DAR4)). D6 refers to an ADC molecule in which six drug molecules are coupled to one single antibody molecule (i.e., drug to antibody molar ratio is 6 (i.e., DAR6)). And D8 refers to an ADC molecule in which eight drug molecules are coupled to one single antibody molecule (i.e., drug to antibody molar ratio is 8 (i.e., DAR8)), i.e., all the four S-S bonds in one antibody molecule are reduced to eight -SH groups and each -SH group attaches one drug molecule. In general, the heterogeneous mixture of ADC molecules generated by conventional conjugation processes is a mixture of D0, D2, D4, D6 and D8. Among them, D0 has no ADC efficacy, and due to their hydrophobicity induced from payload (i.e., drug) molecules, D6 and D8 are considered to be the reason of *in vivo* instability and unpredictable safety and PK profiles. Although antibody-drug conjugate potency in vitro has been shown to be directly dependent on drug loading (Hamblett KJ, et al., Clin Cancer Res. 2004 Oct 15;10(20):7063-70), in-vivo antitumor activity of antibody-drug conjugates with four drugs per molecule (D4) was comparable with conjugates with eight drugs per molecule (D8) at equal mAb doses, even though the conjugates contained half the amount of drug per mAb. Drug-loading also affected plasma clearance, with the D8 conjugate being cleared 3-fold faster than the D4 conjugate and 5-fold faster than a D2 conjugate. Therefore, on one hand, for payloads with outstanding

efficacy (i.e., high activity payload), the conjugate prepared as D2 can control the safety risk of drugs and improve the circulating stability of conjugates in vivo under the premise of ensuring efficacy, on the other hand, for special mechanisms of action and spatial structure, such as nucleic acid loading or immune antagonist, and the like, D2 conjugate can reduce the impact of conjugated large molecular weight payloads on antibody function as much as possible. In general, if the content of D2 is high in the mixture of ADCs, the ADCs are considered to have a high homogeneity. Antibody-drug conjugates with improved homogeneity provide benefits in therapy, for example a higher therapeutic index, improving efficacy and reducing toxicity of the drug. Homogeneous antibody conjugates also provide more accurate and consistent measurements in diagnostic and imaging applications. So, novel processes for preparing ADCs with high homogeneity are highly desirable and long-term pursuit.

[0008] Accordingly, several methods have been developed to improve the homogeneity of antibody-drug conjugates. In this regard, several site-specific labeling technologies have been developed and applied to prepare ADCs for pre-clinical and clinical studies.

[0009] Firstly, the introduction of engineered reactive cysteine residues has become a common approach for site-specific conjugation. like ThioMab technology which developed by Genentech. However, a main limitation of the ThioMab technology is that the thiol group introduction step may cause a wrong disulfide bond formed between the two Fabs in the antibody, which remains a challenge to be addressed (1).

[0010] Another method for site-specific conjugation is through introduction of unnatural amino acids. Special functional groups in these unnatural amino acids enable the site-specific conjugation. However, it is sometimes difficult to produce the modified antibodies and the antibody with unnatural amino acids may induce immunogenicity (2). The hydrophobicity of unnatural amino acids also increases the risk of antibody aggregation (3).

[0011] Enzyme assisted ligation is also an effective strategy for site-specific conjugation. Through genetic engineering, specific amino acid sequences are artificially induced to express in the antibody and these sequences can be recognized by certain enzymes and subsequently specific amino acid residues are modified by the enzyme, to enable site-specific conjugation (4). However, it is worth noting that the immunogenicity may be induced upon modification of the amino acid sequences and the conjugation process of enzyme assisted ligation would be more complex.

[0012] Site specific ADCs can also be generated from glycan remodeling and glycoconjugation. Human IgG molecules have a conserved glycosylation site at each N297 residue in the CH2 domain. the glycosylation site is sufficiently far from the variable region that conjugation to attached glycans is unlikely to impact antigen binding. However, glycosylation is a heterogeneous posttranslational modification, rendering the generation of homo glycans for chemical modification a formidable challenge. Another complication associated with glycoengineering approaches is that the conjugates produced may be immunogenic (4).

[0013] Overall, those technologies involve protein engineering, linker payload modification and/or enzyme catalysis, so that those technologies suffer from several drawbacks, such as lower level of antibody expression, complicated purification, and high cost (5). There remains, however, a risk that engineering free cysteine residues into the antibody molecule may cause rearrangement and scrambling reactions with existing cysteine residues in the molecule during antibody folding and assembly, or result in dimerization through reaction with a free cysteine residue in another antibody molecule, leading to impaired antibody function or aggregation.

[0014] Therefore, there is a continuing need for developing a novel bio-conjugation process which can generate ADCs without gene engineering and linker payloads modification. The present disclosure relates to a process for preparing a composition comprising antibody-drug conjugates (ADCs), wherein the antibody-drug conjugates (ADCs) with drug to antibody molar ratio of 2 (i.e., DAR2) is present in a high level, and wherein the antibody-drug conjugates have improved homogeneity and have more advantages in terms of safety, in vivo stability and preservation of antibody function. Said process is performed through simple manipulation and with reduced cost.

SUMMARY OF INVENTION

[0015] The present disclosure has an object to develop a bio-conjugation process for preparing a composition comprising antibody-drug conjugates (ADCs) with improved homogeneity, wherein the antibody-drug conjugates (ADCs) with drug to antibody molar ratio of 2 (i.e., D2) are present in a high level in the composition, and the process has simple manipulation and reduced cost. As compared with conventional conjugation process involving the reaction of interchain disulfide bonds, the content of antibody-drug conjugates (ADCs) with drug to antibody molar ratio of 2 (i.e., D2) is higher than 65mol% based on the total molar of the produced antibody-drug conjugates (ADCs) (i.e., D0, D2, D4, D6 and D8), and thus, the homogeneity of antibody-drug

conjugates (ADCs) generated from the bio-conjugation process of the present disclosure can be dramatically improved.

[0016] In a first aspect, the present disclosure relates to a process for preparing a composition of antibody-drug conjugates (ADCs), comprising the following steps:

(a) reduction step: incubating a reductant (e.g., Tris(2-carboxyethyl) phosphine (TCEP), THPP (Tris(3-hydroxypropyl)phosphine), diPPBs (2-diphenylphosphanylbenzenesulfonic acid), DTT (Dithiothreitol), DPAA (2-(Diphenylphosphino) acetic acid), DTE (Dithioerythritol), β-Mercaptoethanol, $LiAlH_4$, $Na_2S_2O_3$, KBH_4, Hydrazine, and so on) and the antibody to be conjugated in a buffer system (e.g., TAPS, Bicine, Tris, Tricine, HEPES, TES, MOPS, PIPES, Sodium citrate, Histidine buffer, PB (phosphate buffer), PBS, MES, etc.) to reduce inter-chain disulfide bonds within the antibody, optionally purifying the reduced antibody;

(b) re-oxidation step: adding an oxidant (e.g., Hydrogen peroxide (H_2O_2), nitrate compounds, Potassium chlorate ($KClO_3$), Peroxydisulfuric acid ($H_2S_2O_8$), Peroxymonosulfuric acid (H_2SO_5), NaClO, Sodium dichromate ($Na_2Cr_2O_7$), Permanganate compounds, Sodium perborate, Nitrous oxide, Sodium bismuthate ($NaBiO_3$), ceric sulfate, NAD, DHAA, DNTB, NADP, 2-Aminophenyl disulfide (DDD), etc.) to re-oxidize some of the reduced thiol groups, optionally purifying the re-oxidized antibody;

wherein the step (a) or the step (b) is performed in the presence of transition metal ions (e.g., Zn^{2+}, Mn^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, etc.),

wherein in step (b), the reduced thiol groups which are not blocked by the transition metal ions are re-oxidated;

(c) conjugation step: removing transition metal ions (e.g., by adjusting pH (e.g., adding NaOH, HCl, etc.), by adjusting temperature, by changing buffer (e.g., adding NaCl, sodium citrate, etc.), or by adding a metal chelator (e.g., EDTA, DTPA, Ethylenediamine, 2,2'-Bipyridine, 1,10-phenanthroline, etc.)), and adding an excess amount of payload bearing reactive groups (e.g., maleimide linking drugs, etc.) to react with reduced thiol groups of the re-oxidized antibody; and

(d) recovering step: recovering the resultant antibody-drug conjugates to obtain a composition of antibody-drug conjugates;

wherein the antibody-drug conjugates with drug to antibody molar ratio of 2 (D2) are present in a high level in the composition.

[0017] In the context of the disclosure, the expression that "the step (a) or the step (b) is performed in the presence of transition metal ions" means that one of the step (a) and the step (b) is performed in the presence of transition metal ions, for example, the step (a) is performed in the presence of transition metal ions, or the step (b) is performed in the presence of transition metal ions. However, the present application does not exclude that both step (a) and step (b) are performed in the presence of transition metal ions. For example, the process of the present application can be performed in a one-pot manner, where if the transition metal ions are added in step (a), step (b) will be also performed in the presence of transition metal ions. From the view of economic efficiency, the transition metal ions can be added only once, i.e., in step (a) or in step (b).

[0018] In one embodiment, the transition metal ions (e.g., Zn^{2+}, Mn^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, etc.) are provided by adding a soluble transition metal salt or a solution of the transition metal salt to step (a) or step (b). Free transition metal ions from the soluble transition metal salt or a solution of the transition metal salt can be released in the reaction solution.

[0019] In one embodiment, the process for preparing a composition of antibody-drug conjugates (ADCs) with improved homogeneity comprises the following steps:

(a) reduction step: incubating a reductant (e.g., Tris(2-carboxyethyl) phosphine (TCEP), THPP (Tris(3-hydroxypropyl)phosphine), diPPBs (2-diphenylphosphanylbenzenesulfonic acid), DTT (Dithiothreitol), DPAA (2-(Diphenylphosphino) acetic acid), DTE (Dithioerythritol), β-Mercaptoethanol, $LiAlH_4$, $Na_2S_2O_3$, KBH_4, Hydrazine, and so on) and the antibody to be conjugated in the presence of transition metal ions (e.g., Zn^{2+}, Mn^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, etc.) in a buffer system (e.g., TAPS, Bicine, Tris, Tricine, HEPES, TES, MOPS, PIPES, Sodium citrate, Histidine buffer, PB (phosphate buffer), PBS, MES, etc.) to reduce inter-chain disulfide bonds within the antibody, optionally purifying the reduced antibody;

(b) re-oxidation step: adding an oxidant (e.g., Hydrogen peroxide (H_2O_2), nitrate compounds, Potassium chlorate ($KClO_3$), Peroxydisulfuric acid ($H_2S_2O_8$), Peroxymonosulfuric acid (H_2SO_5), NaClO, Sodium dichromate ($Na_2Cr_2O_7$), Permanganate compounds, Sodium perborate, Nitrous oxide, Sodium bismuthate ($NaBiO_3$), ceric sulfate, NAD, DHAA, DNTB, NADP, 2-Aminophenyl disulfide (DDD), etc.) to re-oxidize the reduced thiol groups which are not blocked by the transition metal ions, optionally purifying the re-oxidized antibody;

(c) conjugation step: removing transition metal ions (e.g., by adjusting pH (e.g., adding NaOH, HCl, etc.), by adjusting temperature, by changing buffer (e.g., adding NaCl, sodium citrate, etc.), or by a metal chelator (e.g., EDTA, DTPA, Ethylenediamine, 2,2'-Bipyridine, 1,10-phenanthroline, etc.)), and adding an excess amount of payload bearing reactive groups (e.g., maleimide linking drugs, etc.) to react with reduced thiol groups of the re-oxidized antibody; and

(d) recovering step: recovering the resultant antibody-drug conjugates to obtain a composition of antibody-drug conjugates;

wherein the antibody-drug conjugates with drug to antibody molar ratio of 2 (D2) are present in a high level in the composition.

[0020] In another embodiment, the process for preparing a composition of antibody-drug conjugates (ADCs) comprises the following steps:

(a) reduction step: incubating a reductant (e.g., Tris(2-carboxyethyl) phosphine (TCEP), THPP (Tris(3-hydroxypropyl)phosphine), diPPBs (2-diphenylphosphanylbenzenesulfonic acid), DTT (Dithiothreitol), DPAA (2-(Diphenylphosphino) acetic acid), DTE (Dithioerythritol), β-Mercaptoethanol, $LiAlH_4$, $Na_2S_2O_3$, KBH_4, Hydrazine, and so on) and the antibody to be conjugated in a buffer system (e.g., TAPS, Bicine, Tris, Tricine, HEPES, TES, MOPS, PIPES, Sodium citrate, Histidine buffer, PB (phosphate buffer), PBS, MES, etc.) to reduce inter-chain disulfide bonds within the antibody, optionally purifying the reduced antibody;

(b) re-oxidation step: adding a soluble transition metal salt or a solution comprising a soluble transition metal salt, and adding an oxidant (e.g., Hydrogen peroxide (H_2O_2), nitrate compounds, Potassium chlorate ($KClO_3$), Peroxydisulfuric acid ($H_2S_2O_8$), Peroxymonosulfuric acid (H_2SO_5), NaClO, Sodium dichromate ($Na_2Cr_2O_7$), Permanganate compounds, Sodium perborate, Nitrous oxide, Sodium bismuthate ($NaBiO_3$), ceric sulfate, NAD, DHAA, DNTB, NADP, 2-Aminophenyl disulfide (DDD), etc.) to re-oxidize the reduced thiol groups which are not blocked by the transition metal ions in the presence of transition metal ions (e.g., Zn^{2+}, Mn^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, etc.), optionally purifying the re-oxidized antibody;

(c) conjugation step: removing transition metal ions (e.g., by adjusting pH (e.g., adding NaOH, HCl, etc.), by adjusting temperature, by changing buffer (e.g., adding NaCl, sodium citrate, etc.), or by adding a metal chelator (e.g., EDTA, DTPA, Ethylenediamine, 2,2'-Bipyridine, 1,10-

phenanthroline, etc.)), and adding an excess amount of payload bearing reactive groups (e.g., maleimide linking drugs, etc.) to react with reduced thiol groups of the re-oxidized antibody; and

(d) recovering step: recovering the resultant antibody-drug conjugates to obtain a composition of antibody-drug conjugates;

wherein the antibody-drug conjugates with drug to antibody molar ratio of 2 (D2) are present in a high level in the composition.

[0021] In the present disclosure, the phrase that "antibody-drug conjugates (ADCs) with drug to antibody molar ratio of 2 (D2) are present in a high level in the composition" means, in the composition of ADCs prepared by the process of the present disclosure, the content of D2 (i.e., antibody-drug conjugates (ADCs) with drug to antibody molar ratio of 2) is more than 64 mol%, based on the total molar of the produced antibody-drug conjugates (ADCs) (i.e., D0, D2, D4, D6 and D8).

[0022] In one embodiment, the reductant in step (a) is TCEP. In one embodiment, in step (a), the molar ratio of reductant/antibody in the reaction solution is in a range of 1 to 20, for example, 2 to 16, preferably 3 to 8. In a specific embodiment, in step (a), the reductant is added at a molar ratio of 8 relative to the antibody, i.e., the molar ratio of reductant/antibody is 8.

[0023] The transition metal ion which is suitable to be used in the bio-conjugation process of the present disclosure may include, but not limited to, Zn^{2+}, Mn^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, and the like. Among others, Zn^{2+} is preferably used due to its easily availability and low cost. For example, suitable transition metal salts may be added in step (a) provided that they are soluble in the reaction solution so that free transition metal ions can be released in the reaction solution. In this regard, $ZnCl_2$, $Zn(NO_3)_2$, $ZnSO_4$, $Zn(CH_3COO)_2$, ZnI_2, $ZnBr_2$, $Zn(ClO_4)_2$, Zinc Formate, and zinc tetrafluoroborate may be mentioned as suitable zinc salts. Likewise, other transition metal salts which are soluble and can release free transition metal ions, such as Mn^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, and the like, in the reaction solution can be mentioned, which include, but not limited to, the salts of Cu, Ni, Co, Fe, Mn, Cr, V, Ti, Sc, Mg, Sr, Y, Zr, Nb, Mo, Ru, Rh, Pd, Ag, Cd, Ga, Ge, and the like.

[0024] In one embodiment, the transition metal ion in step (a) or step (b) is Zn^{2+}. In one embodiment, in step (a) or step (b), the molar ratio of Zn^{2+}/antibody in the reaction solution is in

a range of 0.5 to 50, preferably 1 to 16, more preferably 2 to 8. In a specific embodiment, in step (a) or step (b), the transition metal ions are added at a molar ratio of 4 relative to the antibody, i.e., the molar ratio of transition metal ions/antibody is 4.

[0025] Depending on the transition metal ions, those skilled in the art can select a suitable buffer system for the reaction in step (a), including, but not limited to, TAPS, Bicine, Tris, Tricine, HEPES, TES, MOPS, PIPES, Sodium citrate, Histidine buffer, Phosphate buffer (PB), PBS, MES, and the like, and the pH value of the buffer system is about 5 to 8, preferably about 7.0.

[0026] The transition metal ions will be removed by appropriate methods known in the art, for example, but not limited to, by adjusting pH (e.g., adding NaOH, HCl, etc.), by adjusting temperature, by changing buffer (e.g., adding NaCl, sodium citrate, etc.), or by using chelating reagent such as EDTA, DTPA, Ethylenediamine, 2,2'-Bipyridine, or 1,10-phenanthroline, and then will be optionally filtered out in subsequent dialysis, ultrafiltration, or gel filtration.

[0027] In one embodiment, the oxidant may be selected from, but not limited to, Hydrogen peroxide (H_2O_2), nitrate compounds, Potassium chlorate ($KClO_3$), Peroxydisulfuric acid ($H_2S_2O_8$), Peroxymonosulfuric acid (H_2SO_5), NaClO, Sodium dichromate ($Na_2Cr_2O_7$), Permanganate compounds, Sodium perborate, Nitrous oxide, Sodium bismuthate ($NaBiO_3$), ceric sulfate, dehydroascorbic acid (DHAA), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), or 2-Aminophenyl disulfide (DDD). Those skilled in the art may appreciate that any other oxidants that can oxidize sulfhydryl groups can be used in the present invention. In one embodiment, the oxidant to be added in step (b) is DHAA. In another embodiment, the oxidant to be added in step (b) is DTNB. In an embodiment, in step (b), the molar ratio of oxidant/antibody in the reaction solution is in a range of 0.5 to 160, for example, 10 to 160 (weak oxidant), 5 to 24 (medium oxidant) or 0.5 to 10 (strong oxidant). In a specific embodiment, in step (b), the oxidant is added at a molar ratio of 16 relative to the antibody, i.e., the molar ratio of oxidant/antibody is 16. In one embodiment, DTNB is used as the oxidant at a molar ratio of oxidant/antibody from 1.6 to 7.7. In another embodiment, DHAA is used as the oxidant at a molar ratio of oxidant/antibody of 16 or 40. In yet another embodiment, DDD is used as the oxidant at a molar ratio of oxidant/antibody of 1.82.

[0028] In step (b), in the resultant re-oxidized antibody, it seems that only one set of reduced inter-chain disulfide bonds is blocked by the transition metal ions. In some embodiments, the re-

oxidized antibody is not purified after reoxidation in step (b), and the process for preparing a composition of antibody-drug conjugates (ADCs) with improved homogeneity as described herein may be performed in a one-pot manner. In some embodiments, the re-oxidized antibody is purified after reoxidation in step (b).

[0029] The optimum pH for the reaction will typically between about 5 and about 8, for instance, about 5.5 to about 7.5, or about 6 to about 7.5. The optimal reaction conditions will depend upon the specific reactants employed. In a specific embodiment, the pH in step (a) and step (b) is about 6 to about 7.5, preferably about 7.0, and the pH in step (c) is about 5 to about 8, preferably about 7.0.

[0030] The incubation time and temperature for each step can be determined by those skilled in the art based on the specific antibody to be conjugated. The optimum temperature for the reaction will typically be between about -10 and 37°C. In a specific embodiment, in step (a) (i.e., reduction step), the temperature may be 0°C to 37°C, preferably 2°C to 25°C, e.g., 4°C, 12°C, 22°C or 37°C, and the incubation time may be 0.5h to 90h, for example 0.5h to 72h, preferably 2h to 20h, more preferably 16h to 20h, for example, 2h, 16h, 19h, 45h, or 92h. In step (b) (i.e., re-oxidation step), the temperature may be 0°C to 37°C, preferably 2°C to 25°C or 4 to 12°C, e.g., 0°C, 12°C, 22°C or 37°C, and the incubation time may be 0.5h to 170h, preferably 0.5h to 72h, more preferably 0.5h to 48h, for example, 0.5h, 1h, 2h, 4h, 24h, 47h, or 170h. In step (c) (i.e., conjugation step), the temperature may be 0°C to 37°C, preferably 2°C to 25°C or 4 to 12°C, e.g., 0°C, 4°C or 12°C and the incubation time may be 0.5h to 72h, preferably 8h to 16h, more preferably 1h to 4h.

[0031] There is no specific limitation to the antibody which can be conjugated with a linker-drug by using the bio-conjugation process of the present disclosure. The selection of the antibody depends on the diseases or disorder (e.g., a cancer) to be treated by the antibody-drug conjugates (ADCs). The antibody can specifically bind corresponding antigens expressed on cancer cells (also referred to as tumor-associated antigens (TAA)), viral antigens, or microbial antigens, have antibody-dependent cell-mediated phagocytosis (ADCP) activity, and have antitumor, antiviral, or antimicrobial activity *in vivo*. The interchain S-S bonds in the antibody, after reduced, may serve as the sites for attaching drug-linker complex.

[0032] In some embodiments, the antibody may include, but not limited to, a monoclonal antibody or a polyclonal antibody, or the antibody may be a monospecific antibody or a multi-specific antibody, for example, a bispecific antibody. Specific examples of the antibody include a human antibody, a humanized antibody, or a chimeric antibody. In certain embodiments, the antibody is a monoclonal antibody, for instance, a human antibody or a humanized antibody. As isotype of the antibody of the present disclosure, for example, IgG (IgG1, IgG2, IgG3, or IgG4) can be exemplified. In a specific embodiment, the antibody is an IgG1 monoclonal antibody. For instance, three antibodies exemplified in the examples, Trastuzumab, Rituximab and Cetuximab, are representative IgG1 type antibodies. The results of the examples demonstrate that the bio-conjugation process of the present disclosure is at least applicable to IgG1-type antibodies. Furthermore, the bio-conjugation process of the present disclosure may also be applicable to IgG4-type antibodies.

[0033] As for the payload bearing reactive group to be conjugated to the selected antibody, it generally has a format of drug-linker. There are no specific limitations to the drug and linker which can be used in the bio-conjugation process of the present disclosure, as long as the drug molecule has a desired (e.g., cytotoxic, antitumor, or labelling, etc.) effect and at least one substituted group or a partial structure allowing connection to a linker structure, and the linker contains at least two reactive groups, one of which can covalently bond a drug molecule and the other of which can covalently couple to an antibody.

[0034] A wide variety of diagnostic, therapeutic and labelling agents that are known in the art have been conjugated to antibody molecules. For example, in a broadest sense, the drug to be conjugated may include a diagnostic agent, a drug molecule, for example a cytotoxic agent, a toxin, a radionuclide, a fluorescent agent (for example an amine derivatized fluorescent probe such as 5-dimethylaminonaphthalene-1-(N-(2-aminoethyl))sulfonamide-dansyl ethylenediamine, Oregon Green® 488 cadaverine (catalogue number O-10465, Molecular Probes), dansyl cadaverine, N-(2-aminoethyl)-4-amino-3,6-disulfo-1,8-naphthalimide, dipotassium salt (lucifer yellow ethylenediamine), or rhodamine B ethylenediamine (catalogue number L- 2424, Molecular Probes), or a thiol derivatized fluorescent probe for example BODIPY® FLL-cystine (catalogue number B-20340, Molecular Probes)).

[0035] Most common reactive group capable of bonding to thiol group in ADC preparation is maleimide. Additionally, other electron deficient alkenyl or alkynyl derivatives, mono- or di-derivatives of disulfides, sulfones, bicyclo[1.1.0]butane derivatives, sulfonyl fluorides, pentafluorophenol ester, palladium oxidative-addition complexes, iodoxolones, highly electron deficient arenes, organic bromides, iodides are also frequently used. Those skilled in the art may appreciate that any other group that selectively reacts with sulfhydryl groups can be used.

[0036] Depending on the desired drug and selected linker, those skilled in the art can select suitable method for coupling them together. For example, some conventional coupling methods, such as amine coupling methods, may be used to form the desired drug-linker complex which still contains reactive groups for conjugating to the antibodies through covalent linkage. A drug-maleimide complex (i.e., maleimide linking drug) is taken as an example of the payload bearing reactive group in the present disclosure. The drug may include, but not limited to, cytotoxic reagents, such as chemo-therapeutic agents, immunotherapeutic agents and the like, antiviral agents, or antimicrobial agents. Most common reactive group capable of bonding to thiol group in ADC preparation is maleimide. Additionally, organic bromides, iodides also are frequently used. In a particular embodiment, the payload comprises a maleimide moiety, bromide, or iodide.

[0037] As for step (d), those skilled in the art can select proper purification methods to recover the resultant antibody-drug conjugates. Many ADC purification methods are well known in the art. For example, the resultant antibody-drug conjugates may be purified by using a de-salting column, size exclusion chromatography, and the like.

[0038] With a conjugation process using the same steps without the addition of transition metal ions in step (a) as a negative control (see Example 1, and Fig 1-3), the inventors successfully demonstrated that transition metal ions may be the key factor responsible for higher level of D2 and lower level of D0, D4, D6 and D8 in the resultant ADCs. This process has been verified with several commercial therapeutic antibodies and showed great consistency.

[0039] By using the process of the present disclosure to produce antibody-drug conjugates, the homogeneity of the antibody-drug conjugates is higher than those produced by conventional conjugation processes. Specifically, in the ADCs prepared by the process of the present disclosure, the content of D2 is generally more than 64 mol%, preferably more than 75 mol% or even more than 80 mol%, while the content D2 is normally less than 50 mol% in the ADCs prepared by

conventional conjugation processes. Moreover, the content of D0+D4+D6+D8 in the ADCs prepared by the process of the present disclosure is less than 36mol%, preferably less than 25mol%, more preferably less than 20 mol%, most preferably less than 15%, while the content of D0+D4+D6+D8 is normally more than 40 mol% in the ADCs prepared by conventional conjugation processes.

[0040] The process of the present disclosure bypasses any need of protein engineering or enzyme catalysis, but is based on native inter-chain disulfide bonds and only needs transition metal ions. Therefore, as compared with conventional processes for preparing ADCs in absence of transition metal ions, the process of the disclosure is less complicate, the homogeneity of the resultant antibody-drug conjugates is dramatically improved, and the cost will be highly reduced.

[0041] In a second aspect, the present disclosure relates to a composition of antibody-drug conjugates (ADCs) produced by the process of the first aspect, wherein the content of D2 is generally more than 64 mol%, preferably more than 75 mol% or even more than 80 mol%, and the content of D0+D4+D6+D8 in the ADCs is less than 36mol%, preferably less than 25mol%, more preferably less than 20 mol%, most preferably less than 15%, based on the total molar of the antibody-drug conjugates (ADCs) (i.e., D0, D2, D4, D6 and D8).

[0042] In a third aspect, the present disclosure relates to a pharmaceutical composition comprising a composition of antibody-drug conjugates (ADCs) produced by the process of the first aspect and a pharmaceutically acceptable carrier.

[0043] In an embodiment, the pharmaceutical composition is used for treating a condition or disorder in a subject, wherein the condition or disorder is selected from, but not limited to, a tumor (e.g., a cancer), an autoimmune disease, or an infectious disease.

[0044] In a particular embodiment, the infectious disease is viral or microbial infection.

[0045] In an embodiment, the subject is a mammal, for example, a human.

[0046] In a fourth aspect, the present disclosure relates to use of the composition of antibody-drug conjugates (ADCs) produced by the process of the first aspect in manufacture of a pharmaceutical composition for treating a condition or disorder in a subject.

[0047] In a fifth aspect, the present disclosure relates to a method for treating a condition or disorder in a subject, comprising administrating to the subject an effective amount of the composition of antibody-drug conjugates (ADCs) produced by the process of the first aspect or an effective amount of the pharmaceutical composition of the second aspect.

[0048] In a particular embodiment, the condition or disorder is selected from, but not limited to, a tumor (e.g., a cancer), an autoimmune disease, or an infectious disease.

DESCRIPTION OF DRAWINGS

[0049] The foregoing and other features and advantages of the disclosure will become more apparent from the following detailed description of several embodiments which proceeds with reference to the accompanying figures.

[0050] Fig. 1: The HIC (Hydrophobic interaction chromatography) result of mAb1 (Trastuzumab) (A), mAb2 (Cetuximab) (B), and mAb3 (Rituximab) (C) Random-DAR2 conjugation with MC-VC-PAB-MMAE through conventional conjugation process.

[0051] Fig. 2: The HIC result of mAb1 (Trastuzumab) (A), mAb2 (Cetuximab) (B), and mAb3 (Rituximab) (C) DAR2 conjugation with MC-VC-PAB-MMAE through the conjugation process of the present disclosure.

[0052] Fig. 3: The HIC result of mAb1 (Trastuzumab) DAR2 conjugation with MC-VC-PAB-MMAE (A), MC-MMAF (B), GGFG-Dxd (C), Tesirine (PBD) (D), DL1-VC-PAB -MMAE (E) and DM21 (F) through the conjugation process of the present disclosure.

[0053] Fig. 4 (A-C): The HIC result of mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure under different reductants (A: TCEP; B: diPPBS1; C: DPAA).

[0054] Fig. 4 (D-F): The HIC result of mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure under different ratios of TCEP (D: TCEP, 3.25 eq; E: TCEP, 10 eq; F: TCEP, 16 eq.

[0055] Fig. 5: The HIC result of mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure in different buffers: (A) HEPES; (B) MES; (C) Tris.

[0056] Fig. 6: The HIC result of mAb3 (Rituximab) DAR2 conjugation through the conjugation process of the present disclosure in different buffers: (A) HEPES; (B) MES; (C) Tris.

[0057] Fig. 7: The HIC result of mAb3 (Rituximab) conjugation through a conjugation process

in different buffers: (A) HEPES; (B) MES; (C) Tris but without transition metal ions ($0Zn^{2+}$).

[0058] Fig. 8: The HIC result of a bispecific antibody (mAb4) DAR2 conjugation through the conjugation process of the present disclosure.

[0059] Fig. 9 (A-C): The HIC result of mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure by using TCEP at different temperature for reduction: (A) 4°C, (B) 22°C, (C) 37°C.

[0060] Fig. 9 (D-F): The HIC result of mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure by using TCEP for different durations of reduction at 4°C: (A) 19 hours, (B) 45 hours, (C) 92 hours.

[0061] Fig. 10: The HIC result of mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure by using different Zn^{2+} related salts: (A) $ZnSO_4 \cdot 7H_2O$, (B) $ZnBr_2 \cdot 2H_2O$, (C) $Zn(ClO_4)_2 \cdot 6H_2O$, (D) $Zn(OAc)_2 \cdot 2H_2O$.

[0062] Fig. 11. The HIC result of mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure by using amounts of Zn^{2+} ions: (A) 2 eq, (B) 8 eq, (C) 16 eq.

[0063] Fig. 12: The HIC result of mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure by removing TCEP after the reduction of the antibody.

[0064] Fig. 13: The HIC result of mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure under different oxidants: (A) DHAA, (B) DTNB, (C) DDD.

[0065] Fig. 14 (A-D): The HIC result of mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure at different temperature for re-oxidation: (A) 0°C, (B) 12°C, (C) 22°C, (D) 37°C.

[0066] Fig. 14 (E-H): The HIC result of mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure for different durations of re-oxidation: (E) 1 hour, (F) 2 hours, (G) 4 hours, (H) 24 hours.

[0067] Fig. 15: The HIC result of mAb1 (Trastuzumab) DAR2 conjugation with linker-payload at different temperatures: A: 0 °C; B: 12 °C.

[0068] Fig. 16: The HIC result of mAb1 (Trastuzumab) DAR2 conjugation by adding transition metal ions in the re-oxidation step and performing the re-oxidation at 22°C for 47.5 hours.

[0069] Fig. 17. The HIC result of mAb1 (Trastuzumab) DAR2 conjugation by adding transition metal ions in the re-oxidation step: (A) performing the re-oxidation in PB at 12°C for 170 hours in the presence of transition metal ions, (B) performing the re-oxidation in PB at 12°C for 24 hours without transition metal ions (as negative control), (C) performing the re-oxidation in HEPES+PB at 12°C for 170 hours in the presence of transition metal ions, (D) performing the re-oxidation in HEPES at 12°C for 24 hours without transition metal ions (as negative control).

[0070] Fig. 18: The HIC result of mAb3 (Rituximab) DAR2 conjugation by adding transition metal ions in the re-oxidation step: (A) performing the re-oxidation in PB at 12°C for 170 hours in the presence of transition metal ions, (B) performing the re-oxidation in PB at 12°C for 24 hours without transition metal ions (as negative control).

[0071] Fig. 19: The HIC result of mAb1 (Trastuzumab) DAR2 conjugation with different linker-payloads by adding transition metal ions in the re-oxidation step: (A) GGFG-Dxd, (B) Tesirine (PBD), (C) MC-VC-PAB-MMAE.

[0072] Fig. 20: The HIC result of mAb1 (Trastuzumab) DAR2 conjugation under different antibody concentrations: (A) 5mg/ml Ab, (B) 10mg/ml Ab.

[0073] Fig. 21: The HIC result of mAb1 (Trastuzumab) DAR2 conjugation under different HEPES concentrations: (A) 40mM HEPES, (B) 20mM HEPES.

[0074] Fig. 22: Schematic diagram of the process of the present invention for preparing a composition of ADCs. (A) and (B) show the procedures for a normal antibody (i.e., a monospecific antibody): (A) The transition metal ions (M^{2+}) are added in the reduction step, (B) The transition metal ions (M^{2+}) are added in the re-oxidation step; and (C) shows the procedures for a bispecific antibody, in which the transition metal ions (M^{2+}) are added in the reduction step, but The transition metal ions (M^{2+}) can also be added in the re-oxidation step (not shown).

DETAILED DESCRIPTION OF INVENTION

[0075] While the present disclosure may be embodied in many different forms, disclosed herein are specific illustrative embodiments thereof that exemplify the principles of the disclosure. It should be emphasized that the present disclosure is not limited to the specific embodiments illustrated. Moreover, any section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

[0076] Generally, nomenclature used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid

chemistry and hybridization described herein are those well-known and commonly used in the art. The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. See, e.g., Abbas et al., Cellular and Molecular Immunology, 6th ed., W.B. Saunders Company (2010); Sambrook J. & Russell D. Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2000); Ausubel et al., Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology, Wiley, John & Sons, Inc. (2002); Harlow and Lane Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1998); and Coligan et al., Short Protocols in Protein Science, Wiley, John & Sons, Inc. (2003). The nomenclature used in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art. Moreover, any section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

[0077] DEFINITIONS

[0078] In order to better understand the disclosure, the definitions and explanations of the relevant terms are provided as follows.

[0079] Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. More specifically, as used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an antibody" includes a plurality of antibodies; reference to "a transition metal ion" includes mixtures of transition metal ions, and the like. In this application, the use of "or" means "and/or" unless stated otherwise.

[0080] Throughout this disclosure, unless the context requires otherwise, the words "comprise", "comprises" and "comprising" will be understood to imply the inclusion of a stated

step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but other elements are optional and may or may not be present depending upon whether they affect the activity or action of the listed elements.

[0081] As used herein, the term "about" or "approximately" refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight, or length that varies by as much as 30, 25, 20, 25, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1% to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In particular embodiments, the terms "about" or "approximately" when preceding a numerical value indicates the value plus or minus a range of 15%, 10%, 5%, or 1%.

[0082] Reference throughout this disclosure to "one embodiment," "an embodiment," "a particular embodiment," "a related embodiment," "a certain embodiment," "an additional embodiment," or "a further embodiment" or combinations thereof means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment of the present disclosure. Thus, the appearances of the foregoing phrases in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

[0083] "Antibody-drug conjugate" or ADC refers to a conjugate formed by covalently coupling a drug to an antibody directly or indirectly via one or more suitable linkers. ADC is generally in a format of antibody-linker-drug conjugate. The Antibody-drug conjugates combine ideal properties of both antibodies and cytotoxic drugs by targeting potent cytotoxic drugs to the antigen-expressing tumor cells, thereby enhancing their anti-tumor activity.

[0084] The term "drug" as used herein refers to any cytotoxic molecule which has an antitumor effect and at least one substituted group or a partial structure allowing connection to a linker

structure. The drug may kill cancer cells and/or inhibit growth, proliferation, or metastasis of cancer cells, thereby reducing, alleviating, or eliminating one or more symptoms of a disease or disorder.

[0085] The term "linker" as used herein refers to a reactive molecule which contains at least two reactive groups, one of which can covalently bond a drug molecule and the other of which can covalently couple to an antibody.

[0086] The term "antibody" as used herein encompasses any immunoglobulin, monoclonal antibody, polyclonal antibody, multi-specific antibody, or bispecific (bivalent) antibody that binds to specific antigens. A native intact antibody comprises two heavy chains and two light chains. Each heavy chain consists of a variable region ("HCVR") and a first, second, and third constant region (CH1, CH2 and CH3), while each light chain consists of a variable region ("LCVR") and a constant region (CL). Mammalian heavy chains are classified as α, δ, ε, γ, and μ, and mammalian light chains are classified as λ or κ. The antibody has a "Y" shape, with the stem of the Y consisting of the second and third constant regions of two heavy chains bound together via disulfide bonding. Each arm of the Y includes the variable region and first constant region of a single heavy chain bound to the variable and constant regions of a single light chain. The variable regions of the light and heavy chains are responsible for antigen binding. The variable regions in both chains generally contain three highly variable loops called the complementarity determining regions (CDRs) (light (L) chain CDRs including LCDR1, LCDR2, and LCDR3, heavy (H) chain CDRs including HCDR1, HCDR2, HCDR3). CDR boundaries for antibodies may be defined or identified by the conventions of Kabat, Chothia, or Al-Lazikani (Al-Lazikani, B., Chothia, C., Lesk, A. M., J. Mol. Biol., 273(4), 927 (1997); Chothia, C. et al., J Mol Biol. Dec 5;186(3):651-63 (1985); Chothia, C. and Lesk, A.M., J. Mol. Biol., 196,901 (1987); Chothia, C. et al., Nature. Dec 21-28; 342(6252):877-83 (1989); Kabat E.A. et al., National Institutes of Health, Bethesda, Md. (1991)). The three CDRs are interposed between flanking stretches known as framework regions (FRs), which are more highly conserved than the CDRs and form a scaffold to support the hypervariable loops. Each HCVR and LCVR comprises four FRs, and the CDRs and FRs are arranged from amino terminus to carboxy terminus in the order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The constant regions of the heavy and light chains are not involved in antigen binding, but exhibit various effector functions. Antibodies are assigned to classes based on the

amino acid sequence of the constant region of their heavy chain. The five major classes or isotypes of antibodies are IgA, IgD, IgE, IgG, and IgM, which are characterized by the presence of α, δ, ε, γ, and μ heavy chains, respectively. Several of the major antibody classes are divided into subclasses such as IgG1 (γ1 heavy chain), IgG2 (γ2 heavy chain), IgG3 (γ3 heavy chain), IgG4 (γ4 heavy chain), IgA1 (α1 heavy chain), or IgA2 (α2 heavy chain).

[0087] "Antibody fragments" comprise a portion of a full-length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments; diabodies; linear antibodies; minibodies (Olafsen et al. (2004) Protein Eng. Design & Sel. 17(4):315-323), fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, CDR (complementary determining region), and epitope -binding fragments of any described herein which immuno-specifically bind to cancer cell antigens, viral antigens or microbial antigens, single-chain antibody molecules; and multi-specific antibodies formed from antibody fragments.

[0088] The term "variable domain" with respect to an antibody as used herein refers to an antibody variable region or a fragment thereof comprising one or more CDRs. Although a variable domain may comprise an intact variable region (such as HCVR or LCVR), it is also possible to comprise less than an intact variable region yet still retain the capability of binding to an antigen or forming an antigen-binding site.

[0089] The term "antigen-binding moiety" as used herein refers to an antibody fragment formed from a portion of an antibody comprising one or more CDRs, or any other antibody fragment that binds to an antigen but does not comprise an intact native antibody structure. Examples of antigen-binding moiety include, without limitation, a variable domain, a variable region, a diabody, a Fab, a Fab', a F(ab')2, an Fv fragment, a disulfide stabilized Fv fragment (dsFv), a (dsFv)2, a bispecific dsFv (dsFv-dsFv'), a disulfide stabilized diabody (ds diabody), a multi-specific antibody, a camelized single domain antibody, a nanobody, a domain antibody, and a bivalent domain antibody. An antigen-binding moiety is capable of binding to the same antigen to which the parent antibody binds. In certain embodiments, an antigen-binding moiety may comprise one or more CDRs from a particular human antibody grafted to a framework region from one or more different human antibodies. For more and detailed formats of antigen-binding moiety

are described in Spiess et al, 2015 (Supra), and Brinkman et al., mAbs, 9(2), pp.182–212 (2017), which are incorporated herein by their entirety.

[0090] "Fab" with regard to an antibody refers to that portion of the antibody consisting of a single light chain (both variable and constant regions) associating to the variable region and first constant region of a single heavy chain by a disulfide bond. In certain embodiments, the constant regions of both the light chain and heavy chain are replaced with TCR constant regions.

[0091] "Fc" with regard to an antibody refers to that portion of the antibody consisting of the second (CH2) and third (CH3) constant regions of a first heavy chain bound to the second and third constant regions of a second heavy chain via disulfide bonding. The Fc portion of the antibody is responsible for various effector functions such as ADCC, and CDC, but does not function in antigen binding.

[0092] "Hinge region" in terms of an antibody includes the portion of a heavy chain molecule that joins the CH1 domain to the CH2 domain. This hinge region comprises approximately 25 amino acid residues and is flexible, thus allowing the two N-terminus antigen binding regions to move independently.

[0093] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations which include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies. 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 the hybridoma method first described by Kohler et al. (1975) Nature 256:495, or may be made by recombinant DNA methods (see for example: US 4816567; US 5807715). The monoclonal antibodies may also be isolated

from phage antibody libraries using the techniques described in Clackson et al. (1991) Nature, 352:624-628; Marks et al. (1991) J. Mol. Biol., 222:581-597; for example.

[0094] The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (US 4816567; and Morrison et al. (1984) Proc. Natl. Acad. Sci. USA, 81:6851-6855). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g., Old World Monkey, Ape, etc.) and human constant region sequences.

[0095] An "intact antibody" herein is one comprising a VL and VH domains, as well as a light chain constant domain (CL) and heavy chain constant domains, CH1, CH2 and CH3. The constant domains may be native sequence constant domains (e.g., human native sequence constant domains) or amino acid sequence variant thereof. The intact antibody may have one or more "effector functions" which refer to those biological activities attributable to the Fc constant region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody. Examples of antibody effector functions include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; and down regulation of cell surface receptors such as B cell receptor and BCR.

[0096] Depending on the amino acid sequence of the constant domain of their heavy chains, intact antibodies can be assigned to different "classes". There are five major classes of intact immunoglobulin antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into "subclasses" (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known. Ig forms include hinge-modifications or hingeless forms (Roux et al. (1998) J. Immunol. 161 :4083-4090; Lund et al. (2000) Eur. J. Biochem. 267:7246-7256; US 2005/0048572; US 2004/0229310).

[0097] 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., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively.

[0098] 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.

[0099] 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.

[00100] 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 or 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.

[00101] An "isolated antibody" is one which has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC). For review of methods for assessment of antibody purity, see, e.g., Flatman et al, J. Chromatogr. B 848:79-87 (2007).

[00102] "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 (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

[00103] A "cysteine engineered antibody" or "cysteine engineered antibody variant" is an antibody in which one or more residues of an antibody are substituted with cysteine residues. In accordance with the present disclosure, the thiol group(s) of the cysteine engineered antibodies can be conjugated to calicheamicin to form a THIOMAB™ antibody (i.e., a THIOMAB™ drug conjugate (TDC), wherein in accordance with the present disclosure the drug is a calicheamicin derivative). In particular embodiments, the substituted residues occur at accessible sites of the antibody. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antibody and may be used to conjugate the antibody to the drug moiety to create an immunoconjugate, as described further herein. For example, a THIOMAB™ antibody may be an antibody with a single mutation of a non-cysteine native residue to a cysteine in the light chain (e.g., G64C, K149C or R142C according to Kabat numbering) or in the heavy chain (e.g., D101C or V184C or T205C according to Kabat numbering). In specific examples, a THIOMAB™ antibody has a single cysteine mutation in either the heavy or light chain such that each full-length antibody (i.e., an antibody with two heavy chains and two light chains) has two engineered cysteine residues. Cysteine engineered antibodies and preparatory methods are disclosed by US 2012/0121615 A1 (incorporated by reference herein in its entirety).

[00104] A "disulfide bond" refers to a covalent bond with the structure R-S-S-R'. The amino acid cysteine comprises a thiol group that can form a disulfide bond with a second thiol group, for example from another cysteine residue. The disulfide bond can be formed between the thiol groups of two cysteine residues residing respectively on the two polypeptide chains, thereby forming an interchain bridge or interchain bond.

[00105] The term "specific binding" or "specifically binds" as used herein refers to a non-random binding reaction between two molecules, such as for example between an antibody and an

antigen. In certain embodiments, the polypeptide complex and the bispecific polypeptide complex provided herein specifically bind an antigen with a binding affinity (K_D) of $\leq 10^{-6}$ M (e.g., $\leq 5x10^{-7}$ M, $\leq 2x10^{-7}$ M, $\leq 10^{-7}$ M, $\leq 5x10^{-8}$ M, $\leq 2x10^{-8}$ M, $\leq 10^{-8}$ M, $\leq 5x10^{-9}$ M, $\leq 2x10^{-9}$ M, $\leq 10^{-9}$ M, or $\leq 10^{-10}$ M). K_D as used herein refers to the ratio of the dissociation rate to the association rate (koff/kon), may be determined using surface plasmon resonance methods for example using instrument such as Biacore.

[00106] The term "transition metal", as used herein, refers to the elements of groups 4-11, justified by their typical chemistry, i.e., a large range of complex ions in various oxidation states, colored complexes, and catalytic properties either as the element or as ions (or both). Sc and Y in Group 3 are also generally recognized as transition metals.

[00107] As discussed above, a mixture of antibody-drug conjugates will be generated by the conventional conjugation processes or the bio-conjugation process of the present disclosure. In general, one antibody molecule belonging to IgG1 or IgG4 subclass has 4 inter-chain S-S bonds, each of which is formed with two -SH groups. The antibody molecule can be subjected to partial or complete reduction of one or more interchain S-S bonds to form 2n (n is an integer selected from 1, 2, 3 or 4) reactive -SH groups, and thus, the number of drugs coupling to a single antibody molecule is 2, 4, 6 or 8. In accordance with the number of drugs coupling to a single antibody molecule, the different conjugates containing different number of drug molecules are denominated as D0, D2, D4, D6 and D8. If the number of drugs coupling to a single antibody molecule is 0 (i.e., drug to antibody molar ratio is 0 (i.e., DAR0)), the product is referred to as D0 (i.e., actually representing one single antibody molecule which is not conjugated with any drug). Accordingly, D2 refers to an ADC molecule in which two drug molecules are coupled to one single antibody molecule (i.e., drug to antibody molar ratio is 2 (i.e., DAR2)), where two drug molecules may be coupled to -SH groups generated by reduction of S-S bonds between heavy and light chains via linkers, or may be coupled to -SH groups generated by reduction of S-S bonds between heavy and heavy chains via linkers. D4 refers to an ADC molecule in which four drug molecules are coupled to one single antibody molecule (i.e., drug to antibody molar ratio is 4 (i.e., DAR4)), where four drug molecules may be coupled to four -SH groups generated by reduction of two S-S bonds between heavy and light chains via linkers (such ADC is referred to as D4-1), or four drug molecules may be coupled to four -SH groups generated by reduction of two S-S bonds between

heavy and heavy chains via linkers (such ADC is referred to as D4-2), or two drug molecules may be coupled to two -SH groups generated by reduction of one S-S bond between heavy and light chains via linkers and the other two drug molecules may be coupled to two -SH groups generated by reduction of one S-S bond between heavy and heavy chains vis linkers (such ADC is referred to as D4-3). D6 refers to an ADC molecule in which six drug molecules are coupled to one single antibody molecule (i.e., drug to antibody molar ratio is 6 (i.e., DAR6)), where four drug molecules may be coupled to four -SH groups generated by reduction of two S-S bonds between heavy and light chains via linkers and two drug molecules may be coupled to two -SH groups generated by reduction of one S-S bonds between heavy and heavy chains via linkers (such ADC is referred to as D6-1), or four drug molecules may be coupled to four -SH groups generated by reduction of two S-S bonds between heavy and heavy chains via linkers and two drug molecules may be coupled to two -SH groups generated by reduction of one S-S bonds between heavy and light chains via linkers (such ADC is referred to as D6-2). And D8 refers to an ADC molecule in which eight drug molecules are coupled to one single antibody molecule (i.e., drug to antibody molar ratio is 8 (i.e., DAR8)), i.e., all the four S-S bonds in one antibody molecule are reduced to eight -SH groups and each -SH group attaches one drug molecule. In general, the heterogeneous mixture of ADC molecules generated by conventional conjugation processes or the bio-conjugation process of the present disclosure is a mixture of D0, D2, D4, D6 and D8 (also referred to as "a composition of ADCs"). And thus, the "homogeneity" of antibody-drug conjugates is used to describe the property of dominance of one specific type of antibody-drug conjugate (i.e., one type selected from D0, D2, D4, D6 and D8 conjugates) in one given mixture of antibody-drug conjugates. Although antibody-drug conjugate potency in vitro has been shown to be directly dependent on drug loading (Hamblett KJ, et al., Clin Cancer Res. 2004 Oct 15;10(20):7063-70), in-vivo therapeutical activity (e.g., anti-tumor) of antibody-drug conjugates with four drugs per molecule (D4) is comparable with conjugates with eight drugs per molecule (D8) at equal mAb doses, even though the conjugates contain half the amount of drug per mAb. Drug-loading also affects plasma clearance, with the D8 conjugate being cleared 3-fold faster than the D4 conjugate and 5-fold faster than a D2 conjugate. If the molecule to be conjugated has high toxicity (i.e., high toxic payload), D2 may be desired. In practice, for payloads with outstanding efficacy, on one hand, the conjugate prepared as D2 can control the safety risk of drugs and improve the circulating stability of conjugates *in vivo* under the premise of ensuring efficacy, on the other hand, for special

mechanisms of action and spatial structure, such as nucleic acid loading or immune antagonist, and the like, D2 conjugate can reduce the impact of conjugated large molecular weight payloads on antibody function as much as possible. In general, if the content of D2 is high in the mixture, the ADCs are considered to have a high homogeneity, In the present disclosure, the "homogeneity" of antibody-drug conjugates refers to a high level of D2 in the mixture of antibody-drug conjugates.

[00108] Accordingly, "improved homogeneity" of ADCs, as used herein, refers to a higher level of D2 in the mixture of antibody-drug conjugates generated by the process of the present disclosure as compared with the mixture of ADCs generated by conventional conjugation processes. In the ADCs prepared by the process of the present disclosure, based on the total molar of the produced antibody-drug conjugates (ADCs) (i.e., D0, D2, D4, D6 and D8), the content of D2 is generally more than 65 mol%, for example, more than 70 mol%, preferably more than 75 mol% or even more than 80mol%, while the content of D2 is normally less than 50 mol% in the ADCs prepared by conventional conjugation processes. Moreover, the content of D0+D4+D6+D8 in the ADCs prepared by the process of the present disclosure is less than 35mol%, preferably less than 25mol%, more preferably less than 20 mol%, most preferably less than 15%, while the content of D0+D4+D6+D8 is normally more than 40 mol% in the ADCs prepared by conventional conjugation processes.

[00109] The term "pharmaceutically acceptable" indicates that the designated carrier, vehicle, diluent, excipient(s), and/or salt is generally chemically and/or physically compatible with the other ingredients comprising the formulation, and physiologically compatible with the recipient thereof.

[00110] A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is bioactivity acceptable and nontoxic to a subject. Pharmaceutical acceptable carriers for use in the pharmaceutical compositions disclosed herein may include, for example, pharmaceutically acceptable liquid, gel, or solid carriers, aqueous vehicles, nonaqueous vehicles, antimicrobial agents, isotonic agents, buffers, antioxidants, anesthetics, suspending/dispending agents, sequestering or chelating agents, diluents, adjuvants, excipients, or non-toxic auxiliary substances, other components known in the art, or various combinations thereof.

[00111] The term "subject" includes any human or nonhuman animal, for example, humans.

[00112] The term "cancer", as used herein, refers to a malignant tumor, for example, a tumor caused by a malignant cell growth or proliferation, or metastasis-mediated, solid tumors and non-solid tumors such as leukemia. A "cancer" comprises one or more cancerous cells. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastema, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (e.g., epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer ("NSCLC"), adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, as well as head and neck cancer.

[00113] The term "treatment", "treating" or "treated", as used herein in the context of treating a condition, pertains generally to treatment and therapy, whether of a human or an animal, in which some desired therapeutic effect is achieved, for example, the inhibition of the progress of the condition, and includes a reduction in the rate of progress, a halt in the rate of progress, regression of the condition, amelioration of the condition, and cure of the condition. Treatment as a prophylactic measure (i.e., prophylaxis, prevention) is also included. For cancer, "treating" may refer to dampen or slow the tumor or malignant cell growth, proliferation, or metastasis, or some combination thereof. For tumors, "treatment" includes removal of all or part of the tumor, inhibiting or slowing tumor growth and metastasis, preventing, or delaying the development of a tumor, or some combination thereof.

[00114] The antibody is against tumor-associated antigens (TAA), an antigen of a cell that is responsible for producing autoimmune antibodies, a viral or a microbial antigen. Tumor-associated antigens are known in the art, and can be prepared for use in generating antibodies using methods and information which are well known in the art. In attempts to discover effective cellular targets for cancer diagnosis and therapy, researchers have sought to identify transmembrane or otherwise tumor-associated polypeptides that are specifically expressed on the surface of one or more particular type(s) of cancer cell as compared to on one or more normal non-cancerous cell(s). Often,

such tumor-associated polypeptides are more abundantly expressed on the surface of the cancer cells as compared to on the surface of the non-cancerous cells. The identification of such tumor-associated cell surface antigen polypeptides has given rise to the ability to specifically target cancer cells for destruction via antibody -based therapies.

[00115] Examples of tumor-associated antigens TAA include, but are not limited to, TAA (1)-(53) listed herein. For convenience, information relating to these antigens, all of which are known in the art, is listed herein and includes names, alternative names, GenBank accession numbers and primary reference(s), following nucleic acid and protein sequence identification conventions of the National Center for Biotechnology Information (NCBI) (see WO2017068511A1, which is entirely incorporated herein by reference). Nucleic acid and protein sequences corresponding to TAA (1)-(53) are available in public databases such as GenBank. Tumor-associated antigens targeted by antibodies include all amino acid sequence variants and isoforms possessing at least about 70%, 80%, 85%, 90%, or 95% sequence identity relative to the sequences identified in the cited references, or which exhibit substantially the same biological properties or characteristics as a TAA having a sequence found in the cited references. For example, a TAA having a variant sequence generally is able to bind specifically to an antibody that binds specifically to the TAA with the corresponding sequence listed. The sequences and disclosure in the reference specifically recited herein are expressly incorporated by reference.

[00116] **TUMOR-ASSOCIATED ANTIGENS (TAAs)**

[00117] (1) BMPR1B (bone morphogenetic protein receptor-type 1B, GenBank accession no. NM_001203) ten Dijke, P., et al. Science 264 (5155): 101-104 (1994), Oncogene 14 (11): 1377-1382 (1997)); WO2004063362 (Claim 2); WO2003042661 (Claim 12); US2003134790-A1 (Page 38-39); WO2002102235 (Claim 13; Page 296); WO2003055443 (Page 91-92); WO200299122 (Example 2; Page 528-530); WO2003029421 (Claim 6); WO2003024392 (Claim 2; Fig 112); WO200298358 (Claim 1; Page 183); WO200254940 (Page 100-101); WO200259377 (Page 349-350); WO200230268 (Claim 27; Page 376); WO200148204 (Example; Fig 4) NP_001194 bone morphogenetic protein receptor, type IB/pid=NP_001194.1 - Cross-references: MIM:603248; NP_001194.1; AY065994.

[00118] (2) E16 (LAT1, SLC7A5, GenBank accession no. NM_003486) Biochem. Biophys. Res. Commun. 255 (2), 283-288 (1999), Nature 395 (6699):288-291 (1998), Gaugitsch, H.W., et

al. (1992) J. Biol. Chem. 267 (16): 11267-11273); WO2004048938 (Example 2); WO2004032842 (Example IV); WO2003042661 (Claim 12); WO2003016475 (Claim 1); WO200278524 (Example 2); WO200299074 (Claim 19; Page 127-129); WO200286443 (Claim 27; Pages 222, 393); WO2003003906 (Claim 10; Page 293); WO200264798 (Claim 33; Page 93-95); WO200014228 (Claim 5; Page 133-136); US2003224454 (Fig 3); WO2003025138 (Claim 12; Page 150); NP_003477 solute carrier family 7 (cationic amino acid transporter, y+ system), member 5 /pid=NP_003477.3 - Homo sapiens Cross-references: MIM:600182; NP_003477.3; NM_015923; NM_003486_1.

[00119] (3) STEAP1 (six transmembrane epithelial antigen of prostate, GenBank accession no. NM_012449) Cancer Res. 61 (15), 5857-5860 (2001), Hubert, R.S., et al. (1999) Proc. Natl. Acad. Sci. U.S.A. 96 (25): 14523-14528); WO2004065577 (Claim 6); WO2004027049 (Fig 1L); EP1394274 (Example 11); WO2004016225 (Claim 2); WO2003042661 (Claim 12); US2003157089 (Example 5); US2003185830 (Example 5); US2003064397 (Fig 2); WO200289747 (Example 5; Page 618-619); WO2003022995 (Example 9; Fig 13A, Example 53; Page 173, Example 2; Fig 2A); NP_036581 six transmembrane epithelial antigen of the prostate Cross-references: MIM:604415; NP_036581.1; NM_012449_1.

[00120] (4) 0772P (CA125, MUC16, GenBank accession no. AF361486) J. Biol. Chem. 276 (29):27371-27375 (2001)); WO2004045553 (Claim 14); WO200292836 (Claim 6; Fig 12); WO200283866 (Claim 15; Page 116-121); US2003124140 (Example 16); US 798959. Cross-references: GI:34501467; AAK74120.3; AF361486_1.

[00121] (5) MPF (MPF, MSLN, SMR, megakaryocyte potentiating factor, mesothelin, GenBank accession no. NM_005823), Yamaguchi, N., et al. Biol. Chem. 269 (2), 805-808 (1994), Proc. Natl. Acad. Sci. U.S.A. 96 (20): 11531-11536 (1999), Proc. Natl. Acad. Sci. U.S.A. 93 (1): 136-140 (1996), J. Biol. Chem. 270 (37):21984-21990 (1995)); WO2003101283 (Claim 14); WO2002102235 (Claim 13; Page 287-288); WO2002101075 (Claim 4; Page 308-309); WO200271928 (Page 320-321); WO9410312 (Page 52-57); Cross-references: MIM:601051; NP_005814.2; NM_005823_1.

[00122] (6) Napi3b (NAPI-3B, NPTIIb, SLC34A2, solute carrier family 34 (sodium phosphate), member 2, type II sodium-dependent phosphate transporter 3b, GenBank accession no. NM_006424) J. Biol. Chem. 277 (22): 19665-19672 (2002), Genomics 62 (2):281-284 (1999),

J.A., et al. (1999) Biochem. Biophys. Res. Commun. 258 (3):578-582); WO2004022778 (Claim 2); EP1394274 (Example 11); WO2002102235 (Claim 13; Page 326); EP875569 (Claim 1; Page 17-19); WO200157188 (Claim 20; Page 329); WO2004032842 (Example IV); WO200175177 (Claim 24; Page 139-140); Cross-references: MIM:604217; NP_006415.1; NM_006424_1.

[00123] (7) Sema 5b (FLJ10372, KIAA1445, Mm.42015, SEMA5B, SEMAG, Semaphorin 5b Hlog, sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5B, GenBank accession no. AB040878) Nagase T., et al. (2000) DNA Res. 7 (2): 143-150); WO2004000997 (Claim 1); WO2003003984 (Claim 1); WO200206339 (Claim 1; Page 50); WO200188133 (Claim 1; Page 41-43, 48-58); WO2003054152 (Claim 20); WO2003101400 (Claim 11); Accession: Q9P283; EMBL; AB040878; BAA95969.1. Genew; HGNC: 10737.

[00124] (8) PSCA hlg (2700050C12Rik, C530008O16Rik, RIKEN cDNA 2700050C12, RIKEN cDNA 2700050C12 gene, GenBank accession no. AY358628); Ross et al. (2002) Cancer Res. 62:2546-2553; US2003129192 (Claim 2); US2004044180 (Claim 12); US2004044179 (Claim 11); US2003096961 (Claim 11); US2003232056 (Example 5); WO2003105758 (Claim 12); US2003206918 (Example 5); EP1347046 (Claim 1); WO2003025148 (Claim 20); Cross-references: GE37182378; AAQ88991.1; AY358628_1.

[00125] (9) ETBR (Endothelin type B receptor, GenBank accession no. AY275463); Nakamuta M., et al. Biochem. Biophys. Res. Commun. 177, 34-39, 1991; Ogawa Y., et al. Biochem. Biophys. Res. Commun. 178, 248-255, 1991; Arai H., et al. Jpn. Circ. J. 56, 1303-1307, 1992; Arai H., et al. J. Biol. Chem. 268, 3463-3470, 1993; Sakamoto A., Yanagisawa M., et al. Biochem. Biophys. Res. Commun. 178, 656-663, 1991; Elshourbagy N.A., et al. J. Biol. Chem. 268, 3873-3879, 1993; Haendler B., et al. J. Cardiovasc. Pharmacol. 20, s1-S4, 1992; Tsutsumi M., et al. Gene 228, 43-49, 1999; Strausberg R.L., et al. Proc. Natl. Acad. Sci. U.S.A. 99, 16899-16903, 2002; Bourgeois C, et al. J. Clin. Endocrinol. Metab. 82, 3116-3123, 1997; Okamoto Y., et al. Biol. Chem. 272, 21589-21596, 1997; Verheij J.B., et al. Am. J. Med. Genet. 108, 223-225, 2002; Hofstra R.M.W., et al. Eur. J. Hum. Genet. 5, 180-185, 1997; Puffenberger E.G., et al. Cell 79, 1257-1266, 1994; Attie T., et al., Hum. Mol. Genet. 4, 2407-2409, 1995; Auricchio A., et al. Hum. Mol. Genet. 5 :351-354, 1996; Amiel J., et al. Hum. Mol. Genet. 5, 355-357, 1996; Hofstra R.M.W., et al. Nat. Genet. 12, 445-447, 1996; Svensson P.J., et al. Hum. Genet. 103, 145-148, 1998; Fuchs S., et al.

Mol. Med. 7, 115-124, 2001; Pingault V., et al. (2002) Hum. Genet. Ill, 198-206; WO2004045516 (Claim 1); WO2004048938 (Example 2); WO2004040000 (Claim 151); WO2003087768 (Claim 1); WO2003016475 (Claim 1); WO2003016475 (Claim 1); WO200261087 (Fig 1); WO2003016494 (Fig 6); WO2003025138 (Claim 12; Page 144); WO200198351 (Claim 1; Page 124-125); EP522868 (Claim 8; Fig 2); WO200177172 (Claim 1; Page 297-299); US2003109676; US6518404 (Fig 3); US5773223 (Claim 1a; Col 31-34); WO2004001004.

[00126] (10) MSG783 (RNF124, hypothetical protein FLJ20315, GenBank accession no. NM_017763); WO2003104275 (Claim 1); WO2004046342 (Example 2); WO2003042661 (Claim 12); WO2003083074 (Claim 14; Page 61); WO2003018621 (Claim 1); WO2003024392 (Claim 2; Fig 93); WO200166689 (Example 6); Cross-references: Locus ID: 54894; NP_060233.2; NM_017763_1.

[00127] (11) STEAP2 (HGNC_8639, IPCA-1, PCANAP1, STAMP1, STEAP2, STMP, prostate cancer associated gene 1, prostate cancer associated protein 1, six transmembrane epithelial antigen of prostate 2, six transmembrane prostate protein, GenBank accession no. AF455138); Lab. Invest. 82 (11): 1573-1582 (2002); WO2003087306; US2003064397 (Claim 1; Fig 1); WO200272596 (Claim 13; Page 54-55); WO200172962 (Claim 1; Fig 4B); WO2003104270 (Claim 11); WO2003104270 (Claim 16); US2004005598 (Claim 22); WO2003042661 (Claim 12); US2003060612 (Claim 12; Fig 10); WO200226822 (Claim 23; Fig 2); WO200216429 (Claim 12; Fig 10); Cross-references: GE22655488; AAN04080.1; AF455138_1.

[00128] (12) TrpM4 (BR22450, FLJ20041, TRPM4, TRPM4B, transient receptor potential cation channel, subfamily M, member 4, GenBank accession no. NM_017636) Xu, X.Z., et al. Proc. Natl. Acad. Sci. U.S.A. 98 (19): 10692-10697 (2001), Cell 109 (3):397-407 (2002), J. Biol. Chem. 278 (33):30813-30820 (2003)); US2003143557 (Claim 4); WO200040614 (Claim 14; Page 100-103); WO200210382 (Claim 1; Fig 9A); WO2003042661 (Claim 12); WO200230268 (Claim 27; Page 391); US2003219806 (Claim 4); WO200162794 (Claim 14; Fig 1A-D); Cross-references: MIM:606936; NP_060106.2; NM_017636_1.

[00129] (13) CRIPTO (CR, CR1, CRGF, CRIPTO, TDGF1, teratocarcinoma-derived growth factor, GenBank accession no. NP_003203 or NM_003212) Ciccodicola, A., et al. EMBO J. 8 (7): 1987-1991 (1989), Am. J. Hum. Genet. 49 (3):555-565 (1991)); US2003224411 (Claim 1);

WO2003083041 (Example 1); WO2003034984 (Claim 12); WO200288170 (Claim 2; Page 52-53); WO2003024392 (Claim 2; Fig 58); WO200216413 (Claim 1; Page 94-95, 105); WO200222808 (Claim 2; Fig 1); US5854399 (Example 2; Col 17-18); US5792616 (Fig 2); Cross-references: MIM: 187395; NP_003203.1; NM_003212_1.

[00130] (14) CD21 (CR2 (Complement receptor 2) or C3DR (C3d/Epstein Barr virus receptor) or Hs.73792 GenBank accession no. M26004) Fujisaku et al. (1989) J. Biol. Chem. 264 (4):2118-2125); Weis J.J., et al. J. Exp. Med. 167, 1047-1066, 1988; Moore M., et al. Proc. Natl. Acad. Sci. U.S.A. 84, 9194-9198, 1987; Barel M., et al. Mol. Immunol. 35, 1025-1031, 1998; Weis J.J., et al. Proc. Natl. Acad. Sci. U.S.A. 83, 5639-5643, 1986; Sinha S.K., et al. (1993) J. Immunol. 150, 5311-5320; WO2004045520 (Example 4); US2004005538 (Example 1); WO2003062401 (Claim 9); WO2004045520 (Example 4); WO9102536 (Fig 9.1-9.9); WO2004020595 (Claim 1); Accession: P20023; Q13866; Q14212; EMBL; M26004; AAA35786.1.

[00131] (15) CD79b (CD79B, CD79β, IGb (immunoglobulin-associated beta), B29, GenBank accession no. NM_000626 or 11038674) Proc. Natl. Acad. Sci. U.S.A. (2003) 100 (7):4126-4131, Blood (2002) 100 (9):3068-3076, Muller et al. (1992) Eur. J. Immunol. 22 (6): 1621-1625); WO2004016225 (claim 2, Fig 140); WO2003087768, US2004101874 (claim 1, page 102); WO2003062401 (claim 9); WO200278524 (Example 2); US2002150573 (claim 5, page 15); US5644033; WO2003048202 (claim 1, pages 306 and 309); WO 99/558658, US6534482 (claim 13, Fig 17A/B); WO200055351 (claim 11, pages 1145-1146); Cross-references: MIM: 147245; NP_000617.1; NM_000626_1.

[00132] (16) FcRH2 (IFGP4, IRTA4, SPAP1A (SH2 domain containing phosphatase anchor protein 1a), SPAP1B, SPAP1C, GenBank accession no. NM_030764, AY358130) Genome Res. 13 (10):2265-2270 (2003), Immunogenetics 54 (2):87-95 (2002), Blood 99 (8):2662-2669 (2002), Proc. Natl. Acad. Sci. U.S.A. 98 (17):9772-9777 (2001), Xu, M.J., et al. (2001) Biochem. Biophys. Res. Commun. 280 (3):768-775; WO2004016225 (Claim 2); WO2003077836; WO200138490 (Claim 5; Fig 18D-1-18D-2); WO2003097803 (Claim 12); WO2003089624 (Claim 25); Cross-references: MIM:606509; NP_110391.2; NM_030764_1.

[00133] (17) HER2 (ErbB2, GenBank accession no. M11730) Coussens L., et al. Science (1985) 230(4730): 1132-1139); Yamamoto T., et al. Nature 319, 230-234, 1986; Semba K., et al. Proc. Natl. Acad. Sci. U.S.A. 82, 6497-6501, 1985; Swiercz J.M., et al. J. Cell Biol. 165, 869-880, 2004;

Kuhns J.J., et al. J. Biol. Chem. 274, 36422-36427, 1999; Cho H.-S., et al. Nature 421, 756-760, 2003; Ehsani A., et al. (1993) Genomics 15, 426-429; WO2004048938 (Example 2); WO2004027049 (Fig 11); WO2004009622; WO2003081210; WO2003089904 (Claim 9); WO2003016475 (Claim 1); US2003118592; WO2003008537 (Claim 1); WO2003055439 (Claim 29; Fig 1A-B); WO2003025228 (Claim 37; Fig 5C); WO200222636 (Example 13; Page 95-107); WO200212341 (Claim 68; Fig 7); WO200213847 (Page 71-74); WO200214503 (Page 114-117); WO200153463 (Claim 2; Page 41-46); WO200141787 (Page 15); WO200044899 (Claim 52; Fig 7); WO200020579 (Claim 3; Fig 2); US5869445 (Claim 3; Col 31-38); WO9630514 (Claim 2; Page 56-61); EP 1439393 (Claim 7); WO2004043361 (Claim 7); WO2004022709; WO200100244 (Example 3; Fig 4); Accession: P04626; EMBL; M11767; AAA35808.1. EMBL; M11761; AAA35808.1.

[00134] (18) NCA (CEACAM6, GenBank accession no. M18728); Barnett T., et al. Genomics 3, 59-66, 1988; Tawaragi Y., et al. Biochem. Biophys. Res. Commun. 150, 89-96, 1988; Strausberg R.L., et al. Proc. Natl. Acad. Sci. U.S.A. 99: 16899-16903, 2002; WO2004063709; EP1439393 (Claim 7); WO2004044178 (Example 4); WO2004031238; WO2003042661 (Claim 12); WO200278524 (Example 2); WO200286443 (Claim 27; Page 427); WO200260317 (Claim 2); Accession: P40199; Q14920; EMBL; M29541; AAA59915.1. EMBL; M18728.

[00135] (19) MDP (DPEP1, GenBank accession no. BC017023) Proc. Natl. Acad. Sci. U.S.A. 99 (26): 16899-16903 (2002)); WO2003016475 (Claim 1); WO200264798 (Claim 33; Page 85-87); JP05003790 (Fig 6-8); W09946284 (Fig 9); Cross-references: MIM: 179780; AAH17023.1; BC017023_1.

[00136] (20) IL20Rα (IL20Rα, ZCYTOR7, GenBank accession no. AF184971); Clark H.F., et al. Genome Res. 13, 2265-2270, 2003; Mungall A.J., et al. Nature 425, 805-811, 2003; Blumberg H., et al. Cell 104, 9-19, 2001; Dumoutier L., et al. J. Immunol. 167, 3545-3549, 2001; Parrish-Novak J., et al. J. Biol. Chem. 277, 47517-47523, 2002; Pletnev S., et al. (2003) Biochemistry 42: 12617-12624; Sheikh F., et al. (2004) J. Immunol. 172, 2006-2010; EP1394274 (Example 11); US2004005320 (Example 5); WO2003029262 (Page 74-75); WO2003002717 (Claim 2; Page 63); WO200222153 (Page 45-47); US2002042366 (Page 20-21); WO200146261 (Page 57- 59); WO200146232 (Page 63-65); W09837193 (Claim 1; Page 55-59); Accession: Q9UHF4; Q6UWA9; Q96SH8; EMBL; AF184971; AAF01320.1.

[00137] (21) Brevican (BCAN, BEHAB, GenBank accession no. AF229053); Gary S.C., et al. Gene 256, 139-147, 2000; Clark H.F., et al. Genome Res. 13, 2265-2270, 2003; Strausberg R.L., et al. Proc. Natl. Acad. Sci. U.S.A. 99, 16899-16903, 2002; US2003186372 (Claim 11); US2003186373 (Claim 11); US2003119131 (Claim 1; Fig 52); US2003119122 (Claim 1; Fig 52); US2003119126 (Claim 1); US2003119121 (Claim 1; Fig 52); US2003119129 (Claim 1); US2003119130 (Claim 1); US2003119128 (Claim 1; Fig 52); US2003119125 (Claim 1); WO2003016475 (Claim 1); WO200202634 (Claim 1).

[00138] (22) EphB2R (DRT, ERK, Hek5, EPHT3, Tyro5, GenBank accession no. NM_004442); Chan, J. and Watt, V.M., Oncogene 6 (6), 1057-1061 (1991) Oncogene 10 (5):897-905 (1995), Annu. Rev. Neurosci. 21:309-345 (1998), Int. Rev. Cytol. 196: 177-244 (2000)); WO2003042661 (Claim 12); WO200053216 (Claim 1; Page 41); WO2004065576 (Claim 1); WO2004020583 (Claim 9); WO2003004529 (Page 128-132); WO200053216 (Claim 1; Page 42); Cross-references: MIM: 600997; NP_004433.2; NM_004442_1.

[00139] (23) ASLG659 (B7h, GenBank accession no. AX092328); US20040101899 (Claim 2); WO2003104399 (Claim 11); WO2004000221 (Fig 3); US2003165504 (Claim 1); US2003124140 (Example 2); US2003065143 (Fig 60); WO2002102235 (Claim 13; Page 299); US2003091580 (Example 2); WO200210187 (Claim 6; Fig 10); WO200194641 (Claim 12; Fig 7b); WO200202624 (Claim 13; Fig 1A-1B); US2002034749 (Claim 54; Page 45-46); WO200206317 (Example 2; Page 320-321, Claim 34; Page 321-322); WO200271928 (Page 468-469); WO200202587 (Example 1; Fig 1); WO200140269 (Example 3; Pages 190-192); WO200036107 (Example 2; Page 205-207); WO2004053079 (Claim 12); WO2003004989 (Claim 1); WO200271928 (Page 233-234, 452-453); WO 0116318.

[00140] (24) PSCA (Prostate stem cell antigen precursor, GenBank accession no. AJ297436); Reiter R.E., et al. Proc. Natl. Acad. Sci. U.S.A. 95, 1735-1740, 1998; Gu Z., et al. Oncogene 19, 1288-1296, 2000; Biochem. Biophys. Res. Commun. (2000) 275(3):783-788; WO2004022709; EP1394274 (Example 11); US2004018553 (Claim 17); WO2003008537 (Claim 1); WO200281646 (Claim 1; Page 164); WO2003003906 (Claim 10; Page 288); WO200140309 (Example 1; Fig 17); US2001055751 (Example 1; Fig 1b); WO200032752 (Claim 18; Fig 1); WO9851805 (Claim 17; Page 97); W09851824 (Claim 10; Page 94); WO9840403 (Claim 2; Fig 1B); Accession: 043653; EMBL; AF043498; AAC39607.1.

[00141] (25) GEDA (GenBank accession No. AY260763); AAP14954 lipoma HMGIC fusion-partner-like protein /pid= AAP14954.1 - Homo sapiens Species: Homo sapiens (human) WO2003054152 (Claim 20); WO2003000842 (Claim 1); WO2003023013 (Example 3, Claim 20); US2003194704 (Claim 45); Cross-references: GI:30102449; AAP14954.1; AY260763_1.

[00142] (26) BAFF-R (B cell-activating factor receptor, BLyS receptor 3, BR3, GenBank accession No. AF116456); BAFF receptor /pid=NP_443177.1 - Homo sapiens Thompson, J.S., et al. Science 293 (5537), 2108-2111 (2001); WO2004058309; WO2004011611; WO2003045422 (Example; Page 32-33); WO2003014294 (Claim 35; Fig 6B); WO2003035846 (Claim 70; Page 615-616); WO200294852 (Col 136-137); WO200238766 (Claim 3; Page 133); WO200224909 (Example 3; Fig 3); Cross-references: MIM: 606269; NP_443177.1; NM_052945_1; AF132600.

[00143] (27) CD22 (B-cell receptor CD22-B isoform, BL-CAM, Lyb-8, Lyb8, SIGLEC-2, FLJ22814, GenBank accession No. AK026467); Wilson et al. (1991) J. Exp. Med. 173: 137-146; WO2003072036 (Claim 1; Fig 1); Cross-references: MIM: 107266; NP_001762.1; NM_001771_1.

[00144] (28) CD79a (CD79A, CD79α, immunoglobulin-associated alpha, a B cell-specific protein that covalently interacts with Ig beta (CD79B) and forms a complex on the surface with Ig M molecules, transduces a signal involved in B-cell differentiation), pI: 4.84, MW: 25028 TM: 2 [P] Gene Chromosome: 19q13.2, GenBank accession No. NP_001774.10); WO2003088808; US20030228319; WO2003062401 (claim 9); US2002150573 (claim 4, pages 13-14); W09958658 (claim 13, Fig 16); WO9207574 (Fig 1); US5644033; Ha et al. (1992) J. Immunol. 148(5): 1526-1531; Mueller et al. (1992) Eur. J. Biochem. 22: 1621-1625; Hashimoto et al. (1994) Immunogenetics 40(4):287-295; Preud'homme et al. (1992) Clin. Exp. Immunol. 90(1): 141-146; Yu et al. (1992) J. Immunol. 148(2) 633-637; Sakaguchi et al. (1988) EMBO J. 7(11):3457-3464.

[00145] (29) CXCR5 (Burkitt's lymphoma receptor 1, a G protein-coupled receptor that is activated by the CXCL13 chemokine, functions in lymphocyte migration and humoral defense, plays a role in HIV-2 infection and perhaps development of AIDS, lymphoma, myeloma, and leukemia); 372 aa, pI: 8.54 MW: 41959 TM: 7 [P] Gene Chromosome: 11q23.3, GenBank accession No. NP_001707.1); WO2004040000; WO2004015426; US2003105292 (Example 2); US6555339 (Example 2); WO200261087 (Fig 1); WO200157188 (Claim 20, page 269); WO200172830 (pages 12-13); WO200022129 (Example 1, pages 152-153, Example 2, pages 254-

256); W09928468 (claim 1, page 38); US5440021 (Example 2, col 49-52); W09428931 (pages 56-58); W09217497 (claim 7, Fig 5); Dobner et al. (1992) Eur. J. Immunol. 22:2795- 2799; Barella et al. (1995) Biochem. J. 309:773-779.

[00146] (30) HLA-DOB (Beta subunit of MHC class II molecule (Ia antigen) that binds peptides and presents them to CD4+ T lymphocytes); 273 aa, pI: 6.56 MW: 30820 TM: 1 [P] Gene Chromosome: 6p21.3, GenBank accession No. NP_002111.1); Tonnelle et al. (1985) EMBO J. 4(11):2839-2847; Jonsson et al. (1989) Immunogenetics 29(6):411-413; Beck et al. (1992) J. Mol. Biol. 228:433-441; Strausberg et al. (2002) Proc. Natl. Acad. Sci USA 99: 16899-16903; Servenius et al. (1987) J. Biol. Chem. 262:8759-8766; Beck et al. (1996) J. Mol. Biol. 255: 1-13; Naruse et al. (2002) Tissue Antigens 59:512-519; W09958658 (claim 13, Fig 15); US6153408 (Col 35-38); US5976551 (col 168-170); US6011146 (col 145-146); Kasahara et al. (1989) Immunogenetics 30(1):66-68; Larhammar et al. (1985) J. Biol. Chem. 260(26): 14111-14119.

[00147] (31) P2X5 (Purinergic receptor P2X ligand-gated ion channel 5, an ion channel gated by extracellular ATP, may be involved in synaptic transmission and neurogenesis, deficiency may contribute to the pathophysiology of idiopathic detrusor instability); 422 aa, pI: 7.63, MW: 47206 TM: 1 [P] Gene Chromosome: 17p13.3, GenBank accession No. NP_002552.2); Le et al. (1997) FEBS Lett. 418(1-2): 195-199; WO2004047749; WO2003072035 (claim 10); Touchman et al. (2000) Genome Res. 10: 165-173; WO200222660 (claim 20); WO2003093444 (claim 1); WO2003087768 (claim 1); WO2003029277 (page 82).

[00148] (32) CD72 (B-cell differentiation antigen CD72, Lyb-2, 359 aa, pI: 8.66, MW: 40225 TM: 1 [P] Gene Chromosome: 9p13.3, GenBank accession No. NP_001773.1); WO2004042346 (claim 65); WO2003026493 (pages 51- 52, 57-58); WO200075655 (pages 105-106); Von Hoegen et al. (1990) J. Immunol. 144(12):4870-4877; Strausberg et al. (2002) Proc. Natl. Acad. Sci USA 99: 16899-16903.

[00149] (33) LY64 (Lymphocyte antigen 64 (RP105), type I membrane protein of the leucine rich repeat (LRR) family, regulates B-cell activation and apoptosis, loss of function is associated with increased disease activity in patients with systemic lupus erythematosis); 661 aa, pI: 6.20, MW: 74147 TM: 1 [P] Gene Chromosome: 5q12, GenBank accession No. NP_005573.1); US2002193567; WO9707198 (claim 11, pages 39-42); Miura et al. (1996) Genomics 38(3):299-

304; Miura et al. (1998) Blood 92:2815-2822; WO2003083047; W09744452 (claim 8, pages 57-61); WO200012130 (pages 24-26).

[00150] (34) FcRH1 (Fc receptor-like protein 1, a putative receptor for the immunoglobulin Fc domain that contains C2 type Ig-like and ITAM domains, may have a role in B-lymphocyte differentiation); 429 aa, pI: 5.28, MW: 46925 TM: 1 [P] Gene Chromosome: 1q21-1q22, GenBank accession No. NP_443170.1); WO2003077836; WO200138490 (claim 6, Fig 18E-1-18-E-2); Davis et al. (2001) Proc. Natl. Acad. Sci USA 98(17):9772-9777; WO2003089624 (claim 8); EP1347046 (claim 1); WO2003089624 (claim 7).

[00151] (35) IRTA2 (Immunoglobulin superfamily receptor translocation associated 2, a putative immunoreceptor with possible roles in B cell development and lymphomagenesis; deregulation of the gene by translocation occurs in some B cell malignancies); 977 aa, pI: 6.88 MW: 106468 TM: 1 [P] Gene Chromosome: 1q21, GenBank accession No. Human: AF343662, AF343663, AF343664, AF343665, AF369794, AF397453, AK090423, AK090475, AL834187, AY358085; Mouse:AK089756, AY158090, AY506558; NP_112571.1; WO2003024392 (claim 2, Fig 97); Nakayama et al. (2000) Biochem. Biophys. Res. Commun. 277(1): 124-127; WO2003077836; WO200138490 (claim 3, Fig 18B-1 to 18B-2).

[00152] (36) TENB2 (TMEFF2, tomoregulin, TPEF, HPP1, TR, putative transmembrane proteoglycan, related to the EGF/heregulin family of growth factors and follistatin); 374 aa, NCBI Accession: AAD55776, AAF91397, AAG49451, NCBI RefSeq: NP_057276; NCBI Gene: 23671; OMIM: 605734; SwissProt Q9UIK5; GenBank accession No. AF179274; AY358907, CAF85723, CQ782436 WO2004074320 (SEQ ID NO 810); JP2004113151 (SEQ ID NOS 2, 4, 8); WO2003042661 (SEQ ID NO 580); WO2003009814 (SEQ ID NO 411); EP1295944 (pages 69-70); WO200230268 (page 329); WO200190304 (SEQ ID NO 2706); US2004249130; US2004022727; WO2004063355; US2004197325; US2003232350; US2004005563; US2003124579; Horie et al. (2000) Genomics 67: 146-152; Uchida et al. (1999) Biochem. Biophys. Res. Commun. 266:593-602; Liang et al. (2000) Cancer Res. 60:4907-12; Glynne-Jones et al. (2001) Int J Cancer. Oct 15; 94(2): 178-84.

[00153] (37) PMEL17 (silver homolog; SILV; D12S53E; PMEL17; SI; SIL); ME20; gp100) BC001414; BT007202; M32295; M77348; NM_006928; McGlinchey, R.P. et al. (2009) Proc.

Natl. Acad. Sci. U.S.A. 106 (33), 13731-13736; Kummer, M.P. et al. (2009) J. Biol. Chem. 284 (4), 2296-2306.

[00154] (38) TMEFF1 (transmembrane protein with EGF-like and two follistatin-like domains 1; Tomoregulin-1); H7365; C9orf2; C90RF2; U19878; X83961; NM_080655; NM_003692; Harms, P.W. (2003) Genes Dev. 17 (21), 2624-2629; Gery, S. et al. (2003) Oncogene 22 (18):2723-2727.

[00155] (39) GDNF-Ra1 (GDNF family receptor alpha 1; GFRA1; GDNFR; GDNFRA; RETL1; TRNR1; RET1L; GDNFR-alpha1; GFR-ALPHA-1); U95847; BC014962; NM 145793 NM_005264; Kim, M.H. et al. (2009) Mol. Cell. Biol. 29 (8), 2264-2277; Treanor, J.J. et al. (1996) Nature 382 (6586):80-83.

[00156] (40) Ly6E (lymphocyte antigen 6 complex, locus E; Ly67, RIG-E, SCA-2, TSA-1); NP_002337.1; NM_002346.2; de Nooij-van Dalen, A G. et al. (2003) Int. J. Cancer 103 (6), 768-774; Zammit, D.J. et al. (2002) Mol. Cell. Biol. 22 (3):946-952.

[00157] (41) TMEM46 (shisa homolog 2 (Xenopus laevis); SHISA2); NP_001007539.1; NM_001007538.1; Furushima, K. et al. (2007) Dev. Biol. 306 (2), 480-492; Clark, H.F. et al. (2003) Genome Res. 13 (10):2265-2270.

[00158] (42) Ly6G6D (lymphocyte antigen 6 complex, locus G6D; Ly6-D, MEGT1); NP_067079.2; NM_021246.2; Mallya, M. et al. (2002) Genomics 80 (1): 113-123; Ribas, G. et al. (1999) J. Immunol. 163 (1):278-287.

[00159] (43) LGR5 (leucine-rich repeat-containing G protein-coupled receptor 5; GPR49, GPR67); NP_003658.1; NM_003667.2; Salanti, G. et al. (2009) Am. J. Epidemiol. 170 (5):537-545; Yamamoto, Y. et al. (2003) Hepatology 37 (3):528-533.

[00160] (44) RET (ret proto-oncogene; MEN2A; HSCR1; MEN2B; MTC1; PTC; CDHF12; Hs.168114; RET51; RET-ELE1); NP_066124.1; NM_020975.4; Tsukamoto, H. et al. (2009) Cancer Sci. 100 (10): 1895-1901; Narita, N. et al. (2009) Oncogene 28 (34):3058-3068.

[00161] (45) LY6K (lymphocyte antigen 6 complex, locus K; LY6K; HSJ001348; FLJ35226); NP_059997.3; NM_017527.3; Ishikawa, N. et al. (2007) Cancer Res. 67 (24): 11601-11611; de Nooij-van Dalen, A G. et al. (2003) Int. J. Cancer 103 (6):768-774.

[00162] (46) GPR19 (G protein-coupled receptor 19; Mm.4787); NP_006134.1; NM_006143.2; Montpetit, A. and Sinnett, D. (1999) Hum. Genet. 105 (1-2): 162-164; O'Dowd, B.F. et al. (1996) FEBS Lett. 394 (3):325-329.

[00163] (47) GPR54 (KISS1 receptor; KISS1R; GPR54; HOT7T175; AXOR12); NP_115940.2; NM_032551.4; Navenot, J. M. et al. (2009) Mol. Pharmacol. 75 (6): 1300-1306; Hata, K. et al. (2009) Anticancer Res. 29 (2) :617-623.

[00164] (48) ASPHD1 (aspartate beta-hydroxylase domain containing 1; LOC253982); NP_859069.2; NM_181718.3; Gerhard, D.S. et al. (2004) Genome Res. 14 (10B):2121-2127.

[00165] (49) Tyrosinase (TYR; OCAIA; OCA1A; tyrosinase; SHEP3); NP_000363.1; NM_000372.4; Bishop, D.T. et al. (2009) Nat. Genet. 41 (8):920-925; Nan, H. et al. (2009) Int. J. Cancer 125 (4):909-917.

[00166] (50) TMEM118 (ring finger protein, transmembrane 2; RNFT2; FLJ14627); NP_001103373.1; NM_001109903.1; Clark, H.F. et al. (2003) Genome Res. 13 (10):2265-2270; Scherer, S.E. et al. (2006) Nature 440 (7082):346-351.

[00167] (51) GPR172A (G protein-coupled receptor 172A; GPCR41; FLJ11856; D15Ertd747e); NP_078807.1; NM_024531.3; Ericsson, T.A. et al. (2003) Proc. Natl. Acad. Sci. U.S.A. 100 (l l):6759-6764; Takeda, S. et al. (2002) FEBS Lett. 520 (1-3):97-101.

[00168] (52) CD33, a member of the sialic acid binding, immunoglobulin-like lectin family, is a 67-kDa glycosylated transmembrane protein. CD33 is expressed on most myeloid and monocytic leukemia cells in addition to committed myelomonocytic and erythroid progenitor cells. It is not seen on the earliest pluripotent stem cells, mature granulocytes, lymphoid cells, or nonhematopoietic cells (Sabbath et al., (1985) J. Clin. Invest. 75:756-56; Andrews et al., (1986) Blood 68: 1030-5). CD33 contains two tyrosine residues on its cytoplasmic tail, each of which is followed by hydrophobic residues similar to the immunoreceptor tyrosine-based inhibitory motif (ITIM) seen in many inhibitory receptors.

[00169] (53) CLL-1 (CLEC12A, MICL, and DCAL2), encodes a member of the C-type lectin/C-type lectin-like domain (CTL/CTLD) superfamily. Members of this family share a common protein fold and have diverse functions, such as cell adhesion, cell-cell signaling, glycoprotein turnover, and roles in inflammation and immune response. The protein encoded by

this gene is a negative regulator of granulocyte and monocyte function. Several alternatively spliced transcript variants of this gene have been described, but the full-length nature of some of these variants has not been determined. This gene is closely linked to other CTL/CTLD superfamily members in the natural killer gene complex region on chromosome 12p13 (Drickamer K (1999) Curr. Opin. Struct. Biol. 9 (5):585-90; van Rhenen A, et al, (2007) Blood 110 (7):2659-66; Chen CH, et al. (2006) Blood 107 (4): 1459-67; Marshall AS, et al. (2006) Eur. J. Immunol. 36 (8):2159-69; Bakker AB, et al. (2005) Cancer Res. 64 (22): 8443-50; Marshall AS, et al. (2004) J. Biol. Chem. 279 (15): 14792-802). CLL-1 has been shown to be a type II transmembrane receptor comprising a single C-type lectin-like domain (which is not predicted to bind either calcium or sugar), a stalk region, a transmembrane domain and a short cytoplasmic tail containing an ITIM motif.

[00170] **THE CONJUGATION PROCESS OF THE PRESENT INVENTION**

[00171] In an aspect, the present disclosure relates to a process for preparing a composition of antibody-drug conjugates (ADCs), comprising the following steps:

(a) reduction step: incubating a reductant and the antibody in a buffer system to reduce inter-chain disulfide bonds within the antibody, optionally purifying the reduced antibody;

(b) re-oxidation step: adding an oxidant to re-oxidize some of the reduced thiol groups, optionally purifying the re-oxidized antibody;

wherein the step (a) or the step (b) is performed in the presence of transition metal ions, and wherein in step (b), the reduced thiol groups which are not blocked by the transition metal ions are re-oxidated,

(c) conjugation step: removing transition metal ions and adding an excess amount of payload bearing reactive groups to react with reduced thiol groups of the re-oxidized antibody; and

(d) recovering step: recovering the resultant antibody-drug conjugates to obtain a composition of antibody-drug conjugates (ADCs).

[00172] In the context of the disclosure, the expression that "the step (a) or the step (b) is performed in the presence of transition metal ions" means that one of the step (a) and the step (b) is performed in the presence of transition metal ions, for example, the step (a) is performed in the presence of transition metal ions, or the step (b) is performed in the presence of transition metal ions. However, the present application does not exclude that both step (a) and step (b) are

performed in the presence of transition metal ions. For example, the process of the present application can be performed in a one-pot manner, where if the transition metal ions are added in step (a), step (b) will be also performed in the presence of transition metal ions. From the view of economic efficiency, the transition metal ions can be added only once, i.e., in step (a) or in step (b).

[00173] In one embodiment, the process for preparing a composition of antibody-drug conjugates (ADCs) with improved homogeneity comprises the following steps:

(a) reduction step: incubating a reductant and the antibody to be conjugated in the presence of transition metal ions in a buffer system to reduce inter-chain disulfide bonds within the antibody, optionally purifying the reduced antibody;

(b) re-oxidation step: adding an oxidant to re-oxidize the reduced thiol groups which are not blocked by the transition metal ions, optionally purifying the re-oxidized antibody;

(c) conjugation step: removing transition ions and adding an excess amount of payload bearing reactive groups (e.g., maleimide linking drugs, etc.) to react with reduced thiol groups of the re-oxidized antibody; and

(d) recovering step: recovering the resultant antibody-drug conjugates to obtain a composition of antibody-drug conjugates;

wherein the antibody-drug conjugates (ADCs) with drug to antibody molar ratio of 2 (D2) are present in a high level in the composition.

[00174] In another embodiment, the process for preparing a composition of antibody-drug conjugates (ADCs) comprises the following steps:

(a) reduction step: incubating a reductant and the antibody to be conjugated in a buffer system to reduce inter-chain disulfide bonds within the antibody, optionally purifying the reduced antibody;

(b) re-oxidation step: adding a soluble transition metal salt or a solution comprising a soluble transition metal salt, and adding an oxidant to re-oxidize the reduced thiol groups which are not blocked by the transition metal ions in the presence of transition metal ions, optionally purifying the re-oxidized antibody;

(c) conjugation step: removing transition ions, and adding an excess amount of payload bearing reactive groups to react with reduced thiol groups of the re-oxidized antibody; and

(d) recovering step: recovering the resultant antibody-drug conjugates to obtain a composition of antibody-drug conjugates;

wherein the antibody-drug conjugates with drug to antibody molar ratio of 2 (D2) are present in a high level in the composition.

[00175] In the present disclosure, the phrase that "antibody-drug conjugates (ADCs) with drug to antibody molar ratio of 2 (D2) are present in a high level in the composition" means, in the ADCs prepared by the process of the present disclosure, the content of D2 (i.e., antibody-drug conjugates (ADCs) with drug to antibody molar ratio of 2) is generally more than 64 mol%, preferably more than 75 mol% or even more than 80mol%, based on the total molar of the produced antibody-drug conjugates (ADCs) (i.e., D0, D2, D4, D6 and D8).

[00176] In one embodiment, the reductant may be selected from, but not limited to, Tris(2-carboxyethyl) phosphine (TCEP), THPP (Tris(3-hydroxypropyl)phosphine), diPPBs (2-diphenylphosphanylbenzenesulfonic acid), DTT (Dithiothreitol), DPAA (2-(Diphenylphosphino) acetic acid), DTE (Dithioerythritol), β-Mercaptoethanol, $LiAlH_4$, $Na_2S_2O_3$, KBH_4, or Hydrazine. In one embodiment, the reductant in step (a) is TCEP. In one embodiment, in step (a), the molar ratio of reductant/antibody in the reaction solution is in a range of 1 to 20, for example, 2 to 16, preferably 3 to 8. In a specific embodiment, in step (a), the reductant is added at a molar ratio of 8 relative to the antibody, i.e., the molar ratio of reductant/antibody is 8.

[00177] In one embodiment, the transition metal ion in step (a) or step (b) is Zn^{2+}. In one embodiment, in step (a) or step (b), the molar ratio of Zn^{2+}/antibody in the reaction solution is in a range of 0.5 to 50, preferably 1 to 16, more preferably 2 to 8. In a specific embodiment, in step (a) or step (b), the transition metal ions are added at a molar ratio of 4 relative to the antibody, i.e., the molar ratio of transition metal ions/antibody is 4. Zinc salts which are water soluble are suitable for the process of the present disclosure. For example, $ZnCl_2$ may be added in step (a) as the Zn^{2+} source.

[00178] Depending on the transition metal ions, those skilled in the art can select suitable buffer system for the reaction in step (a), including, but not limited to, TAPS, Bicine, Tris, Tricine,

HEPES, TES, MOPS, PIPES, Sodium citrate, Histidine buffer, PB (Phosphate buffer), PBS, MES, and the like. In a specific embodiment, the buffer system used in step (a) is PB buffer, and the pH value of the buffer system is about 5 to 8, preferably about 7.0.

[00179] The transition metal ions will be removed by appropriate methods known in the art, for example, but not limited to, by adjusting pH (e.g., adding NaOH, HCl, etc.), by adjusting temperature, by changing buffer (e.g., adding NaCl, sodium citrate, etc.), or by using chelating reagent such as EDTA, DTPA, Ethylenediamine, 2,2'-Bipyridine, or 1,10-phenanthroline, and then will be optionally filtered out in subsequent dialysis, ultrafiltration, or gel filtration.

[00180] In one embodiment, the oxidant may be selected from, but not limited to, Hydrogen peroxide (H_2O_2), nitrate compounds, Potassium chlorate ($KClO_3$), Peroxydisulfuric acid ($H_2S_2O_8$), Peroxymonosulfuric acid (H_2SO_5), NaClO, Sodium dichromate ($Na_2Cr_2O_7$), Permanganate compounds, Sodium perborate, Nitrous oxide, Sodium bismuthate ($NaBiO_3$), ceric sulfate, dehydroascorbic acid (DHAA), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP) , or 2-Aminophenyl disulfide (DDD). Those skilled in the art may appreciate that any other oxidants that can oxidize sulfhydryl groups can be used in the present invention. In one embodiment, the oxidant to be added in step (b) is DHAA. In another embodiment, the oxidant to be added in step (b) is DTNB. In an embodiment, in step (b), the molar ratio of oxidant /antibody in the reaction solution is in a range of 0.5 to 160, for example, 10 to 160 (weak oxidant), 5 to 24 (medium oxidant) or 0.5 to 10 (strong oxidant). In a specific embodiment, in step (b), the oxidant is added at a molar ratio of 16 relative to the antibody, i.e., the molar ratio of oxidant/antibody is 16. In one embodiment, DTNB is used as the oxidant at a molar ratio of oxidant/antibody from 1.6 to 7.7. In another embodiment, DHAA is used as the oxidant at a molar ratio of oxidant/antibody of 16 or 40. In yet another embodiment, DDD is used as the oxidant at a molar ratio of oxidant/antibody of 1.82.

[00181] The optimum pH for the reaction will typically between about 5 and about 8, for instance, about 5.5 to about 7.5, or about 6 to about 7.5. The optimal reaction conditions will depend upon the specific reactants employed. In a specific embodiment, the pH in step (a) and step (b) is about 6 to about 7.5, preferably about 7.0, and the pH in step (c) is about 5 to about 8, preferably about 6.5-7.0.

[00182] In an embodiment, the buffer is PB (phosphate buffer), pH 6.90.

[00183] The incubation time and temperature for each step can be determined by those skilled in the art based on the specific antibody to be conjugated. The optimum temperature for the reaction will typically be between about -10 and 37°C. The reaction occurs, for instance, at a temperature between about 0 and 20°C overnight.

[00184] Those skilled in the art should understand that the incubation time period and temperature in step (a) may depend on specific antibodies to be conjugated. The determination of the incubation time period and temperature based on specific antibodies is within the abilities of ordinary skilled persons in the art. For example, the antibody to be conjugated is typically incubated with the reductant in the presence of transition metal ions at 4°C overnight, or at 12°C for 20 hours. In a specific embodiment, in step (a) (i.e., reduction step), the temperature may be 0°C to 37°C, preferably 2°C to 25°C, e.g., 4°C, 12°C, 22°C or 37°C, and the incubation time may be 0.5h to 90h, for example 0.5h to 72h, preferably 2h to 20h, more preferably 16h to 20h, for example, 2h,16h, 20h, 45h, or 90h. In step (b) (i.e., re-oxidation step), the temperature may be 0°C to 37°C, preferably 2°C to 25°C or 4 to 12°C, e.g., 0°C, 12°C, 22°C or 37°C, and the incubation time may be 0.5h to 170h, preferably 0.5h to 72h, more preferably 0.5h to 48h, for example, 0.5h, 1h, 2h, 4h, 24h, 47h, or 170h. In step (c) (i.e., conjugation step), the temperature may be 0°C to 37°C, preferably 2°C to 25°Cor 4 to 12°C, e.g., 0°C, 4°C or 12°C and the incubation time may be 0.5h to 72h, preferably 8h to 16h, more preferably 1h to 4h.

[00185] In some embodiments, the antibody to be conjugated, the transition metal ions and the reductant may be present in the reaction mixture in a ratio of 1:2:4 in molar concentration, but not limited to this ratio. In one embodiment, 0.02 mM antibody is incubated with 0.08 mM TCEP and 0.04 mM $ZnCl_2$ at 4°C overnight.

[00186] There is no specific limitation to the antibody to be conjugated. According to the antigens associated with the disease or disorder (e.g., specific tumor-associated antigens, viral antigens, or microbial antigens), those skilled in the art can select suitable antibody useful in the bio-conjugation process of the present disclosure. In some embodiments, the antibody is an antibody which binds to one or more tumor-associated antigens or cell-surface receptors as described elsewhere herein. The antibody may include, but not limited to, a monoclonal antibody, a polyclonal antibody, a monospecific antibody, a multi-specific antibody, or an antibody derivate.

[00187] In an embodiment, the antibody is a monoclonal antibody. In another embodiment, the antibody is a human antibody, a humanized antibody, or a chimeric antibody. In a further embodiment, the antibody is an antibody fragment, e.g., a Fv, Fab, Fab', scFv, diabody, or F(ab')2 fragment. In another embodiment, the antibody is a substantially full-length antibody, e.g., an IgG1 antibody, IgG4 antibody or other antibody class or isotype as defined herein. In a specific embodiment, the antibody is an IgG1 antibody.

[00188] In some other embodiments, the antibody is selected from Trastuzumab, Rituximab, Cetuximab, or antibodies against any one of the following antigens: BMPR1B, E16, STEAP1, MUC16, MPF, Napi2b, Sema 5b, PSCA hlg, ETBR, MSG783, STEAP2, TrpM4, CRIPTO, CD21, CD79b, FcRH2, HER2, NCA, MDP, IL20Ra, Brevican, EphB2R, ASLG659, PSCA, GEDA, BAFF-R, CD22, CD79a, CXCR5, HLA-DOB, P2X5, CD72, LY64, FcRH1, FcRH5, TENB2, PMEL17, TMEFF1, GDNF-Ra1, Ly6E, TMEM46, Ly6G6D, LGR5, RET, Ly6K, GPR19, GPR54, ASPHD1, Tyrosinase, TMEM118, GPR172A, CD33 and CLL-1.

[00189] Useful Polyclonal antibody is a heterogeneous population of antibody molecules derived from the sera of immunized animals. Various procedures well known in the art may be used to produce polyclonal antibodies to an antigen-of-interest. For example, to produce polyclonal antibodies, various host animals can be immunized by injection with an antigen of interest or derivative thereof, including but not limited to rabbits, mice, rats, and guinea pigs.

[00190] Useful monoclonal antibody is a homogeneous population of antibodies to a particular antigen (e.g., a cancer cell antigen, a viral antigen, a microbial antigen covalently linked to a second molecule). A monoclonal antibody (mAb) to an antigen-of-interest can be prepared by using any technique known in the art, for example, producing antibody molecules by continuous cell lines in culture. Said technique includes, but are not limited to, the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256, 495497), the human-B cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4: 72), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, and IgD and any subclass thereof. The hybridoma producing the mAbs of use in this invention may be cultivated *in vitro* or *in vivo*.

[00191] Useful monoclonal antibodies include, but are not limited to, human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80, 7308-7312; Kozbor et al., 1983, Immunology Today 4, 72-79; and Olsson et al., 1982, Meth. Enzymol. 92, 3-16).

[00192] Additionally, recombinant antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are useful Ligands. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine monoclonal and a human immunoglobulin constant region. (See, e.g., Cabilly et al., U.S. Pat. No. 4,816,567; and Boss et al., U.S. Pat. No. 4,816,397, which are incorporated herein by reference in their entirety.) Humanized antibodies are antibody molecules from non-human species having one or more complementarity determining regions (CDRs) from the non-human species and a framework region from a human immunoglobulin molecule. (See, e.g., Queen, U.S. Pat. No. 5,585,089, which is incorporated herein by reference in its entirety.) Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in International Publication No. WO 87/02671; European Patent Publication No. 184,187; European Patent Publication No. 171,496; European Patent Publication No. 173,494; International Publication No. WO 86/01533; U.S. Pat. No. 4,816,567; European Patent Publication No.125,023; Berter et al., 1988, Science 240:1041-1043; Liu et al., 1987, Proc. Natl. Acad. Sci. USA 84:3439-3443; Liu et al., 1987, J. Immunol. 139:3521-3526; Sun et al., 1987, Proc. Natl. Acad. Sci. USA 84:214-218; Nishimura et al., 1987, Canc. Res. 47:999-1005; Wood et al., 1985, Nature 314:446-449; and Shaw et al., 1988, J. Natl. Cancer Inst. 80:1553-1559; Morrison, 1985, Science 229:1202-1207; Oi et al., 1986, BioTechniques 4:214; U.S. Pat. No. 5,225,539; Jones et al., 1986, Nature 321:552-525; Verhoeyan et al. (1988) Science 239:1534; and Beidler et al., 1988, J. Immunol. 141:4053-4060; each of which is incorporated herein by reference in its entirety.

[00193] In a specific embodiment, known antibodies for the treatment or prevention of cancer are used in the invention. Antibodies immune-specific for a cancer cell antigen can be obtained commercially or produced by any method known to one of skill in the art, such as, e.g., chemical

synthesis or recombinant expression techniques. The nucleotide sequence encoding antibodies immuno-specific for a cancer cell antigen can be obtained, e.g., from the GenBank database or a database like it, the literature publications, or by routine cloning and sequencing. Examples of antibodies available for the treatment of cancer include, but are not limited to, HERCEPTIN (Trastuzumab; Genentech, CA) which is a humanized anti-HER2 monoclonal antibody for the treatment of patients with metastatic breast cancer (Stebbing, J., Copson, E., and O'Reilly, S., "Herceptin (trastuzamab) in advanced breast cancer" Cancer Treat Rev. 26, 287-90, 2000); RITUXAN (rituximab; Genentech) which is a chimeric anti-CD20 monoclonal antibody for the treatment of patients with non-Hodgkin's lymphoma; Erbitux (Cetuximab; Merck) which is an IgG1 monoclonal antibody against EGF receptors and used for the treatment of colorectal cancer; OvaRex (AltaRex Corporation, MA) which is a murine antibody for the treatment of ovarian cancer; Panorex (Glaxo Wellcome, NC) which is a murine IgG2a antibody for the treatment of colorectal cancer; BEC2 (ImClone Systems Inc., NY) which is murine IgG antibody for the treatment of lung cancer; IMC-C225 (Imclone Systems Inc., NY) which is a chimeric IgG antibody for the treatment of head and neck cancer; Vitaxin (MedImmune, Inc., MD) which is a humanized antibody for the treatment of sarcoma; Campath I/H (Leukosite, MA) which is a humanized IgG1 antibody for the treatment of chronic lymphocytic leukemia (CLL); Smart MI95 (Protein Design Labs, Inc., CA) which is a humanized IgG antibody for the treatment of acute myeloid leukemia (AML); LymphoCide (Immunomedics, Inc., NJ) which is a humanized IgG antibody for the treatment of non-Hodgkin's lymphoma; Smart ID10 (Protein Design Labs, Inc., CA) which is a humanized antibody for the treatment of non-Hodgkin's lymphoma; Oncolym (Techniclone, Inc., CA) which is a murine antibody for the treatment of non-Hodgkin's lymphoma; Allomune (BioTransplant, CA) which is a humanized anti-CD2 mAb for the treatment of Hodgkin's Disease or non-Hodgkin's lymphoma; anti-VEGF (Genentech, Inc., CA) which is humanized antibody for the treatment of lung and colorectal cancers; CEAcide (Immunomedics, NJ) which is a humanized anti-CEA antibody for the treatment of colorectal cancer; IMC-1C11 (ImClone Systems, NJ) which is an anti-KDR chimeric antibody for the treatment of colorectal cancer, lung cancers, and melanoma; and Cetuximab (ImClone, NJ) which is an anti-EGFR chimeric antibody for the treatment of epidermal growth factor positive cancers.

[00194] Other antibodies useful in the treatment of cancer include, but are not limited to, antibodies against the following antigens: CA125 (ovarian), CA15-3 (carcinomas), CA19-9

(carcinomas), L6 (carcinomas), Lewis Y (carcinomas), Lewis X (carcinomas), alpha fetoprotein (carcinomas), CA 242 (colorectal), placental alkaline phosphatase (carcinomas), prostate specific antigen (prostate), prostatic acid phosphatase (prostate), epidermal growth factor (carcinomas), MAGE-1 (carcinomas), MAGE-2 (carcinomas), MAGE-3 (carcinomas), MAGE 4 (carcinomas), anti-transferrin receptor (carcinomas), p97 (melanoma), MUC1-KLH (breast cancer), CEA (colorectal), gp100 (melanoma), MART1 (melanoma), PSA (prostate), IL-2 receptor (T-cell leukemia and lymphomas), CD20 (non-Hodgkin's lymphoma), CD52 (leukemia), CD33 (leukemia), CD22 (lymphoma), human chorionic gonadotropin (carcinoma), CD38 (multiple myeloma), CD40 (lymphoma), mucin (carcinomas), P21 (carcinomas), MPG (melanoma), and Neu oncogene product (carcinomas). Some specific useful antibodies include, but are not limited to, BR96 mAb (Trail, P. A., Willner, D., Lasch, S. J., Henderson, A. J., Hofstead, S. J., Casazza, A. M., Firestone, R. A., Hellström, I., Hellström, K. E., "Cure of Xenografted Human Carcinomas by BR96-Doxorubicin Immunoconjugates" Science 1993, 261, 212-215), BR64 (Trail, Pa., Willner, D, Knipe, J., Henderson, A. J., Lasch, S. J., Zoeckler, M. E., Trailsmith, M. D., Doyle, T. W., King, H. D., Casazza, A. M., Braslawsky, G. R., Brown, J. P., Hofstead, S. J., Greenfield, Ill. S., Firestone, R. A., Mosure, K., Kadow, D. F., Yang, M. B., Hellstrom, K E., and Hellstrom, I. "Effect of Linker Variation on the Stability, Potency, and Efficacy of Carcinoma-reactive BR64-Doxorubicin Immunoconjugates" Cancer Research 1997, 57, 100-105), mAbs against the CD40 antigen, such as S2C6 mAb (Francisco, J. A., Donaldson, K. L., Chace, D., Siegall, C. B., and Wahl, A. F. "Agonistic properties and in vivo antitumor activity of the anti-CD-40 antibody, SGN-14" Cancer Res. 2000, 60, 3225-3231), mAbs against the CD70 antigen, such as 1F6 mAb, and mAbs against the CD30 antigen, such as AC10 (Bowen, M. A., Olsen, K. J., Cheng, L., Avila, D., and Podack, E. R. "Functional effects of CD30 on a large granular lymphoma cell line YT" J. Immunol., 151, 5896-5906, 1993). Many other internalizing antibodies that bind to tumor associated antigens can be used in this invention, and have been reviewed (Franke, A. E., Sievers, E. L., and Scheinberg, D. A., "Cell surface receptor-targeted therapy of acute myeloid leukemia: a review" Cancer Biother Radiopharm. 2000, 15,459-76; Murray, J. L., "Monoclonal antibody treatment of solid tumors: a coming of age" Semin Oncol. 2000, 27, 64-70; Breitling, F., and Dubel, S., Recombinant Antibodies, John Wiley, and Sons, New York, 1998).

[00195] In another specific embodiment, known antibodies for the treatment or prevention of an autoimmune disease are used in accordance with the process of the disclosure. Antibodies

immune-specific for an antigen of a cell that is responsible for producing autoimmune antibodies can be obtained from any organization (e.g., a university scientist or a company such as Genentech) or produced by any method known to one of skill in the art such as, e.g., chemical synthesis or recombinant expression techniques. In another embodiment, useful ligand antibodies that are immune-specific for the treatment of autoimmune diseases include, but are not limited to, Anti-Nuclear Antibody; Anti-dsDNA; Anti-ssDNA, Anti Cardiolipin Antibody IgM, IgG; Anti-Phospholipid Antibody IgK, IgG; Anti-SM Antibody; Anti-Mitochondrial Antibody; Thyroid Antibody; Microsomal Antibody, Thyroglobulin Antibody, Anti-SCL-70; Anti-Jo; Anti-U1RNP; Anti-La/SSB; Anti SSA; Anti SSB; Anti-Perital Cells Antibody; Anti-Histones; Anti-RNP; C-ANCA; P-ANCA; Anti-centromere; Anti-Fibrillarin, and Anti GBM Antibody.

[00196] In another specific embodiment, useful antibodies that are immuno-specific for a viral or a microbial antigen are monoclonal antibodies. For instance, antibodies that are immuno-specific for a viral antigen or microbial antigen may be humanized or human monoclonal antibodies. As used herein, the term "viral antigen" includes, but is not limited to, any viral peptide, polypeptide protein (e.g. HIV gp120, HIV nef, RSV F glycoprotein, influenza virus neuraminidase, influenza virus hemagglutinin, HTLV tax, herpes simplex virus glycoprotein (e.g., gB, gC, gD, and gE) and hepatitis B surface antigen) that is capable of eliciting an immune response. As used herein, the term "microbial antigen" includes, but is not limited to, any microbial peptide, polypeptide, protein, saccharide, polysaccharide, or lipid molecule (e.g., a bacterial, fungi, pathogenic protozoa, or yeast polypeptide including, e.g., LPS and capsular polysaccharide 5/8) that can elicit an immune response.

[00197] Antibodies immuno-specific for a viral or microbial antigen can be obtained commercially, for example, from Genentech (San Francisco, Calif.) or produced by any method known to one of skill in the art such as, e.g., chemical synthesis or recombinant expression techniques. The nucleotide sequence encoding antibodies that are immuno-specific for a viral or microbial antigen can be obtained, e.g., from the GenBank database or a database like it, the literature publications, or by routine cloning and sequencing.

[00198] In a specific embodiment, useful antibodies are those that are useful for the treatment or prevention of viral or microbial infection. Examples of antibodies available useful for the treatment of viral infection or microbial infection include, but are not limited to, SYNAGIS

(MedImmune, Inc., MD) which is a humanized anti-respiratory syncytial virus (RSV) monoclonal antibody useful for the treatment of patients with RSV infection; PRO542 (Progenics) which is a CD4 fusion antibody useful for the treatment of HIV infection; OSTAVIR (Protein Design Labs, Inc., CA) which is a human antibody useful for the treatment of hepatitis B virus; PROTVIR (Protein Design Labs, Inc., CA) which is a humanized IgG1 antibody useful for the treatment of cytomegalovirus (CMV); and anti-LPS antibodies.

[00199] Other antibodies useful in the treatment of infectious diseases include, but are not limited to, antibodies against the antigens from pathogenic strains of bacteria (Streptococcus pyogenes, Streptococcus pneumoniae, Neisseria gonorrheae, Neisseria meningitidis, Corynebacterium diphtheriae, Clostridium botulinum, Clostridium perfringens, Clostridium tetani, Hemophilus influenzae, Klebsiella pneumoniae, Klebsiella ozaenas, Klebsiella rhinoscleromotis, Staphylococcus aureus, Vibrio colerae, Escherichia coli, Pseudomonas aeruginosa, Campylobacter (Vibrio) fetus, Aeromonas hydrophila, Bacillus cereus, Edwardsiella tarda, Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Salmonella typhimurium, Treponema pallidum, Treponema pertenue, Treponema carateneum, Borrelia vincentii, Borrelia burgdorferi, Leptospira icterohemorrhagiae, Mycobacterium tuberculosis, Pneumocystis carinii, Francisella tularensis, Brucella abortus, Brucella suis, Brucella melitensis, Mycoplasma spp., Rickettsia prowazeki, Rickettsia tsutsugumushi, Clamydia spp.); pathogenic fungi (Coccidioides immitis, Aspergillus fumigatus, Candida albicans, Blastomyces dermatitidis, Cryptococcus neoformans, Histoplasma capsulatum); protozoa (Entomoeba histolytica, Toxoplasma gondii, Trichomonas tenas, Trichomonas hominis, Trichomonas vaginalis, Tryoanosoma gambiense, Trypanosoma rhodesiense, Trypanosoma cruzi, Leishmania donovani, Leishmania tropica, Leishmania braziliensis, Pneumocystis pneumonia, Plasmodium vivax, Plasmodium falciparum, Plasmodium malaria); or Helminiths (Enterobius vermicularis, Trichuris trichiura, Ascaris lumbricoides, Trichinella spiralis, Strongyloides stercoralis, Schistosoma japonicum, Schistosoma mansoni, Schistosoma haematobium, and hookworms).

[00200] Other antibodies useful in this invention for treatment of viral disease include, but are not limited to, antibodies against antigens of pathogenic viruses, including as examples and not by limitation: Poxyiridae, Herpesviridae, Herpes Simplex virus 1, Herpes Simplex virus 2,

Adenoviridae, Papovaviridae, Enteroviridae, Picornaviridae, Parvoviridae, Reoviridae, Retroviridae, influenza viruses, parainfluenza viruses, mumps, measles, respiratory syncytial virus, rubella, Arboviridae, Rhabdoviridae, Arenaviridae, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis E virus, Non-A/Non-B Hepatitis virus, Rhinoviridae, Coronaviridae, Rotoviridae, and Human Immunodeficiency Virus.

[00201] The antibodies suitable for use in the bio-conjugation process provided herein can be produced by any method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or by recombinant expression, and, for example, are produced by recombinant expression techniques.

[00202] As for the payload bearing reactive group to be conjugated to the selected antibody, it generally has a format of drug-linker. There are no specific limitations to the drug and linker which can be used in the bio-conjugation process of the present disclosure, as long as the drug molecule has an antitumor, antiviral or antimicrobial effect and contains at least one substituted group or a partial structure allowing connection to a linker structure, and the linker contains at least two reactive groups, one of which can covalently bond a drug molecule and the other of which can covalently couple to an antibody.

[00203] Depending on the desired drug and selected linker, those skilled in the art can select suitable method for coupling them together. For example, some conventional coupling methods, such as amine coupling methods, may be used to form the desired drug-linker complex which still contains reactive groups for conjugating to the antibodies through covalent linkage. A drug-maleimide complex (i.e., maleimide linking drug) is taken as an example of the payload bearing reactive group in the present disclosure.

[00204] In an embodiment, the drug may include, but not limited to, cytotoxic reagents, such as chemo-therapeutic agents, immunotherapeutic agents and the like, antiviral agents or antimicrobial agents. In an embodiment, the drug to be conjugated with an antibody may be selected from, but not limited to, MMAE (monomethyl auristatin E), MMAD (monomethyl auristatin D), MMAF (monomethyl auristatin F), and the like.

[00205] Most common reactive group capable of bonding to thiol group in ADC preparation is maleimide. Additionally, organic bromides, iodides also are frequently used.

[00206] Drug loading is represented by the number of drug moieties per antibody in a molecule of ADC. For some antibody-drug conjugates, the drug loading may be limited by the number of attachment sites on the antibody. For example, where the attachment is a cysteine thiol, as in certain exemplary embodiments described herein, the drug loading may range from 0 to 8 drug moieties per antibody. In certain embodiments, higher drug loading, e.g. p $\geq$5, may cause aggregation, insolubility, toxicity, or loss of cellular permeability of certain antibody-drug conjugates. In certain embodiments, the average drug loading for an antibody-drug conjugate ranges from 1 to about 8; from about 2 to about 6; or from about 2 to about 5.

[00207] It is to be understood that where more than one nucleophilic group reacts with a drug, then the resulting product is a mixture of antibody-drug conjugate compounds with a distribution of one or more drug moieties attached to an antibody. The average number of drugs per antibody may be calculated from the mixture by a dual ELISA antibody assay, which is specific for antibody and specific for the drug. Individual antibody-drug conjugate molecules may be identified in the mixture by mass spectroscopy and separated by HPLC, e.g. hydrophobic interaction chromatography (see, e.g., McDonagh et al. (2006) Prot. Engr. Design & Selection 19(7):299-307; Hamblett et al. (2004) Clin. Cancer Res. 10:7063-7070; Hamblett, K.J., et al. "Effect of drug loading on the pharmacology, pharmacokinetics, and toxicity of an anti-CD30 antibody-drug conjugate", Abstract No. 624, American Association for Cancer Research, 2004 Annual Meeting, March 27-31, 2004, Proceedings of the AACR, Volume 45, March 2004; Alley, S.C., et al. "Controlling the location of drug attachment in antibody-drug conjugates", Abstract No. 627, American Association for Cancer Research, 2004 Annual Meeting, March 27-31, 2004, Proceedings of the AACR, Volume 45, March 2004). In certain embodiments, a homogeneous antibody-drug conjugate with a single loading value may be isolated from the conjugation mixture by electrophoresis or chromatography.

[00208] Improving homogeneity of ADCs requires isolation of ADC with certain drug loading or selective attachment of drug moieties to an antibody in the conjugation process. However, the isolation of ADCs with certain drug loading will result in complex manipulation and high cost. With a conjugation process using the same steps without the addition of transition metal ions as a negative control (see US7659241B2), the inventors successfully demonstrated that transition metal ions were the key factor responsible for higher level of D2 and lower level of D0, D4, D6 and D8

in the resultant ADCs. By using the process of the present disclosure to produce antibody-drug conjugates, the homogeneity of the antibody-drug conjugates is higher than those produced by conventional conjugation processes. Specifically, in the composition of ADCs prepared by the process of the present disclosure, the content of D2 is generally more than 64 mol%, preferably more than 75 mol% or even more than 80mol%, while the content D2 is normally less than 50 mol% in the ADCs prepared by conventional conjugation processes without transition metal ions. Moreover, the content of D0+D4+D6+D8 in the ADCs prepared by the process of the present disclosure is less than 36mol%, preferably less than 25mol%, more preferably less than 20 mol%, most preferably less than 15%, while the content of D0+D4+D6+D8 is normally more than 40 mol% in the ADCs prepared by conventional conjugation processes.

[00209] In an embodiment, the resultant antibody-drug conjugates are recovered or further purified by any suitable method, such as using a de-salting column, size exclusion chromatography, ultrafiltration, dialysis, UF-DF, and the like.

[00210] ANTIBODY-DRUG CONJUGATES WITH IMPROVED HOMOGENEITY

[00211] In an aspect, the present disclosure relates to a composition of antibody-drug conjugates (ADCs) produced by the process of the present invention, wherein the content of D2 is generally more than 64 mol%, preferably more than 75 mol% or even more than 80mol%, and the content of D0+D4+D6+D8 in the ADCs is less than 36mol%, preferably less than 25mol%, more preferably less than 20 mol%, most preferably less than 15%, based on total molar of D0, D2, D4, D6 and D8.

[00212] In an embodiment, the homogeneity of the antibody-drug conjugates generated by the process of the first aspect is measured, and compared with the homogeneity of corresponding control antibody-drug conjugates generated by conventional conjugation processes.

[00213] Various analytical methods can be used to determine the yields and isomeric mixtures of the antibody-drug conjugates. For example, in one embodiment, Hydrophobic interaction chromatography (HIC) is the analytical method used to determine yields and isomeric mixtures from resultant antibody-drug conjugates (e.g., for D2 conjugates). This technique is able to separate antibodies loaded with various numbers of drugs. The drug loading level can be determined based on the ratio of absorbances, e.g., at 250 nm and 280 nm. For example, if a drug

can absorb at 250 nm while the antibody absorbs at 280 nm. The 250/280 ratio therefore increases with drug loading. Using the bio-conjugation process described herein, generally antibodies with even numbers of drugs were observed to be conjugated to the antibody since reduction of disulfides yields even numbers of free cysteine thiols.

[00214] As compared with corresponding control antibody-drug conjugates generated by conventional conjugation processes, the antibody-drug conjugates generated by the process of the present invention have improved homogeneity, which is represented by the increased content of D2 in the resultant ADCs. In an embodiment, as shown in Tables 2 to 3, the content of D2 in the antibody-drug conjugates produced by the process of the present disclosure is higher than 75 mol%, preferably higher than 80 mol%, whereas the content of D2 in the antibody-drug conjugates produced by conventional conjugation processes is usually less than 50 mol% (Table 1).

[00215] A PHARMACEUTICAL COMPOSITION

[00216] In a further aspect, the present disclosure relates to a pharmaceutical composition comprising a composition of antibody-drug conjugates (ADCs) produced by the process of the present invention and a pharmaceutically acceptable carrier. The pharmaceutical composition is suitable for veterinary or human administration. In the pharmaceutical composition, the composition of antibody-drug conjugates (ADCs) produced by the process of the present invention is present in an effective amount, for example, in an amount effective for treating a condition or disorder in a subject.

[00217] The pharmaceutical compositions of the present invention can be in any form that allows for the pharmaceutical composition to be administered to an animal. For example, the pharmaceutical composition can be in the form of a solid, liquid or gas (aerosol). Typical routes of administration include, without limitation, oral, topical, parenteral, sublingual, rectal, vaginal, ocular, and intranasal. Parenteral administration includes subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques. For example, the compositions are administered parenterally. Pharmaceutical compositions of the invention can be formulated so as to allow ADCs of the invention to be bioavailable upon administration of the pharmaceutical composition to an animal. Pharmaceutical compositions can take the form of one or more dosage units, where for example, a tablet can be a single dosage unit, and a container of ADCs of the invention in aerosol form can hold a plurality of dosage units.

[00218] Materials used in preparing the pharmaceutical compositions can be non-toxic in the amounts used. It will be evident to those of ordinary skill in the art that the optimal dosage of the active ingredient(s) in the pharmaceutical composition will depend on a variety of factors. Relevant factors include, without limitation, the type of animal (e.g., human), the particular form of the ADCs of the invention, the manner of administration, and the composition employed.

[00219] Suitable components may include, for example, antioxidants, fillers, binders, disintegrants, buffers, preservatives, lubricants, flavorings, thickeners, coloring agents, emulsifiers, or stabilizers such as sugars and cyclodextrins. Suitable antioxidants may include, for example, methionine, ascorbic acid, EDTA, sodium thiosulfate, platinum, catalase, citric acid, cysteine, thioglycerol, thioglycolic acid, thiosorbitol, butylated hydroxanisol, butylated hydroxytoluene, and/or propyl gallate. As disclosed herein, inclusion of one or more antioxidants such as methionine in a pharmaceutical composition provided herein decreases oxidation of the polypeptide complex or the bispecific polypeptide complex. This reduction in oxidation prevents or reduces loss of binding affinity, thereby improving protein stability and maximizing shelf-life. Therefore, in certain embodiments, compositions are provided that comprise the polypeptide complex or the bispecific polypeptide complex disclosed herein and one or more antioxidants such as methionine.

[00220] To further illustrate, pharmaceutical acceptable carriers may include, for example, aqueous vehicles such as sodium chloride injection, Ringer's injection, isotonic dextrose injection, sterile water injection, or dextrose and lactated Ringer's injection, non-aqueous vehicles such as fixed oils of vegetable origin, cottonseed oil, corn oil, sesame oil, or peanut oil, antimicrobial agents at bacteriostatic or fungistatic concentrations, isotonic agents such as sodium chloride or dextrose, buffers such as phosphate or citrate buffers, antioxidants such as sodium bisulfate, local anesthetics such as procaine hydrochloride, suspending and dispersing agents such as sodium carboxymethylcelluose, hydroxypropyl methylcellulose, or polyvinylpyrrolidone, emulsifying agents such as Polysorbate 80 (TWEEN-80), sequestering or chelating agents such as EDTA (ethylenediaminetetraacetic acid) or EGTA (ethylene glycol tetraacetic acid), ethyl alcohol, polyethylene glycol, propylene glycol, sodium hydroxide, hydrochloric acid, citric acid, or lactic acid. Antimicrobial agents utilized as carriers may be added to pharmaceutical compositions in multiple-dose containers that include phenols or cresols, mercurials, benzyl alcohol, chlorobutanol,

methyl and propyl p-hydroxybenzoic acid esters, thimerosal, benzalkonium chloride and benzethonium chloride. Suitable excipients may include, for example, water, saline, dextrose, glycerol, or ethanol. Suitable non-toxic auxiliary substances may include, for example, wetting or emulsifying agents, pH buffering agents, stabilizers, solubility enhancers, or agents such as sodium acetate, sorbitan monolaurate, triethanolamine oleate, or cyclodextrin.

[00221] The pharmaceutical compositions can be a liquid solution, suspension, emulsion, pill, capsule, tablet, sustained release formulation, or powder. Oral formulations can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, polyvinyl pyrollidone, sodium saccharine, cellulose, magnesium carbonate, etc.

[00222] In certain embodiments, the pharmaceutical compositions are formulated into an injectable composition. The injectable pharmaceutical compositions may be prepared in any conventional form, such as for example liquid solution, suspension, emulsion, or solid forms suitable for generating liquid solution, suspension, or emulsion. Preparations for injection may include sterile and/or non-pyretic solutions ready for injection, sterile dry soluble products, such as lyophilized powders, ready to be combined with a solvent just prior to use, including hypodermic tablets, sterile suspensions ready for injection, sterile dry insoluble products ready to be combined with a vehicle just prior to use, and sterile and/or non-pyretic emulsions. The solutions may be either aqueous or non-aqueous.

[00223] In certain embodiments, unit-dose parenteral preparations are packaged in an ampoule, a vial or a syringe with a needle. All preparations for parenteral administration should be sterile and not pyretic, as is known and practiced in the art.

[00224] In certain embodiments, a sterile, lyophilized powder is prepared by dissolving the ADCs as disclosed herein in a suitable solvent. The solvent may contain an excipient which improves the stability or other pharmacological components of the powder or reconstituted solution, prepared from the powder. Excipients that may be used include, but are not limited to, water, dextrose, sorbital, fructose, corn syrup, xylitol, glycerin, glucose, sucrose or other suitable agents. The solvent may contain a buffer, such as citrate, sodium or potassium phosphate or other such buffer known to those of skill in the art at, in one embodiment, about neutral pH. Subsequent sterile filtration of the solution followed by lyophilization under standard conditions known to those of skill in the art provides a desirable formulation. In one embodiment, the resulting solution

will be apportioned into vials for lyophilization. Each vial can contain a single dosage or multiple dosages of the ADCs provided herein or composition thereof. Overfilling vials with a small amount above that needed for a dose or set of doses (e.g., about 10%) is acceptable so as to facilitate accurate sample withdrawal and accurate dosing. The lyophilized powder can be stored under appropriate conditions, such as at about 4 °C to room temperature.

[00225] Reconstitution of a lyophilized powder with water for injection provides a formulation for use in parenteral administration. In one embodiment, for reconstitution the sterile and/or non-pyretic water or other liquid suitable carrier is added to lyophilized powder. The precise amount depends upon the selected therapy being given, and can be empirically determined.

[00226] Additionally, the antibody-drug conjugates or the pharmaceutical composition of the present invention may be manufactured into a kit, including an insert which indicates the information for the application, such as the indications, the amount in use, the route to be administrated, and the like.

[00227] USE OF THE ANTIBODY-DRUG CONJUGATES WITH IMPROVED HOMOGENEITY

[00228] In a further aspect, the present disclosure relates to the use of the composition of antibody-drug conjugates with improved homogeneity prepared by the process of the present invention in the manufacture of a pharmaceutical composition or a kit for treating a condition or disorder in a subject.

[00229] The subject may be a mammal, for example, a human.

[00230] The condition or disorder to be treated may be a tumor (e.g., a cancer), autoimmune disease, or infectious disease. In specific embodiments, the infectious disease may be viral or microbial infection.

[00231] In an additional aspect, the present disclosure relates to a method for treating a subject having a condition or disorder, comprising: administrating a therapeutically effective amount of the composition of ADCs with improved homogeneity prepared by the process of the present invention or a therapeutically effective amount of the pharmaceutical composition of the present invention to a subject in need thereof, thereby treating or preventing the condition or disorder.

[00232] In certain embodiments, the subject has been identified as having a condition or disorder likely to respond to the ADCs provided herein.

[00233] The subject may be a mammal, for example, a human.

[00234] The condition or disorder to be treated may be a tumor (e.g., a cancer), autoimmune disease, or infectious disease. In specific embodiments, the infectious disease may be viral or microbial infection.

[00235] The therapeutically effective amount of the composition of ADCs provided herein will depend on various factors known in the art, such as for example body weight, age, past medical history, present medications, state of health of the subject and potential for cross-reaction, allergies, sensitivities and adverse side-effects, as well as the administration route and extent of disease development. Dosages may be proportionally reduced or increased by one of ordinary skill in the art (e.g., physician or veterinarian) as indicated by these and other circumstances or requirements.

[00236] In certain embodiments, the composition of ADCs or pharmaceutical composition provided herein may be administered at a therapeutically effective dosage of about 0.01 mg/kg to about 100 mg/kg (e.g., about 0.01 mg/kg, about 0.5 mg/kg, about 1 mg/kg, about 2 mg/kg, about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg, about 50 mg/kg, about 55 mg/kg, about 60 mg/kg, about 65 mg/kg, about 70 mg/kg, about 75 mg/kg, about 80 mg/kg, about 85 mg/kg, about 90 mg/kg, about 95 mg/kg, or about 100 mg/kg). In certain of these embodiments, the composition of ADCs or pharmaceutical composition provided herein are administered at a dosage of about 50 mg/kg or less, and in certain of these embodiments the dosage is 10 mg/kg or less, 5 mg/kg or less, 1 mg/kg or less, 0.5 mg/kg or less, or 0.1 mg/kg or less. In certain embodiments, the administration dosage may change over the course of treatment. For example, in certain embodiments, the initial administration dosage may be higher than subsequent administration dosages. In certain embodiments, the administration dosage may vary over the course of treatment depending on the reaction of the subject.

[00237] Dosage regimens may be adjusted to provide the optimum desired response (e.g., a therapeutic response). For example, a single dose may be administered, or several divided doses may be administered over time.

[00238] The ADCs or pharmaceutical composition provided herein may be administered by any route known in the art, such as for example parenteral (e.g., subcutaneous, intraperitoneal, intravenous, including intravenous infusion, intramuscular, or intradermal injection) or non-parenteral (e.g., oral, intranasal, intraocular, sublingual, rectal, or topical) routes.

[00239] In certain embodiments, the condition or disorder treated by the ADCs or pharmaceutical composition provided herein is cancer or a cancerous condition, autoimmune disease or infectious disease.

[00240] The cancer may be antigen positive carcinomas including those of the lung, breast, colon, ovaries, and pancreas, for example, the cancers associated with tumor-associated antigens listed in (1)-(53) under the heading of "TUMOR-ASSOCIATED ANTIGENS (TAAs)".

[00241] Other particular types of cancers that can be treated with the ADCs or pharmaceutical composition provided herein include, but are not limited to, solid tumors, including but not limited to: fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon cancer, colorectal cancer, kidney cancer, pancreatic cancer, bone cancer, breast cancer, ovarian cancer, prostate cancer, esophogeal cancer, stomach cancer, oral cancer, nasal cancer, throat cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, uterine cancer, testicular cancer, small cell lung carcinoma, bladder carcinoma, lung cancer, epithelial carcinoma, glioma, glioblastoma multiforme, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, skin cancer, melanoma, neuroblastoma, retinoblastoma; blood-borne cancers, including but not limited to: acute lymphoblastic leukemia "ALL", acute lymphoblastic B-cell leukemia, acute lymphoblastic T-cell leukemia, acute myeloblastic leukemia "AML", acute promyelocytic leukemia "APL", acute monoblastic leukemia, acute erythroleukemic leukemia, acute megakaryoblastic leukemia, acute myelomonocytic leukemia, acute nonlymphocyctic leukemia, acute undifferentiated leukemia, chronic myelocytic leukemia

"CML", chronic lymphocytic leukemia "CLL", hairy cell leukemia, multiple myeloma; Lymphomas: B cell lymphoma, optionally Hodgkin lymphoma or non-Hodgkin lymphoma, wherein the non-Hodgkin lymphoma comprises: Diffuse large B-cell lymphoma (DLBCL), Follicular lymphoma, Marginal zone B-cell lymphoma (MZL), Mucosa-Associated Lymphatic Tissue lymphoma (MALT), Small lymphocytic lymphoma (chronic lymphocytic leukemia, CLL), or Mantle cell lymphoma (MCL), Acute Lymphoblastic Leukemia (ALL), or Waldenstrom's Macroglobulinemia (WM).

[00242] The autoimmune disease may include, but not limited to, Active Chronic Hepatitis, Addison's Disease, Allergic Alveolitis, Allergic Reaction, Allergic Rhinitis, Alport's Syndrome, Anaphlaxis, Ankylosing Spondylitis, Anti-phosholipid Syndrome, Arthritis, Ascariasis, Aspergillosis, Atopic Allergy, Atropic Dermatitis, Atropic Rhinitis, Behcet's Disease, Bird-Fancier's Lung, Bronchial Asthma, Caplan's Syndrome, Cardiomyopathy, Celiac Disease, Chagas' Disease, Chronic Glomerulonephritis, Cogan's Syndrome, Cold Agglutinin Disease, Congenital Rubella Infection, CREST Syndrome, Crohn's Disease, Cryoglobulinemia, Cushing's Syndrome, Dermatomyositi, Discoid Lupus, Dressler's Syndrome, Eaton-Lambert Syndrome, Echovirus Infection, Encephalomyelitis, Endocrine opthalmopathy, Epstein-Barr Virus Infection, Equine Heaves, Erythematosis, Evan's Syndrome, Felty's Syndrome, Fibromyalgia, Fuch's Cyclitis, Gastric Atrophy, Gastrointestinal Allergy, Giant Cell Arteritis, Glomerulonephritis, Goodpasture's Syndrome, Graft v. Host Disease, Graves' Disease, Guillain-Barre Disease, Hashimoto's Thyroiditis, Hemolytic Anemia, Henoch-Schonlein Purpura, Idiopathic Adrenal Atrophy, Idiopathic Pulmonary Fibritis, IgA Nephropathy, Inflammatory Bowel Diseases, Insulin-dependent Diabetes Mellitus, Juvenile Arthritis, Juvenile Diabetes Mellitus (Type I), Lambert-Eaton Syndrome, Laminitis, Lichen Planus, Lupoid Hepatitis, Lupus, Lymphopenia, Meniere's Disease, Mixed Connective Tissue Disease, Multiple Sclerosis, Myasthenia Gravis, Pernicious Anemia, Polyglandular Syndromes, Presenile Dementia, Primary Agammaglobulinemia, Primary Biliary Cirrhosis, Psoriasis, Psoriatic Arthritis, Raynauds Phenomenon, Recurrent Abortion, Reiter's Syndrome, Rheumatic Fever, Rheumatoid Arthritis, Sampter's Syndrome, Schistosomiasis, Schmidt's Syndrome, Scleroderma, Shulman's Syndrome, Sjorgen's Syndrome, Stiff-Man Syndrome, Sympathetic Ophthalmia, Systemic Lupus Erythematosis, Takayasu's Arteritis, Temporal Arteritis, Thyroiditis, Thrombocytopenia, Thyrotoxicosis, Toxic Epidermal

Necrolysis, Type B Insulin Resistance, Type I Diabetes Mellitus, Ulcerative Colitis, Uveitis, Vitiligo, Waldenstrom's Macroglobulemia, Wegener's Granulomatosis.

[00243] Particular types of infectious diseases that can be treated with the ADCs or pharmaceutical composition of the disclosure include, but are not limited to, Bacterial Diseases: Diptheria, Pertussis, Occult Bacteremia, Urinary Tract Infection, Gastroenteritis, Cellulitis, Epiglottitis, Tracheitis, Adenoid Hypertrophy, Retropharyngeal Abcess, Impetigo, Ecthyma, Pneumonia, Endocarditis, Septic Arthritis, Pneumococcal, Peritonitis, Bactermia, Meningitis, Acute Purulent Meningitis, Urethritis, Cervicitis, Proctitis, Pharyngitis, Salpingitis, Epididymitis, Gonorrhea, Syphilis, Listeriosis, Anthrax, Nocardiosis, Salmonella, Typhoid Fever, Dysentery, Conjuntivitis, Sinusitis, Brucellosis, Tullaremia, Cholera, Bubonic Plague, Tetanus, Necrotizing Enteritis, Actinomycosis, Mixed Anaerobic Infections, Syphilis, Relapsing Fever, Leptospirosis, Lyme Disease, Rat Bite Fever, Tuberculosis, Lymphadenitis, Leprosy, Chlamydia, Chlamydial Pneumonia, Trachoma, Inclusion Conjunctivitis; Systemic Fungal Diseases: Histoplamosis, Coccicidiodomycosis, Blastomycosis, Sporotrichosis, Cryptococcsis, Systemic Candidiasis, Aspergillosis, Mucormycosis, Mycetoma, Chromomycosis; Rickettsial Diseases: Typhus, Rocky Mountain Spotted Fever, Ehrlichiosis, Eastern Tick-Borne Rickettsioses, Rickettsialpox, Q Fever, Bartonellosis; Parasitic Diseases: Malaria, Babesiosis, African Sleeping Sickness, Chagas' Disease, Leishmaniasis, Dum-Dum Fever, Toxoplasmosis, Meningoencephalitis, Keratitis, Entamebiasis, Giardiasis, Cryptosporidiasis, Isosporiasis, Cyclosporiasis, Microsporidiosis, Ascariasis, Whipworm Infection, Hookworm Infection, Threadworm Infection, Ocular Larva Migrans, Trichinosis, Guinea Worm Disease, Lymphatic Filariasis, Loiasis, River Blindness, Canine Heartworm Infection, Schistosomiasis, Swimmer's Itch, Oriental Lung Fluke, Oriental Liver Fluke, Fascioliasis, Fasciolopsiasis, Opisthorchiasis, Tapeworm Infections, Hydatid Disease, Alveolar Hydatid Disease; Viral Diseases: Measles, Subacute sclerosing panencephalitis, Common Cold, Mumps, Rubella, Roseola, Fifth Disease, Chickenpox, Respiratory syncytial virus infection, Croup, Bronchiolitis, Infectious Mononucleosis, Poliomyelitis, Herpangina, Hand-Foot-and-Mouth Disease, Bornholm Disease, Genital Herpes, Genital Warts, Aseptic Meningitis, Myocarditis, Pericarditis, Gastroenteritis, Acquired Immunodeficiency Syndrome (AIDS), Rey's Syndrome, Kawasaki Syndrome, Influenza, Bronchitis, Viral "Walking" Pneumonia, Acute Febrile Respiratory Disease, Acute pharyngoconjunctival fever, Epidemic keratoconjunctivitis, Herpes Simplex Virus 1 (HSV-1), Herpes Simples Virus 2 (HSV-2), Shingles, Cytomegalic

Inclusion Disease, Rabies, Progressive Multifocal Leukoencephalopathy, Kuru, Fatal Familial Insomnia, Creutzfeldt-Jakob Disease, Gerstmann-Straussler-Scheinker Disease, Tropical Spastic Paraparesis, Western Equine Encephalitis, California Encephalitis, St. Louis Encephalitis, Yellow Fever, Dengue, Lymphocytic choriomeningitis, Lassa Fever, Hemorrhagic Fever, Hantvirus Pulmonary Syndrome, Marburg Virus Infections, Ebola Virus Infections, Smallpox.

[00244] In one embodiment, the present disclosure includes a method for treating disease or disorder in a subject, comprising administering to the subject an effective amount of the ADCs or pharmaceutical composition provided herein and another therapeutic agent.

[00245] In some embodiments, the therapeutic agent is an anti-cancer agent. Suitable anticancer agents include, but are not limited to, methotrexate, taxol, L-asparaginase, mercaptopurine, thioguanine, hydroxyurea, cytarabine, cyclophosphamide, ifosfamide, nitrosoureas, cisplatin, carboplatin, mitomycin, dacarbazine, procarbizine, topotecan, nitrogen mustards, cytoxan, etoposide, 5-fluorouracil, BCNU, irinotecan, camptothecins, bleomycin, doxorubicin, idarubicin, daunorubicin, dactinomycin, plicamycin, mitoxantrone, asparaginase, vinblastine, vincristine, vinorelbine, paclitaxel, and docetaxel.

[00246] In some embodiments, the therapeutic agent is an anti-autoimmune disease agent. Suitable anti-autoimmune disease agents include, but are not limited to, cyclosporine, cyclosporine A, mycophenylate mofetil, Sirolimus, tacrolimus, etanercept, prednisone, azathioprine, methotrexate cyclophosphamide, prednisone, aminocaproic acid, chloroquine, hydroxychloroquine, hydrocortisone, dexamethasone, chlorambucil, DHEA, danazol, bromocriptine, meloxicam, and infliximab.

[00247] In some embodiments, the therapeutic agent is anti-infectious disease agent. In one embodiment, the anti-infectious disease agent is, but not limited to, antibacterial agents: [beta]-Lactam Antibiotics: Penicillin G, Penicillin V, Cloxacilliin, Dicloxacillin, Methicillin, Nafcillin, Oxacillin, Ampicillin, Amoxicillin, Bacampicillin, Azlocillin, Carbenicillin, Mezlocillin, Piperacillin, Ticarcillin; Aminoglycosides: Amikacin, Gentamicin, Kanamycin, Neomycin, Netilmicin, Streptomycin, Tobramycin; Macrolides: Azithromycin, Clarithromycin, Erythromycin, Lincomycin, Clindamycin; Tetracyclines: Demeclocycline, Doxycycline, Minocycline, Oxytetracyclinem, Tetracycline; Quinolones: Cinoxacin, Nalidixic Acid; Fluoroquinolones: Ciprofloxacin, Enoxacin, Grepafloxacin, Levofloxacin, Lomefloxacin, Norfloxacin, Ofloxacin,

Sparfloxacin, Trovafloxicin; Polypeptides: Bacitracin, Colistin, Polymyxin B; Sulfonamides: Sulfisoxazole, Sulfamethoxazole, Sulfadiazine, Sulfamethizole, Sulfacetamide; Miscellaneous Antibacterial Agents: Trimethoprim, Sulfamethazole, Chloramphenicol, Vancomycin, Metronidazole, Quinupristin, Dalfopristin, Rifampin, Spectinomycin, Nitrofurantoin; Antiviral Agents: General Antiviral Agents: Idoxuradine, Vidarabine, Trifluridine, Acyclovir, Famcicyclovir, Pencicyclovir, Valacyclovir, Gancicyclovir, Foscarnet, Ribavirin, Amantadine, Rimantadine, Cidofovir, Antisense Oligonucleotides, Immunoglobulins, Inteferons; Drugs for HIV infection: Zidovudine, Didanosine, Zalcitabine, Stavudine, Lamivudine, Nevirapine, Delavirdine, Saquinavir, Ritonavir, Indinavir, Nelfinavir.

[00248] In a specific embodiment, in order to demonstrate the process of the disclosure, three antibodies, Trastuzumab, Rituximab and Cetuximab (produced in WuXi Biologics, according to the published corresponding protein sequences, via standard method for preparing monoclonal antibodies, respectively), were chosen to perform conjugation with MC-VC-PAB-MMAE (HY-15575, MedChemExpress), MC-MMAF (HY-15579， MedChemExpress), GGFG-Dxd (HY-13631E， MedChemExpress), or Tesirine (C210323012-FP, WuXiBiologics).

[00249] The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. All specific compositions, materials, and methods described below, in whole or in part, fall within the scope of the present invention. These specific compositions, materials, and methods are not intended to limit the invention, but merely to illustrate specific embodiments falling within the scope of the invention. One skilled in the art may develop equivalent compositions, materials, and methods without the exercise of inventive capacity and without departing from the scope of the invention. It will be understood that many variations can be made in the procedures herein described while still remaining within the bounds of the present invention. It is the intention of the inventors that such variations are included within the scope of the invention.

EXAMPLES

[00250] Now the present disclosure will be illustrated in detail with reference to the following examples. However, those skilled in the art should understand that the following examples are only provided for illustration, but not intended to limit the present disclosure in any way.

[00251] Example 1. Conjugate Mc-VC-PAB-MMAE to mAb1, mAb2, mAb3 with conventional conjugation process

[00252] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, commercially available from Genentech, in the present invention, it was produced by WuXi Biologics according to standard molecular biological method, and its sequence information is shown in Table A), mAb2 (Cetuximab, 120SD151029H13X01, commercially available from Merck, in the present invention, it was produced by WuXi Biologics according to standard molecular biological method, and its sequence information is shown in Table A) and mAb3 (Rituximab, 254SD131223D01X01E01, commercially available from Genentech, in the present invention, it was produced by WuXi Biologics according to standard molecular biological method, and its sequence information is shown in Table A) in PB buffer (40mM, pH 6.90; Na_2HPO_4 24.4mM, NaH_2PO_4 15.6mM) were individually reduced by TCEP (C4706-2G, Sigma-Aldrich) (TCEP/mAb molar ratio of about 1.3, e.g., TCEP/mAb1-3 is 1.3/1.5/1.3, respectively) at 4 °C for about 20h (e.g., 20/23/23h). A solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress, abbreviated as MC-VC-MMAE) (10 mg/mL in DMA, a linker-payload/mAb molar ratio of 10.0) in DMA (Dimethylacetamide, ARK2190-1L, SAFC) was added into and mixed with the eluted solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 4 °C for 1-2 hours (e.g., 2/1/2 hours) to obtain intermediate mAb conjugates.

[00253] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich) (10mM stock in ddH_2O, a NAC/mAb molar ratio of 16) was added into the conjugation reaction solution to quench conjugation at 4 °C for 0.5 hours.

[00254] The products were submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting columns (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

[00255] The recovered ADCs were characterized, respectively. The DAR values were shown in Table 1 and Fig. 1(A-C).

[00256] Table 1. DAR values of ADCs produced with conventional conjugation process

	mAb1 Random-DAR2 conjugation	mAb2 Random-DAR2 conjugation	mAb3 Random-DAR2 conjugation

DAR0%	31.10	31.96	29.96
DAR2%	**46.92**	**46.86**	**48.91**
DAR4%	19.07	18.47	18.08
DAR6%	2.91	2.71	3.05
DAR8%	0.00	0.00	0.00
DAR	1.88	1.84	1.88

Note:
a. "Random-DAR2 conjugation" means that two molecules of payload are conjugated to one set of reduced thiol groups randomly, without transition metal addition and re-oxidation reaction.
b. The ADCs with DAR0, DAR2, DAR4, DAR6 and DAR8 are also referred to as D0, D2, D4, D6 and D8, respectively.
c. The percent of ADCs with DAR0, DAR2, DAR4, DAR6 and DAR8 is expressed as molar percent (mol%).

[00257] Example 2. Conjugate different antibodies (e.g., mAb1, mAb2 and mAb3) with a linker payload (MC-VC-PAB-MMAE) in phosphate buffer (PB) and in presence of transition metal ions to form high ratio DAR2 ADCs

[00258] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics), mAb2 (Cetuximab, 120SD151029H13X01, produced by WuXi Biologics) and mAb3 (Rituximab, 254SD131223D01X01E01, produced by WuXi Biologics) in PB buffer (40mM, pH 6.90; Na_2HPO_4 24.4mM, NaH_2PO_4 15.6mM) were individually reduced by TCEP (C4706-2G, Sigma-Aldrich, TCEP/mAb molar ratio of 3) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich, Zn(II)/mAb molar ratio of 4) at 12 °C for 20h.

[00259] DHAA (261565-1G, Sigma-Aldrich) solution (DHAA/mAb molar ratio of 12) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 3.5/3/3 hours.

[00260] This intermediate product was purified using a spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher) by PB buffer (40mM, pH 7.09; molar ratio).

[00261] EDTA (EDTA·2Na, 10009719, SCR) (50mM stock in ddH2O) final concentration up to 5mM and solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) in DMA (Dimethylacetamide, ARK2190-1L, SAFC) (10 mg/mL in DMA, a linker-payload/mAb

molar ratio of 10.0) were added into and mixed with the eluted solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12°C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payload.

[00262] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting columns (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

[00263] The recovered ADCs were characterized, respectively. The DAR values were shown in Table 2 and Fig. 2.

[00264] **Table 2.** DAR values of ADCs produced with the conjugation process of the present application by using different antibodies.

	mAb1 DAR2 conjugation (Mc-VC-MMAE)	mAb2 DAR2 conjugation (Mc-VC-MMAE)	mAb3 DAR2 conjugation (Mc-VC-MMAE)
DAR0%	10.16	9.74	8.84
DAR2%	**85.38**	**82.11**	**85.11**
DAR4%	4.46	5.52	3.80
DAR6%	0.00	2.63	2.24
DAR8%	0.00	0.00	0.00
DAR	1.89	2.02	1.99

[00265] The results of Table 2 showed that in the composition of ADCs produced by the conjugation process of the present application by using 3 different antibodies, DAR2% (molar percent) was the highest, DAR0% and DAR4% were low, and DAR6 ADCs and DAR8 ADCs were not produced or were produced in a much lower precent.

[00266] **Example 3. Conjugate various linker payloads with mAb1 in phosphate buffer (PB) and in presence of transition metal ions to form high ratio DAR2 ADCs**

[00267] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics) in PB buffer (40mM, pH 6.90; Na_2HPO_4 24.4mM, NaH_2PO_4 15.6mM) was reduced by TCEP (C4706-2G, Sigma-Aldrich, TCEP/mAb molar ratio of 3/3/3/3/8/8) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich, Zn(II)/mAb molar ratio of 4) at 12 °C for 20h. The reduced antibody solution was directly submitted to next step of re-oxidation without TCEP removal.

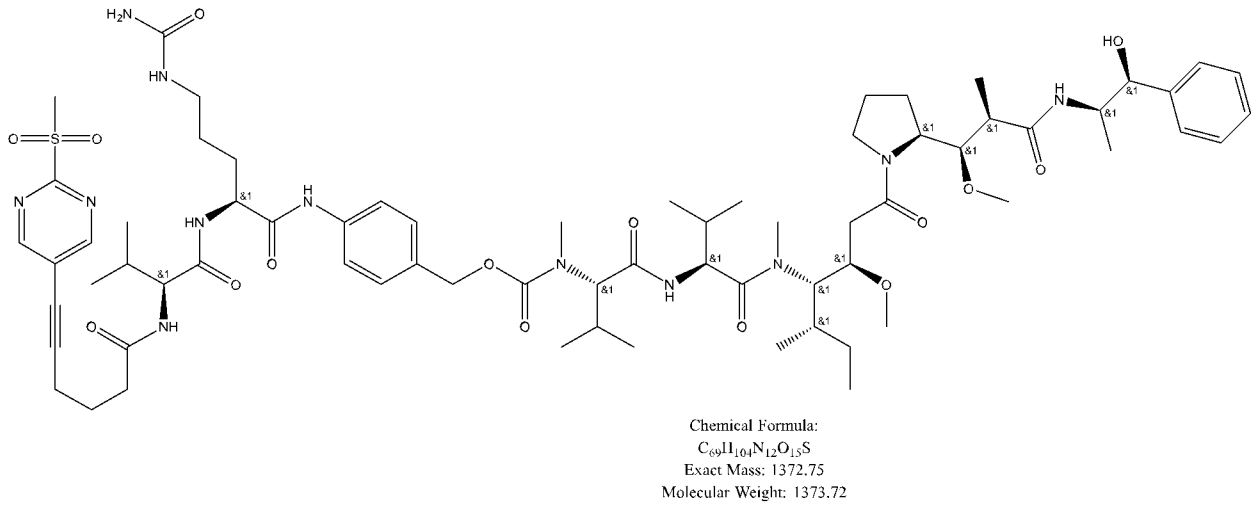
[00268] DHAA (261565-1G, Sigma-Aldrich) solution (10mM stock in ddH_2O, DHAA/mAb molar ratio of 12) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 3/3.5/4 /3hours for MC-VC-PAB-MMAE (HY-15575, MedChemExpress); MC-MMAF (HY-15579, MedChemExpress); Tesirine (PBD) (C210323012-FP, WuXiBiologics); and GGFG-Dxd (HY-13631E, MedChemExpress) conjugation groups, respectively.

[00269] DTNB (D8130-500MG, Sigma-Aldrich, DTNB /mAb molar ratio of 7.6) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 0.5 hours for DM21 or DL1-VC-PAB -MMAE groups.

[00270] This intermediate product was purified using a spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher) by PB buffer (40mM, pH 6.90; Na_2HPO_4 24.4mM, NaH_2PO_4 15.6mM).

[00271] EDTA (EDTA-2Na, 10009719, SCR; 50mM stock in ddH_2O, final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress; MC-MMAF, HY-15579, MedChemExpress; Tesirine (PBD), C210323012-FP, WuXiBiologics; DM21, HY-13631E, MedChemExpress; or DL1-VC-PAB -MMAE, WuXiBiologics) in DMA (Dimethylacetamide, ARK2190-1L, SAFC) (10 mg/mL in DMA, a linker-payload/mAb molar ratio of 10.0/10.0/7.0/5.0/15.0) or a solution of linker-payload (GGFG-Dxd, HY-13631E, MedChemExpress) in DMSO (Dimethy sulfoxide, D4540-1L, Sigma-Aldrich; 10 mg/mL in DMSO, a linker-payload/mAb molar ratio of 6.0) were added into and mixed with the eluted solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hour or 23 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00272] The molecular structure of DL1-VC-PAB -MMAE is represented by formular (I):



formular (I)

[00273] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich) (10mM stock in ddH_2O, a NAC/mAb molar ratio of 16) was added into the conjugation reaction solution to quench conjugation at 4 °C for 0.5 hours.

[00274] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting columns (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

[00275] The recovered ADCs were characterized, respectively. The DAR values were shown in Table 3 and Fig. 3(A-F).

[00276] **Table 3**. DAR values of ADCs produced with the conjugation process of the present application by using different linker-payloads

	mAb1 DAR2 conjugation (Mc-VC-PAB-MMAE)	mAb1 DAR2 conjugation (Mc-MMAF)	mAb1 DAR2 conjugation (GGEF-DXD)	mAb1 DAR2 conjugation (TESIRINE)	mAb1 DAR2 conjugation (DL1-VC-PAB-MMAE)	mAb1 DAR2 conjugation (DM21)
DAR0%	10.16	11.17	8.46	9.10	12.29	8.29
DAR2%	**85.38**	**81.15**	**82.14**	**79.74**	**75.84**	**82.70**
DAR4%	4.46	5.41	9.40	11.16	8.15	7.10
DAR6%	0.00	2.27	0.00	0.00	3.72	1.91
DAR8%	0.00	0.00	0.00	0.00	0.00	0.00
DAR	1.89	1.98	2.02	2.04	2.07	2.05

[00277] The results of Table 3 showed that in the composition of ADCs produced by the conjugation process of the present application by using 6 different linker-payloads, DAR2% (molar percent) was the highest (higher than 75 mole%), DAR0% and DAR4% were low, and DAR6 ADCs and DAR8 ADCs were not produced or were produced in a much lower precent.

[00278] It is known in the art that the 6 linker-payloads used in this example may represent the ones commonly used in the bio-conjugation field. Therefore, the results of Table 3 may also demonstrate that the conjugation process of the present application can be widely used to various antibodies and various linker-payloads.

[00279] Example 4-1. Produce high ratio DAR2 ADCs with mAb1 and different reductants

[00280] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, commercially available from Genentech, in the present invention, it was produced by WuXi Biologics according to standard molecular biological method, and its sequence information is shown in Table A), in PB buffer (40mM, pH 6.90; Na_2HPO_4 24.4mM, NaH_2PO_4 15.6mM) or HEPES buffer (40mM, pH 6.99) were individually reduced by diPPBS1 (2-diphenylphosphanylbenzenesulfonic acid, diPPBS1/mAb molar ratio of 35) or TCEP (C4706-2G, Sigma-Aldrich, TCEP/mAb molar ratio of 3.25) or DPAA (DPAA/mAb molar ratio of 10) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich, Zn(II)/mAb molar ratio of 4) at 12°C for about 20h.

[00281] DHAA (261565-1G, Sigma-Aldrich) solution (DHAA/mAb molar ratio of 12) or DTNB (D8130-500MG, Sigma-Aldrich; DTNB/mAb molar ratio of 2.2) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for about 3 hours.

[00282] This intermediate product was purified using a spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher) by PB buffer (40mM, pH 6.90; molar ratio).

[00283] EDTA (EDTA·2Na, 10009719, SCR) (50mM stock in ddH2O) final concentration up to 5mM and solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) (10 mg/mL in DMA, a linker-payload/mAb molar ratio of 10.0) in DMA (Dimethylacetamide, ARK2190-1L, SAFC) were added into and mixed with the eluted solution to thereby form a

conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payload.

[00284] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting columns (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

[00285] The recovered ADCs were characterized. The DAR values were shown in Table 4-1 and Fig. 4 (A-C).

[00286] Table 4-1. DAR values of ADCs produced with different reductants by the conjugation process of the present application.

	mAb1 DAR2 conjugation **(diPPBS1, 35 eq)**	mAb1 DAR2 conjugation **(TCEP, 3.25 eq)**	mAb1 DAR2 conjugation **(DPAA, 10eq)**
DAR0%	9.09	8.61	15.08
DAR2%	**73.62**	**84.23**	**64.09**
DAR4%	14.64	5.45	16.73
DAR6%	2.66	1.71	4.09
DAR8%	0.00	0.00	0.00
DAR	2.22	2.01	2.20

[00287] The results of Table 4-1 showed that the composition of ADCs produced by the conjugation process of the present application under different reductants (Fig. 4 (A-C): A: TCEP; B: diPPBS1; C: DPAA) could get a significantly high DAR2 ratio.

[00288] Example 4-2. Produce high ratio DAR2 ADCs with mAb1 and TCEP at different ratios

[00289] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, commercially available from Genentech, in the present invention, it was produced by WuXi Biologics according to standard molecular biological method, and its sequence information is shown in Table A), in HEPES buffer (40mM, pH 6.99) were individually reduced TCEP (C4706-2G, Sigma-Aldrich) (TCEP/mAb molar ratio of 3.25/10/16) 12°C in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich, Zn(II)/mAb molar ratio of 4) for about 20h. The reduced antibody solution was directly submitted

to next step of re-oxidation without TCEP removal or purified to remove TCEP for the next step of re-oxidation.

[00290] DTNB (D8130-500MG, Sigma-Aldrich; DTNB/mAb molar ratio of 2.2) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 2 /26 /26 hours.

[00291] This intermediate products were not purified or purified using a spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher) by PB buffer (40mM, pH 6.90; molar ratio).

[00292] EDTA (EDTA·2Na, 10009719, SCR; 50mM stock in ddH2O, final concentration up to 5mM) and solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) in DMA (Dimethylacetamide, ARK2190-1L, SAFC) (10 mg/mL in DMA, a linker-payload/mAb molar ratio of 10.0) were added into and mixed with the eluted solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payload.

[00293] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich) (NAC/mAb molar ratio of 18) was added into the conjugation reaction solution to quench conjugation at 12 °C for 0.5 hours.

[00294] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting columns (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

[00295] The recovered ADCs were characterized. The DAR values were shown in Table 4-2 and Fig. 4 (D-F).

[00296] **Table 4-2**. DAR values of ADCs produced with different ratios of TCEP reductant by the conjugation process of the present application.

	mAb1 DAR2 conjugation **(TCEP, 3.25 eq)**	mAb1 DAR2 conjugation **(TCEP, 10 eq)**	mAb1 DAR2 conjugation **(TCEP, 16 eq)**
DAR0%	8.61	15.21	14.39
DAR2%	**84.23**	**72.46**	**68.43**
DAR4%	5.45	9.10	12.87

DAR6%	1.71	3.23	4.31
DAR8%	0.00	0.00	0.00
DAR	2.01	2.01	2.14

[00297] The results of Table 4-2 showed that the composition of ADCs produced by the conjugation process of the present application under different ratios of TCEP (Fig. 4 (D-F): (D) TCEP, 3.25 eq; (E) TCEP, 10 eq; (F) TCEP, 16 eq) could get a significantly high DAR2 ratio, and it would be not necessary to use higher mole ratio of TCEP/mAb: 3.25 eq TCEP was desirable.

[00298] **Example 5. Produce high ratio DAR2 ADCs with mAb1 in various buffers**

[00299] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics) in HEPES buffer (40mM, pH 6.99; H3375-500G, Sigma-Aldrich) / MES buffer (40mM, pH 6.51, M3671-250G, Sigma-Aldrich) / Tris buffer (40mM, pH 7.01; T1503-1KG, Sigma-Aldrich) was reduced by TCEP (C4706-2G, Sigma-Aldrich) (TCEP/mAb molar ratio of 3.25) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich, Zn(II)/mAb molar ratio of 4) at 12 °C for 18h. The reduced antibody solution was directly submitted to next step of re-oxidation without TCEP removal.

[00300] DTNB (D8130-500MG, Sigma-Aldrich; DTNB/mAb molar ratio of 2.2) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 2 hours.

[00301] EDTA (EDTA-2Na, 10009719, SCR; final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) were added into the solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00302] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich; NAC/mAb molar ratio of 16) was added into the conjugation reaction solution to quench conjugation at 12 °C for 0.5 hours.

[00303] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting columns (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

[00304] The recovered ADCs were characterized. The DAR values were shown in Table 5 and Fig. 5.

[00305] **Table 5**. mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present application in different buffers

	mAb1 DAR2 conjugation (**HEPES**)	mAb1 DAR2 conjugation (**MES**)	mAb1 DAR2 conjugation (**Tris**)
DAR0%	8.61	10.37	11.85
DAR2%	**84.23**	**86.02**	**85.40**
DAR4%	5.45	2.96	2.31
DAR6%	1.71	0.65	0.44
DAR8%	0.00	0.00	0.00
DAR	2.01	1.88	1.83

[00306] The results of Table 5 showed that the composition of ADCs produced by the conjugation process of the present application in different buffers (Fig. 5: (A) **HEPES**; (B) **MES**; (C) **Tris**) could get a significantly high DAR2 ratio, indicating that routine buffers can be used for the conjugation process of the present application.

[00307] **Example 6. Produce high ratio DAR2 ADCs with mAb3 in various buffers**

[00308] 5mg/mL mAb3 (Rituximab, 254SD131223D01X01E01, produced by WuXi Biologics) in HEPES buffer (40mM, pH 6.99; H3375-500G, Sigma-Aldrich) / MES buffer (40mM, pH 6.51, M3671-250G, Sigma-Aldrich) / Tris buffer (40mM, pH 7.01; T1503-1KG, Sigma-Aldrich) was reduced by TCEP (C4706-2G, Sigma-Aldrich) (TCEP/mAb molar ratio of 3.25) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich, Zn(II)/mAb molar ratio of 4) at 12 °C for 20h. The reduced antibody solution was directly submitted to next step of re-oxidation without TCEP removal.

[00309] DTNB (D8130-500MG, Sigma-Aldrich) solution (DTNB/mAb molar ratio of 2.2) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 2 hours.

[00310] EDTA (EDTA-2Na, 10009719, SCR; final concentration up to 5mM) and solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) were added into the solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00311] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich) (NAC/mAb molar ratio of 18) was added into the conjugation reaction solution to quench conjugation at 12 °C for 0.5 hours.

[00312] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting columns (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

[00313] The recovered ADCs were characterized. The DAR values were shown in Table 6 and Fig. 6.

[00314] **Table 6**. mAb3 (Rituximab) DAR2 conjugation through the conjugation process of the present application in different buffers

	mAb3 DAR2 conjugation (**HEPES**)	mAb3 DAR2 conjugation (**MES**)	mAb3 DAR2 conjugation (**Tris**)
DAR0%	10.14	9.13	9.60
DAR2%	**81.63**	**82.46**	**81.91**
DAR4%	5.90	5.95	6.17
DAR6%	2.27	2.45	2.33
DAR8%	0.00	0.00	0.00
DAR	2.01	2.03	2.02

[00315] The results of Table 6 showed that the composition of ADCs produced by the conjugation process of the present application in different buffers (Fig. 6: (A) **HEPES**; (B) **MES**; (C) **Tris**) could get a significantly high DAR2 ratio, indicating that routine buffers can be used for the conjugation process of the present application.

[00316] **Example 7. Produce ADCs with mAb3 in various buffers without transition metal ions**

[00317] 5mg/mL mAb3 (Rituximab, 254SD131223D01X01E01, produced by WuXi Biologics) in HEPES buffer (40mM, pH 6.99; H3375-500G, Sigma-Aldrich) / MES buffer (40mM, pH 6.51, M3671-250G, Sigma-Aldrich) / Tris buffer (40mM, pH 7.01; T1503-1KG, Sigma-Aldrich) was reduced by TCEP (C4706-2G, Sigma-Aldrich; TCEP/mAb molar ratio of 3.25) at 12 °C for 20h. The reduced antibody solution was directly submitted to next step of re-oxidation without TCEP removal.

[00318] DTNB (D8130-500MG, Sigma-Aldrich) solution was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 2 hours.

[00319] EDTA (EDTA-2Na, 10009719, SCR; final concentration up to 5mM) and solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) were added into the solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours.

[00320] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich; NAC/mAb molar ratio of 18) was added into the conjugation reaction solution to quench conjugation at 12 °C for 0.5 hours.

[00321] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting columns (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

[00322] The purified ADCs were characterized. The DAR values were shown in Table 7 and Fig. 7.

[00323] **Table 7**. mAb3 (Rituximab) conjugation through a conjugation process in different buffers without transition metal ions ($0Zn^{2+}$)

	mAb3 conjugation (**HEPES**)	mAb3 conjugation (**MES**)	mAb3 conjugation (**Tris**)
DAR0%	72.55	55.60	60.75
DAR2%	**23.09**	**32.54**	**29.82**
DAR4%	4.36	10.55	8.42
DAR6%	0.00	1.31	1.01
DAR8%	0.00	0.00	0.00
DAR	0.64	1.15	0.99

[00324] The results of Table 7 showed that the composition of ADCs produced by a conjugation process of in different buffers (Fig. 7: (A) HEPES; (B) MES; (C) Tris) without transition metal ions could not get a high DAR2 ratio, indicating that the transition metal ions (e.g., Zn^{2+}) are critical for the conjugation process of the present application.

[00325] **Example 8. Produce high ratio DAR2 ADC with a bispecific antibody (mAb4) in phosphate buffer (PB)**

[00326] 9.53 mg/mL mAb4 (W329001-U5T5.E17R-57.uIgG1, produced by WuXi Biologics, sequence information shown in Table A) in PB buffer (40mM, pH 7.12; Na2HPO4 24.4mM, NaH2PO4 15.6mM) was reduced by TCEP (C4706-2G, Sigma-Aldrich) (TCEP/mAb molar ratio of 10) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich; Zn(II)/mAb molar ratio of 4) at 4 °C for 20 h. The reduced antibody solution was directly submitted to next step re-oxidation without TCEP removal.

[00327] DTNB (261565-1G, Sigma-Aldrich) solution (DTNB/mAb molar ratio of 11) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 0.5 hours.

[00328] EDTA (EDTA·2Na, 10009719, SCR; 50mM stock in ddH2O, final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) in DMA (Dimethylacetamide, ARK2190-1L, SAFC) (10 mg/mL in DMA, a linker-payload/mAb molar ratio of 10.0) were added into and mixed with the eluted solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 0.5 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00329] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich) (10mM stock in ddH2O, a NAC/mAb molar ratio of 40) was added into the conjugation reaction solution to quench conjugation at 12 °C for 0.5 hours.

[00330] The product was submitted to a buffer exchange into its storage buffer (20 mM histidine-ace buffer, pH 5.5) using ultrafiltration tubes (30 K, UFC803024, Thermo Fisher).

[00331] The recovered ADCs were characterized. The DAR values were shown in Table 8 and Fig. 8.

[00332] **Table 8**. A bispecific antibody (mAb4) DAR2 conjugation through the conjugation process of the present application

	mAb4 conjugation (Mc-VC-MMAE)
DAR0%	15.63
DAR2%	**83.46**
DAR4%	0.91

DAR	1.71

[00333] The results of Table 8 showed that in addition to monospecific antibodies, the conjugation process of the present application is applicable to a bispecific antibody, and could produce a composition of ADCs with a significantly high DAR2 ratio.

[00334] Example 9. The temperature and duration for reduction

[00335] 5mg/mL mAb1 (Trastuzumab，838-1W220125, produced by WuXi Biologics) in HEPES buffer (40mM, pH 6.99; H3375-500G, Sigma-Aldrich) was reduced by TCEP (C4706-2G, Sigma-Aldrich) (TCEP/mAb molar ratio of 3.25 at 4°C/19h)/ (TCEP/mAb molar ratio of 3.0 at 22°C/19h)/ (TCEP/mAb molar ratio of 2.8 at 37°C/2h) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich, Zn(II)/mAb molar ratio of 4), and the reduced antibody solution was directly submitted to next step of re-oxidation without TCEP removal.

[00336] DTNB (D8130-500MG, Sigma-Aldrich; DTNB/mAb molar ratio of 2.2) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 2 hours.

[00337] EDTA (EDTA-2Na, 10009719, SCR; final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) were added into the solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00338] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich) (NAC/mAb molar ratio of 18) was added into the conjugation reaction solution to quench conjugation at 12 °C for 0.5 hours.

[00339] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting columns (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

[00340] The recovered ADCs were characterized. The DAR values were shown in Table 9-1 and Fig. 9 (A-C).

[00341] **Table 9-1.** mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure at different temperatures for reduction

	mAb1	**mAb1**	**mAb1**

	4°C	22°C	37°C
DAR0%	8.43	20.19	21.22
DAR2%	**83.46**	**77.22**	**75.33**
DAR4%	5.80	2.59	3.46
DAR6%	2.32	0.00	0.00
DAR8%	0.00	0.00	0.00
DAR	2.04	1.65	1.64

[00342] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics) in HEPES buffer (40mM, pH 6.99; H3375-500G, Sigma-Aldrich) was reduced by TCEP (C4706-2G, Sigma-Aldrich; TCEP/mAb molar ratio of 3.25) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich; Zn(II)/mAb molar ratio of 4) at 4°C at 19h/45h/92h, The reduced antibody solution was directly submitted to next step of re-oxidation without TCEP removal.

[00343] DTNB (D8130-500MG, Sigma-Aldrich; DTNB/mAb molar ratio of 2.2) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 2 hours.

[00344] EDTA (EDTA-2Na, 10009719, SCR; final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) were added into the solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00345] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich; NAC/mAb molar ratio of 18) was added into the conjugation reaction solution to quench conjugation at 12 °C for 0.5 hours.

[00346] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting columns (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

[00347] The recovered ADCs were characterized. The DAR values were shown in Table 9-2 and Fig. 9 (D-F).

[00348] **Table 9-2.** mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure for different durations of reduction

	mAb1 19h	**mAb1 45h**	**mAb1 92h**
DAR0%	8.43	8.35	7.12
DAR2%	**83.46**	**88.18**	**82.23**
DAR4%	5.80	2.71	7.78
DAR6%	2.32	0.76	2.88

DAR8%	0.00	0.00	0.00
DAR	2.04	1.92	2.13

[00349] The results of Tables 9-1 and 9-2 showed that it would be desirable to perform the reduction at about 4°C, room temperature or 37°C for about 2 to about 45 hours.

[00350] **Example 10. Different Zn^{2+} related salts**

[00351] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics) in HEPES buffer (40mM, pH 6.99; H3375-500G, Sigma-Aldrich) was reduced by TCEP (C4706-2G, Sigma-Aldrich) (TCEP/mAb molar ratio of 3.25) in the presence of $ZnSO_4 \cdot 7H_2O$ (Z0251-100G, Sigma-Aldrich), $ZnBr_2 \cdot 2H_2O$ (546739-100G, Sigma-Aldrich), $Zn(ClO_4)_2 \cdot 6H_2O$ (401439-100G, Sigma-Aldrich) or $Zn(OAc)_2 \cdot 2H_2O$ (96459-250G, Sigma-Aldrich) (Zn(II)/mAb molar ratio of 4), respectively, at 12 °C for 20h. The reduced antibody solution was directly submitted to next step of re-oxidation without TCEP removal.

[00352] DTNB (D8130-500MG, Sigma-Aldrich; DTNB/mAb molar ratio of 2.2) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 2 hours.

[00353] EDTA (EDTA-2Na, 10009719, SCR; final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) were added into the solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00354] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich) (NAC/mAb molar ratio of 16) was added into the conjugation reaction solution to quench conjugation at 12 °C for 0.5 hours.

[00355] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using ultrafiltration tubes (30K, UFC803024, Thermo Fisher).

[00356] The recovered ADCs were characterized. The DAR values were shown in Table 10 and Fig. 10.

[00357] **Table 10**. mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure by using different Zn^{2+} related salts

	mAb1 $ZnSO_4 \cdot 7H_2O$	**mAb1 $ZnBr_2 \cdot 2H_2O$**	**mAb1 $Zn(ClO_4)_2 \cdot 6H_2O$**	**mAb1 $Zn(OAc)_2 \cdot 2H_2O$**
DAR0%	11.64	10.69	10.11	10.58
DAR2%	**76.00**	**76.78**	**76.83**	**75.38**
DAR4%	9.09	9.42	9.23	10.01
DAR6%	3.27	3.11	3.83	4.03

DAR8%	0.00	0.00	0.00	0.00
DAR	2.08	2.10	2.14	2.15

[00358] The results of Table 10 showed that there would be no limitation to the type of Zn^{2+} related salts, provided that free Zn^{2+} ions can be released into the reaction solution.

[00359] **Example 11. Different mole ratios of Zn^{2+} ions**

[00360] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics) in PB buffer (40mM, pH 6.90; Na_2HPO_4 24.4mM, NaH_2PO_4 15.6mM) or HEPES buffer (40mM, pH 6.99; H3375-500G, Sigma-Aldrich) was reduced by TCEP (C4706-2G, Sigma-Aldrich) (TCEP/mAb molar ratio of 8/8/3.25) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich; Zn(II)/mAb molar ratio of 2/8/16) at 4°C/17h or 12 °C/20h. The reduced antibody solution was directly submitted to next step of re-oxidation without TCEP removal.

[00361] DTNB (D8130-500MG, Sigma-Aldrich; DTNB/mAb molar ratio of 7.6/7.6/2.2) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 1 or 2 hours.

[00362] EDTA (EDTA-2Na, 10009719, SCR; final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) were added into the solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00363] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich) (NAC/mAb molar ratio of 16) was added into the conjugation reaction solution to quench conjugation at 12 °C for 0.5 hours.

[00364] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using ultrafiltration tubes (30K, UFC803024, Thermo Fisher).

[00365] The recovered ADCs were characterized. The DAR values were shown in Table 11 and Fig. 11.

[00366] **Table 11**. mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure by using different amounts of Zn2+ ions

	mAb1 2eq Zn	**mAb1 8eq Zn**	**mAb1 16eq Zn**
DAR0%	22.55	15.83	13.30
DAR2%	**71.49**	**68.89**	**76.18**
DAR4%	4.18	10.94	7.90
DAR6%	1.78	4.34	2.62
DAR8%	0.00	0.00	0.00

DAR	1.70	2.08	2.00

[00367] The results of Table 11 showed that it would be desirable to use 2 eq-16eq Zn^{2+} ions (i.e., the mole ratio of Zn^{2+} ions/mAb was 2 to 16).

[00368] Example 12. The reduced antibody solution was submitted to re-oxidation step after TCEP removal

[00369] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics) in HEPES buffer (40mM, pH 6.99; H3375-500G, Sigma-Aldrich) was reduced by TCEP (C4706-2G, Sigma-Aldrich) (TCEP/mAb molar ratio of 8) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich (Zn(II)/mAb molar ratio of 4) at 4 °C for 17h. The reduced antibody solution was submitted to re-oxidation step after TCEP removal.

[00370] For TCEP removal, this intermediate product was purified using a spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher) by PB buffer (40mM, pH 6.90; Na_2HPO_4 24.4mM, NaH_2PO_4 15.6mM).

[00371] DHAA (261565-1G, Sigma-Aldrich) solution (DHAA/mAb molar ratio of 8) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 2 hours.

[00372] This intermediate product was purified using a spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher) by PB buffer (40mM, pH 6.90; Na_2HPO_4 24.4mM, NaH_2PO_4 15.6mM).

[00373] EDTA (EDTA-2Na, 10009719, SCR; final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) were added into and mixed with the eluted solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00374] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich) (NAC/mAb molar ratio of 16) was added into the conjugation reaction solution to quench conjugation at 12 °C for 0.5 hours.

[00375] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using ultrafiltration tubes (30K, UFC803024, Thermo Fisher).

[00376] The recovered ADCs were characterized. The DAR values were shown in Table 12 and Fig. 12.

[00377] **Table 12**. mAb1 (Trastuzumab) DAR2 conjugation by removing TCEP after the

reduction of the antibody

	mAb1 8eq TCEP
DAR0%	9.71
DAR2%	**84.83**
DAR4%	5.46
DAR6%	0.00
DAR8%	0.00
DAR	1.91

[00378] The data of Table 12, as compared with the data of Table 3, showed that there was no impact to remove the excess TCEP after the reduction of the antibody.

[00379] **Example 13. Produce high ratio DAR2 ADCs with different oxidants**

[00380] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics), in PB buffer (40mM, pH 6.90; Na_2HPO_4 24.4mM, NaH_2PO_4 15.6mM) or HEPES buffer (40mM, pH 6.99; H3375-500G, Sigma-Aldrich) were individually reduced by TCEP (C4706-2G, Sigma-Aldrich; TCEP/mAb molar ratio of about 3.25) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich, Zn(II)/mAb molar ratio of 4) at 12 °C for 20h.

[00381] DHAA (261565-1G, Sigma-Aldrich) solution (DHAA/mAb molar ratio of 12) / DTNB solution (D8130-500MG, Sigma-Aldrich; DTNB/mAb molar ratio of 2.2) / DDD solution (2-Aminophenyl disulfide, Sigma-Aldrich; DDD/mAb molar ratio of 1.82) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 22 °C for 2 hours.

[00382] EDTA (EDTA·2Na, 10009719, SCR; 50mM stock in ddH2O, final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) in DMA (Dimethylacetamide, ARK2190-1L, SAFC) (10 mg/mL in DMA, a linker-payload/mAb molar ratio of 10.0) were added into the solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payload.

[00383] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich) (10mM stock in ddH2O, a NAC/mAb molar ratio of 18) was added into the conjugation reaction solution to quench conjugation at 4 °C for 0.5 hours.

[00384] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting columns (40 kDa, 0.5 mL, REF: 87766, Lot

#SJ251704, Manufacturer: Thermo fisher).

[00385] The recovered ADCs were characterized. The DAR values were shown in Table 13 and Fig. 13.

[00386] **Table 13.** DAR values of ADCs produced with different oxidants by the conjugation process of the present application.

	mAb1 12eq DHAA	**mAb1 2.2eq DTNB**	**mAb1 1.82eq DDD**
DAR0%	10.16	11.38	10.23
DAR2%	**85.38**	**77.02**	**83.23**
DAR4%	4.46	8.65	5.64
DAR6%	0.00	2.95	0.91
DAR8%	0.00	0.00	0.00
DAR	1.89	2.06	1.94

[00387] The results of Table 13 indicated that many oxidants commonly used for oxidation of antibodies (e.g., oxidation of free thiol groups in the antibodies) can be used in the conjugation process of the present disclosure.

[00388] **Example 14. Temperature and duration for re-oxidation**

[00389] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics) in PB buffer (40mM, pH 6.90; Na_2HPO_4 24.4mM, NaH_2PO_4 15.6mM) or HEPES buffer (40mM, pH 6.99; H3375-500G, Sigma-Aldrich) was reduced by TCEP (C4706-2G, Sigma-Aldrich) (TCEP/mAb molar ratio of about 3.0) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich,) (Zn(II)/mAb molar ratio of 4) at 12°C/20h. The reduced antibody solution was directly submitted to next step of re-oxidation without TCEP removal.

[00390] DTNB (D8130-500MG, Sigma-Aldrich; DTNB/mAb molar ratio of about 2.0) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 4 °C /12 °C/ 22 °C/ 37 °C for 2 hours.

[00391] EDTA (EDTA-2Na, 10009719, SCR; final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) were added into the solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00392] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich) (NAC/mAb molar ratio of 18) was added into the conjugation reaction solution to quench conjugation at 12 °C for 0.5 hours.

[00393] The product was submitted to a buffer exchange into its storage buffer (20mM

histidine-ace buffer, pH 5.5) using desalting columns (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

[00394] The recovered ADCs were characterized. The DAR values were shown in Table 14-1 and Fig. 14 (A-D).

[00395] **Table 14-1**. mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure at different temperatures for re-oxidation

	mAb1 re-oxidation at 0°C	**mAb1 re-oxidation at 12°C**	**mAb1 re-oxidation at 22°C**	**mAb1 re-oxidation at 37°C**
DAR0%	15.12	15.58	16.02	9.55
DAR2%	**76.80**	**76.80**	**77.33**	**79.35**
DAR4%	6.94	6.51	5.65	8.54
DAR6%	1.14	1.12	0.99	2.56
DAR8%	0.00	0.00	0.00	0.00
DAR	1.88	1.86	1.83	2.08

[00396] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics) in HEPES buffer (40mM, pH 6.99; H3375-500G, Sigma-Aldrich was reduced by TCEP (C4706-2G, Sigma-Aldrich; TCEP/mAb molar ratio of 3.25) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich, Zn(II)/mAb molar ratio of 4) at 12°C/20h. The reduced antibody solution was directly submitted to next step of re-oxidation without TCEP removal.

[00397] DTNB solution (D8130-500MG, Sigma-Aldrich; DTNB /mAb molar ratio of 2.2) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 0.5 or 2 or 4 or 24 hours.

[00398] EDTA (EDTA-2Na, 10009719, SCR; final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) were added into the solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00399] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich) (NAC/mAb molar ratio of 18) was added into the conjugation reaction solution to quench conjugation at 12 °C for 0.5 hours.

[00400] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using desalting columns (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

[00401] The recovered ADCs were characterized. The DAR values were shown in Table 14-2 and Fig. 14 (E-H).

[00402] **Table 14-2**. mAb1 (Trastuzumab) DAR2 conjugation through the conjugation process of the present disclosure at 12 °C for different durations for re-oxidation

	mAb1 re-oxidation 0.5 H	**mAb1 re-oxidation 2H**	**mAb1 re-oxidation 4H**	**mAb1 re-oxidation 24H**
DAR0%	11.36	17.89	18.93	21.91
DAR2%	**80.10**	**67.96**	**68.68**	**69.39**
DAR4%	6.41	10.86	9.81	7.40
DAR6%	2.14	3.28	2.58	1.31
DAR8%	0.00	0.00	0.00	0.00
DAR	1.99	1.99	1.92	1.76

[00403] The results of Tables 14-1 and 14-2 showed that it was desirable to perform the re-oxidation at room temperature for about 1 to 24 hours.

[00404] **Examples 15. Temperature for conjugation**

[00405] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics) in PB buffer (40mM, pH 6.90; Na2HPO4 24.4mM, NaH2PO4 15.6mM) was reduced by TCEP (C4706-2G, Sigma-Aldrich; TCEP/mAb molar ratio of 3) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich; Zn(II)/mAb molar ratio of 4) at 12 °C for 20h. The reduced antibody solution was directly submitted to next step of re-oxidation without TCEP removal.

[00406] DNTB (22582-5G, Sigma-Aldrich) solution (DNTB/mAb molar ratio of 1.8) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 3 hours. The re-oxidated antibody solution was directly submitted to next step of conjugating payload bearing reactive groups without purification of the re-oxidated antibody.

[00407] EDTA (EDTA-2Na, 10009719, SCR; 50mM stock in ddH2O, final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) in DMA (Dimethylacetamide, ARK2190-1L, SAFC) (10 mg/mL in DMA, a linker-payload/mAb molar ratio of 10.0) were added to the solution, thereby forming a conjugation reaction solution. The conjugation reaction was kept at 12 °C or 0 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00408] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using ultrafiltration tubes (30K, UFC803024, Thermo Fisher).

[00409] The recovered ADCs were characterized. The DAR values were shown in Table 15 and Fig. 15.

[00410] **Table 15**. mAb1 (Trastuzumab) DAR2 conjugation at different temperatures

	mAb1 conjugation (Mc-VC-MMAE) at 12 °C	**mAb1 conjugation (Mc-VC-MMAE) at 0°C**
DAR0%	15.96	14.90
DAR2%	**67.35**	**71.49**
DAR4%	12.51	10.32
DAR6%	3.59	2.86
DAR8%	0.59	0.43
DAR	2.11	2.05

[00411] The results of Tables 15 showed that it was desirable to perform the conjugation at 0°C or 12°C.

[00412] **Example 16. Transition metal ions are added in the re-oxidation step and the re-oxidation is performed at 22°C for 47.5 hours**

[00413] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics) in PB buffer (40mM, pH 6.90; Na2HPO4 24.4mM, NaH2PO4 15.6mM) was reduced by TCEP (C4706-2G, Sigma-Aldrich; TCEP/mAb molar ratio of 8) at 12 °C for 18h. The reduced antibody solution was purified to remove TCEP for the next step of re-oxidation.

[00414] For TCEP removal, this intermediate product was purified using a spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher) by PB buffer (40mM, pH 6.90; Na_2HPO_4 24.4mM, NaH_2PO_4 15.6mM).

[00415] DTNB (D8130-500MG, Sigma-Aldrich) solution (5mM stock in 40mM PB buffer, DTNB/mAb molar ratio of 1) and $ZnCl_2$ (14422-500G, Sigma-Aldrich; Zn(II)/mAb molar ratio of 4) were added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 22°C for 47.5 hours.

[00416] EDTA (EDTA-2Na, 10009719, SCR; 50mM stock in ddH2O, final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) were added into the solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00417] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using ultrafiltration tubes (30K, UFC803024, Thermo Fisher).

[00418] The recovered ADCs were characterized. The DAR values were shown in Table 16 and

Fig. 16.

[00419] **Table 16**. mAb1 (Trastuzumab) DAR2 conjugation by adding transition metal ions in the re-oxidation step.

	mAb1 re-oxidation at 22°C for 47.5h
DAR0%	14.02
DAR2%	**72.45**
DAR4%	10.25
DAR6%	3.29
DAR8%	0.00
DAR	2.06

[00420] The results of Tables 16 showed that adding the transition metal ions in the re-oxidation step could produce a composition of ADCs having high ratio of DAR2.

[00421] **Example 17. Transition metal ions are added in the re-oxidation step and the re-oxidation is performed at 12°C for 170 hours**

[00422] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics) in HEPES buffer (40mM, pH 6.99; H3375-500G, Sigma-Aldrich) / PB buffer (40mM, pH 6.90; Na2HPO4 24.4mM, NaH2PO4 15.6mM) was reduced by TCEP (C4706-2G, Sigma-Aldrich; TCEP/mAb molar ratio of 8) at 12 °C for 18h. The reduced antibody solution was purified to remove TCEP for the next step of re-oxidation. For TCEP removal, this intermediate product was purified using a spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher) by PB buffer (40mM, pH 6.90; Na_2HPO_4 24.4mM, NaH_2PO_4 15.6mM).

[00423] $ZnCl_2$ (14422-500G, Sigma-Aldrich, Zn(II)/mAb molar ratio of 4) was added to the reduced antibody solution and reacted at 12°C for 0.5h. DTNB (D8130-500MG, Sigma-Aldrich) solution (DTNB/mAb molar ratio of 2.04/2.12) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 170 hours.

[00424] EDTA (EDTA-2Na, 10009719, SCR; 50mM stock in ddH2O, final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) were added into the solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00425] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich) (10mM stock in ddH2O, a NAC/mAb molar ratio of 18) was added into the conjugation reaction solution to quench conjugation at 12 °C for 0.5 hours.

[00426] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using ultrafiltration tubes (30K, UFC803024, Thermo Fisher).

[00427] The recovered ADCs were characterized. The DAR values were shown in Table 17 and Fig. 17.

[00428] **Table 17**. mAb1 (Trastuzumab) DAR2 conjugation by adding transition metal ions in the re-oxidation step, wherein performing the re-oxidation at 12°C for 24 hours without transition metal ions is as negative control.

	mAb1 re-oxidation PB/4Zn^{2+} 170h/12°C	**mAb1 re-oxidation PB/0Zn^{2+} 24h/12°C**	**mAb1 re-oxidation HEPES+PB/4Zn^{2+} 170h/12°C**	**mAb1 re-oxidation HEPES/0Zn^{2+} 24h/12°C**
DAR0%	6.72	100.00	6.33	100.00
DAR2%	**71.20**	**0.00**	**83.60**	**0.00**
DAR4%	16.09	0.00	7.38	0.00
DAR6%	5.99	0.00	2.70	0.00
DAR8%	0.00	0.00	0.00	0.00
DAR	2.43	0.00	2.13	0.00

[00429] The results of Table 17 showed that for mAb1, if no transition metal ions were added in the conjugation process of the present application (i.e., in reduction step or in re-oxidation step), there would no production of ADCs.

[00430] **Example 18. Transition metal ions are added in the re-oxidation step and the re-oxidation is performed at 12°C for 170 hours (mAb3)**

[00431] 5mg/mL mAb3 (Rituximab, 254SD131223D01X01E01, produced by WuXi Biologics) in PB buffer (40mM, pH 6.90; Na2HPO4 24.4mM, NaH2PO4 15.6mM) were reduced by TCEP (C4706-2G, Sigma-Aldrich; TCEP/mAb molar ratio of 8) at 12 °C for 18h. The reduced antibody solution was purified to remove TCEP for the next step of re-oxidation. For TCEP removal, this intermediate product was purified using a spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher) by PB buffer (40mM, pH 6.90; Na_2HPO_4 24.4mM, NaH_2PO_4 15.6mM).

[00432] $ZnCl_2$ (14422-500G, Sigma-Aldrich, Zn(II)/mAb molar ratio of 4) was added to the reduced antibody solution and reacted at 12°C for 0.5h. DTNB (D8130-500MG, Sigma-Aldrich) solution (5mM stock in 40mM PB buffer, DTNB/mAb molar ratio of 2.50) was added into the

reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 170 hours.

[00433] EDTA (EDTA-2Na, 10009719, SCR; 50mM stock in ddH2O, final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) were added into the solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 ℃ for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00434] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich) (10mM stock in ddH2O, a NAC/mAb molar ratio of 18) was added into the conjugation reaction solution to quench conjugation at 12 ℃ for 0.5 hours.

[00435] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using ultrafiltration tubes (30K, UFC803024, Thermo Fisher).

[00436] he recovered ADCs were characterized. The DAR values were shown in Table 18 and Fig. 18.

[00437] **Table 18**. mAb3 (Rituximab) DAR2 conjugation by adding transition metal ions in the re-oxidation step, wherein performing the re-oxidation at 12°C for 24 hours without transition metal ions is as negative control.

	mAb3 re-oxidation PB/4Zn^{2+} 170h/12℃	**mAb3 re-oxidation PB/0Zn^{2+} 24h/12℃**
DAR0%	12.77	98.12
DAR2%	**73.35**	**1.88**
DAR4%	9.41	0.00
DAR6%	4.48	0.00
DAR8%	0.00	0.00
DAR	2.11	0.04

[00438] The results of Table 18 showed that for mAb3, if no transition metal ions were added in the conjugation process of the present application (i.e., in reduction step or in re-oxidation step), only a small amount of D2 was produced.

[00439] **Example 19. Transition metal ions are added in the re-oxidation step and the re-oxidation is performed at 12°C for 170 hours (mAb1 + various Linker payloads)**

[00440] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics) in HEPES buffer (40mM, pH 6.99; H3375-500G, Sigma-Aldrich) / PB buffer (40mM, pH 6.90; Na2HPO4 24.4mM, NaH2PO4 15.6mM) was reduced by TCEP (C4706-2G, Sigma-Aldrich;

TCEP/mAb molar ratio of 8) at 12 °C for 18h. The reduced antibody solution was purified to remove TCEP for the next step of re-oxidation. For TCEP removal, this intermediate product was purified using a spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher) by PB buffer (40mM, pH 6.90; Na_2HPO_4 24.4mM, NaH_2PO_4 15.6mM).

[00441] $ZnCl_2$ (14422-500G, Sigma-Aldrich, Zn(II)/mAb molar ratio of 4) was added to the reduced antibody solution and reacted at 12°C for 0.5h. DTNB (D8130-500MG, Sigma-Aldrich) solution (DTNB/mAb molar ratio of 2.12) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 170 hours.

[00442] EDTA (EDTA-2Na, 10009719, SCR; 50mM stock in ddH2O, final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575; GGFG-Dxd, HY-13631E, MedChemExpress; or Tesirine (PBD), C210323012-FP, WuXiBiologics) were added into the solution to thereby form a conjugation reaction solution. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00443] NAC (Acetylcysteine, A9165-100G, Sigma-Aldrich; 10mM stock in ddH2O, a NAC/mAb molar ratio of 18) was added into the conjugation reaction solution to quench conjugation at 12 °C for 0.5 hours.

[00444] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using ultrafiltration tubes (30K, UFC803024, Thermo Fisher).

[00445] The recovered ADCs were characterized. The DAR values were shown in Table 19 and Fig. 19.

[00446] Table 19. The HIC result of mAb1 (Trastuzumab) DAR2 conjugation with different linker-payloads by adding transition metal ions in the re-oxidation step.

	mAb1-GGFG-Dxd conjugation re-oxidation 170h (PB)	**mAb1-PBD conjugation re-oxidation 170h (PB)**	**mAb1- MC-VC-MMAE conjugation re-oxidation 170h (HEPES)**
DAR0%	6.72	10.34	6.33
DAR2%	**71.20**	**65.66**	**83.60**
DAR4%	16.09	20.60	7.38
DAR6%	5.99	3.40	2.70
DAR8%	0.00	0.00	0.00
DAR	2.43	2.34	2.13

[00447] The results of Tables 19 showed that a composition with high ratio of DAR2 could be produced by adding transition metal ions in the re-oxidation step.

[00448] Example 20. The concentration of the antibody

[00449] 5mg/mL and 10mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics) in HEPES buffer (40mM, pH 6.99; H3375-500G, Sigma-Aldrich was reduced by TCEP (C4706-2G, Sigma-Aldrich; TCEP/mAb molar ratio of 3.25) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich, Zn(II)/mAb molar ratio of 4) at 12°C/20H. The reduced antibody solution was directly submitted to next step of re-oxidation without TCEP removal.

[00450] DNTB (22582-5G, Sigma-Aldrich) solution (DNTB/mAb molar ratio of 2.2) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 2 hours. The re-oxidated antibody solution was directly submitted to next step of conjugating payload bearing reactive groups without purification of the re-oxidated antibody.

[00451] EDTA (EDTA-2Na, 10009719, SCR; 50mM stock in ddH2O, final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) in DMA (Dimethylacetamide, ARK2190-1L, SAFC) (10 mg/mL in DMA, a linker-payload/mAb molar ratio of 10.0) were added into the solution to form a conjugation reaction solution. The conjugation reaction was kept at 12°C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00452] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using desalting columns (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

[00453] The recovered ADCs were characterized. The DAR values were shown in Table 20 and Fig. 20.

[00454] Table 20. mAb1 (Trastuzumab) DAR2 conjugation under different antibody concentrations

	mAb1 5mg/ml	**mAb1 10mg/ml**
DAR0%	13.50	9.35
DAR2%	**79.11**	**81.05**
DAR4%	5.85	7.31
DAR6%	1.54	2.30
DAR8%	0	0
DAR	1.91	2.05

[00455] The results of Table 20 showed that a composition with high ratio of DAR2 could be produced at different antibody concentrations.

[00456] **Example 21. Concentration of HEPES buffer**

[00457] 5mg/mL mAb1 (Trastuzumab, 838-1W220125, produced by WuXi Biologics) in HEPES buffer (20mM and 40mM, pH 6.99; H3375-500G, Sigma-Aldrich) was reduced by TCEP (C4706-2G, Sigma-Aldrich; TCEP/mAb molar ratio of 3.25) in the presence of $ZnCl_2$ (14422-500G, Sigma-Aldrich, Zn(II)/mAb molar ratio of 4) at 12°C/20h. The reduced antibody solution was directly submitted to next step of re-oxidation without TCEP removal.

[00458] DNTB (22582-5G, Sigma-Aldrich) solution (DNTB/mAb molar ratio of 2.2) was added into the reduced antibody solution. The reactions were mixed properly, and the reaction vials were stood at 12 °C for 2 hours. The re-oxidated antibody solution was directly submitted to next step of conjugating payload bearing reactive groups without purification of the re-oxidated antibody.

[00459] EDTA (EDTA-2Na, 10009719, SCR; 50mM stock in ddH2O, final concentration up to 5mM) and a solution of linker-payload (MC-VC-PAB-MMAE, HY-15575, MedChemExpress) in DMA (Dimethylacetamide, ARK2190-1L, SAFC) (10 mg/mL in DMA, a linker-payload/mAb molar ratio of 10.0) were added into the solution to form a conjugation reaction solution. The conjugation reaction was kept at 12°C for 1 hours to obtain an intermediate mAb conjugated with 2 linker-payloads/mAb.

[00460] The product was submitted to a buffer exchange into its storage buffer (20mM histidine-ace buffer, pH 5.5) using desalting columns (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

[00461] The recovered ADCs were characterized. The DAR values were shown in Table 21 and Fig. 21.

[00462] **Table 21**. mAb1 (Trastuzumab) DAR2 conjugation under different HEPES concentrations

	mAb1 40mM HEPES	**mAb1 20mM HEPES**
DAR0%	14.74	12.92
DAR2%	**82.11**	**80.02**
DAR4%	2.69	5.49
DAR6%	0.47	1.57

DAR8%	0	0
DAR	1.78	1.91

[00463] The results of Table 21 showed that change of HEPES concentrations would have minor impact on the ratio of DAR2 in the resultant composition of ADCs.

[00464] **Table A.** Sequence information of mAb1 (Trastuzumab), mAb2 (Cetuximab), mAb3 (Rituximab) and mAb4 (a bispecific antibody, i.e., **wuxibody** W329001-U5T5.E17R-57.uIgG1).

Name	**Sequence of light chain and heavy chain**
mAb1 (Trastuzumab)	**Light chain (SEQ ID No: 1):** DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYS GVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPS VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSK DSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
	Heavy chain (SEQ ID No: 2): EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTN GYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDY WGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
mAb2 (Cetuximab)	**Light chain (SEQ ID No: 3):** DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESISG IPSRFSGSGSGTDFTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELKRTVAA PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
	Heavy chain (SEQ ID No: 4): QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWS GGNTDYNTPFTSRLSINKDNSKSQVFFKMNSLQSNDTAIYYCARALTYYDYEFA YWGQGTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA LHNHYTQKSLSLSPGK
mAb3 (Rituximab)	**Light chain chimeric (SEQ ID No: 5):** QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGV PVRFSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIKRTVAAPSV

	FIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKD STYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
	Heavy chain (SEQ ID No: 6): QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYP GNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWY FNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK AEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEW ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK
mab 4, i.e., wuxibody W329001-U5T5.E17R-57.uIgG1	**Heavy chain 1 (SEQ ID No: 7):** EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWIN TYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPHYYGSSH WYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNT KVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVV DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGK
	Heavy chain 2 (SEQ ID No: 8): QVQLVQSGAEVKKPGASVKVSCKASGYTFTGYYMHWVRQAPGQGLEWMGWI NPNSGGTNYAQKFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCARSPNPYYY DSSGYYYPGAFDIWGQGTMVTVLEDLKNVFPPEVAVFEPSEAEISHTQKATLVCLA TGFYPDHVELSWWVNGKEVHSGVCTDPQPLKEQPALQDSRYALSSRLRVSATFW QNPRNHFRCQVQFYGLSENDEWTQDRAKPVTQIVSAEAWGRASDKTHTCPPCPA PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVCTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG SFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
	Light chain 1 (SEQ ID No: 9): DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGV PSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTVAAPSVFIF PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSL SSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
	Light chain 2 (SEQ ID No: 10): QPGLTQPPSVSVAPGQTARITCGGNNIGSKSVHWYQQKPGQAPVLVVYDDSDRPSGI PERFSGSNSGNTATLTISRVEAGDEADYYCQVWDSSSDHYVFGTGTKVTVLPDIQNPD CAVYQLRDSKSSDKSVCLFTDFDSQTQVSQSKDSDVYITDKCVLDMRSMDFKSNSAVA WSQKSDFACANAFQNSIIPECTFFPS

[00465] Those skilled in the art will further appreciate that the present invention may be embodied in other specific forms without departing from the spirit or central attributes thereof. In that the foregoing description of the present invention discloses only exemplary embodiments thereof, it is to be understood that other variations are contemplated as being within the scope of the present invention. Accordingly, the present invention is not limited to the particular embodiments that have been described in detail herein. Rather, reference should be made to the appended claims as indicative of the scope and content of the invention.

[00466] References:

(1) Junutula Jagath R, Raab Helga,Clark Suzanna et al. Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. [J] .Nat Biotechnol, 2008, 26: 925-32.

(2) Hallam Trevor J, Smider Vaughn V,Unnatural amino acids in novel antibody conjugates. [J] .Future Med Chem, 2014, 6: 1309-24.

(3) Hallam Trevor J, Wold Erik,Wahl Alan et al. Antibody conjugates with unnatural amino acids. [J] .Mol Pharm, 2015, 12: 1848-62.

(4) Agarwal Paresh, Bertozzi Carolyn R, Site-specific antibody-drug conjugates: the nexus of bioorthogonal chemistry, protein engineering, and drug development. [J] .Bioconjug Chem, 2015, 26: 176-92.

(5) Sarrett Samantha M, Rodriguez Cindy, Rymarczyk Grzegorz et al. Lysine-Directed Site-Selective Bioconjugation for the Creation of Radioimmunoconjugates. [J]. Bioconjug Chem, 2022, 33: 1750-1760.

What is claimed

1. A process for preparing a composition of antibody-drug conjugates (ADCs), comprising the following steps:

(a) reduction step: incubating a reductant, and the antibody in a buffer system to reduce inter-chain disulfide bonds within the antibody, optionally purifying the reduced antibody;

(b) re-oxidation step: adding an oxidant to re-oxidize some of the reduced thiol groups, optionally purifying the re-oxidized antibody;

wherein the step (a) or the step (b) is performed in the presence of transition metal ions,

wherein in step (b), the reduced thiol groups which are not blocked by the transition metal ions are re-oxidated;

(c) conjugation step: removing transition metal ions and adding an excess amount of payload bearing reactive groups to react with reduced thiol groups of the re-oxidized antibody; and

(d) recovering step: recovering the resultant antibody-drug conjugates to obtain a composition of antibody-drug conjugates (ADCs).

2. The process according to claim 1, wherein the transition metal ions are selected from Zn^{2+}, Mn^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, or the combination thereof, preferably the transition metal ion is Zn^{2+}.

3. The process according to claim 1, wherein the buffer system used in step (a) is selected from TAPS, Bicine, Tris, Tricine, HEPES, TES, MOPS, PIPES, Sodium citrate, Histidine buffer, PB (phosphate buffer), PBS, or MES, and the pH value of the buffer system is about 5 to 8, preferably about 7.0.

4. The process according to claim 1, wherein in step (a), the reductant is added at a molar ratio of 1 to 20 relative to the antibody.

5. The process according to claim 1, wherein the transition metal ions are added at a molar ratio of 0.5 to 50 relative to the antibody.

6. The process according to claim 1, wherein the reductant in step (a) is selected from Tris(2-carboxyethyl) phosphine (TCEP), THPP (Tris(3-hydroxypropyl)phosphine), diPPBs (2-

diphenylphosphanylbenzenesulfonic acid), DTT (Dithiothreitol), DPAA (2-(Diphenylphosphino) acetic acid), DTE (Dithioerythritol), β-Mercaptoethanol, $LiAlH_4$, $Na_2S_2O_3$, KBH_4, or Hydrazine.

7. The process according to claim 1, wherein the oxidant in step (b) is selected from Hydrogen peroxide (H_2O_2), nitrate compounds, Potassium chlorate ($KClO_3$), Peroxydisulfuric acid ($H_2S_2O_8$), Peroxymonosulfuric acid (H_2SO_5), NaClO, Sodium dichromate ($Na_2Cr_2O_7$), Permanganate compounds, Sodium perborate, Nitrous oxide, Sodium bismuthate ($NaBiO_3$), ceric sulfate, dehydroascorbic acid (DHAA), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), or 2-Aminophenyl disulfide (DDD), preferably DHAA or DTNB.

8. The process according to claim 1, wherein in step (b), the oxidant is added at a molar ratio of 0.5 to 160 relative to the antibody.

9. The process according to claim 1, wherein in step (c), the transition metal ions are removed by adjusting pH, by adjusting temperature, by changing buffer, or by adding metal chelator, wherein the metal chelator is EDTA, DTPA, Ethylenediamine, 2,2'-Bipyridine, or 1,10-phenanthroline.

10. The process according to claim 1, wherein the payload comprises a maleimide moiety, bromide, iodide, electron deficient alkenyl or alkynyl, mono- or di- derivatives of disulfides, sulfones, bicyclo[1.1.0]butane derivatives, sulfonyl fluorides, pentafluorophenol ester, palladium oxidative-addition complexes, iodoxolones, or highly electron deficient arenes.

11. The process according to any one of claims 1 to 10, wherein the antibody is a monoclonal antibody or a polyclonal antibody, or the antibody is a monospecific antibody or a multi-specific antibody, for example, a bispecific antibody.

12. The process according to claim 11, wherein the antibody is a human antibody, a humanized antibody, a chimeric antibody or an antigen-binding moiety thereof.

13. The process according to any one of claims 1 to 12, wherein the payload comprises a diagnostic agent, a therapeutic agent or a labelling agent.

14. The process according to any one of claims 1 to 13, wherein the composition comprises D2 in a content higher than 64 mol%, preferably higher than 75 mol% or even higher than 80mol%, on the basis of total molar of D0, D2, D4, D6 and D8,

wherein D0 represents the antibody molecule which is not conjugated with any drug,

D2 represents an ADC molecule in which two drug molecules are coupled to one single antibody molecule,

D4 represents an ADC molecule in which four drug molecules are coupled to one single antibody molecule,

D6 represents an ADC molecule in which six drug molecules are coupled to one single antibody molecule, and

D8 represents an ADC molecule in which eight drug molecules are coupled to one single antibody molecule.

15. The process according to claim 14, wherein the composition comprises D0, D4, D6 and D8 in a total content less than 36mol%, preferably less than 25mol%, more preferably less than 20 mol%, most preferably less than 15%, on the basis of total molar of D0, D2, D4, D6 and D8.

16. A composition of antibody-drug conjugates (ADCs) prepared by the process of any one of claims 1 to 15, wherein the composition comprises D2 in a content higher than 64 mol%, preferably higher than 75 mol% or even higher than 80mol%, on the basis of total molar of D0, D2, D4, D6 and D8.

17. The composition according to claim 16, wherein the composition comprises D0, D4, D6 and D8 in a total content less than 36mol%, preferably less than 25mol%, more preferably less than 20 mol%, most preferably less than 15%, on the basis of total molar of D0, D2, D4, D6 and D8.

18. A pharmaceutical composition comprising an effective amount of the composition

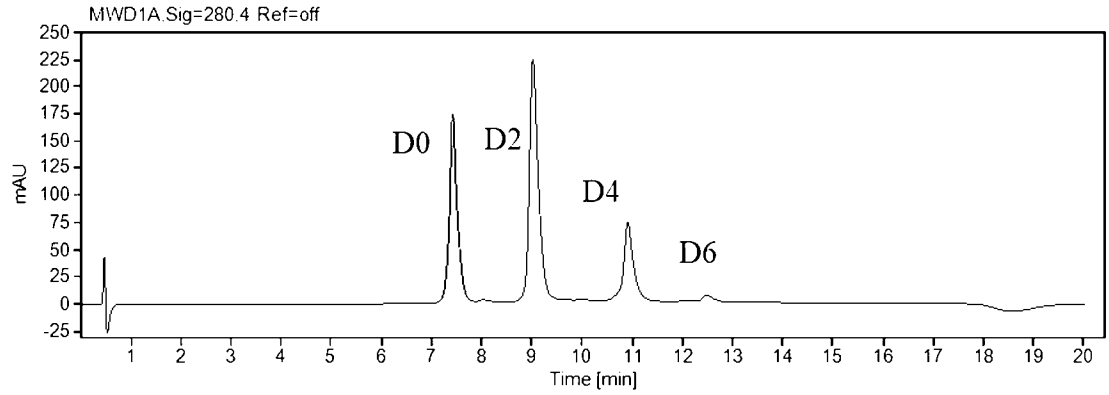
according to claim 16 or 17 and a pharmaceutically acceptable carrier.

19. A method for treating a condition or disorder in a subject, comprising a step of administrating to the subject a therapeutically effective amount of the composition according to claim 16 or 17 or the pharmaceutical composition according to claim 18.

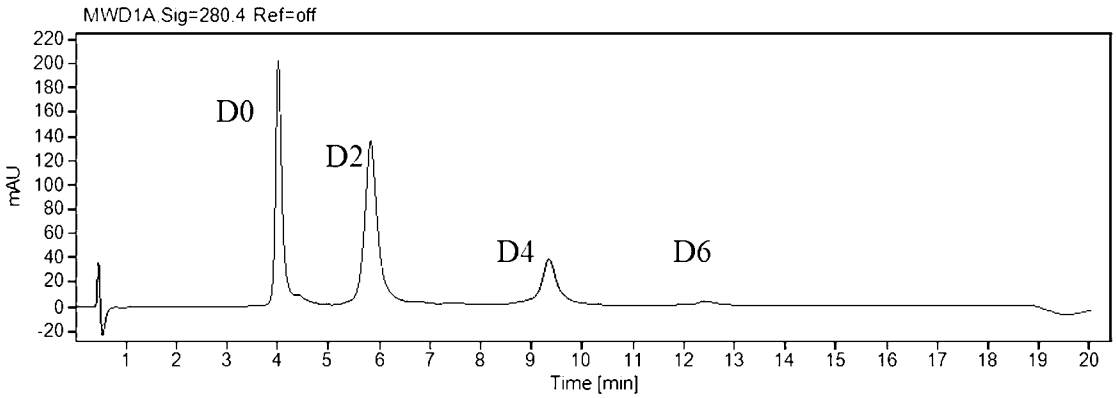
20. The method of claim 19, wherein the condition or disorder is a tumor (e.g., a cancer), autoimmune disease, or infectious disease (e.g., viral or microbial infection).

21. The method of claim 20, wherein the subject is a mammal, preferably a human.

A **mAb1 Random-DAR2 conjugation（Mc-VC-MMAE）**



B **mAb2 Random-DAR2 conjugation（Mc-VC-MMAE）**



C **mAb3 Random-DAR2 conjugation（Mc-VC-MMAE）**

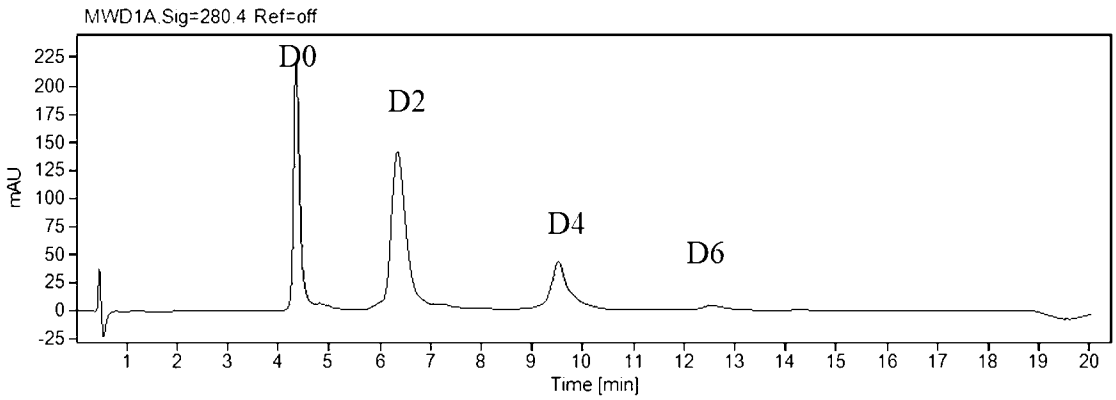
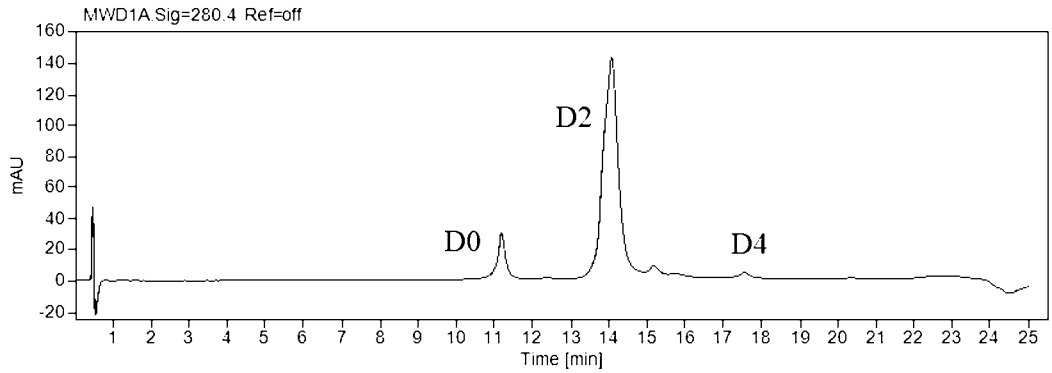
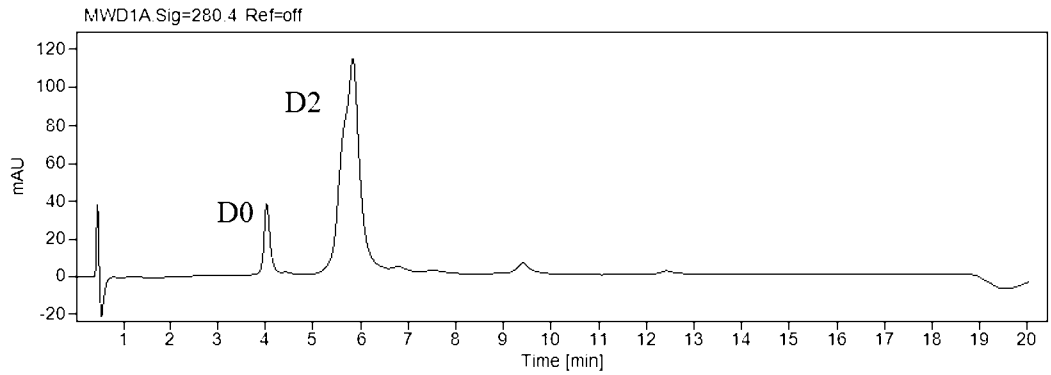


Fig. 1

A **mAb1 DAR2 conjugation（Mc-VC-MMAE）**



B **mAb2 DAR2 conjugation（Mc-VC-MMAE）**



C **mAb3 DAR2 conjugation（Mc-VC-MMAE）**

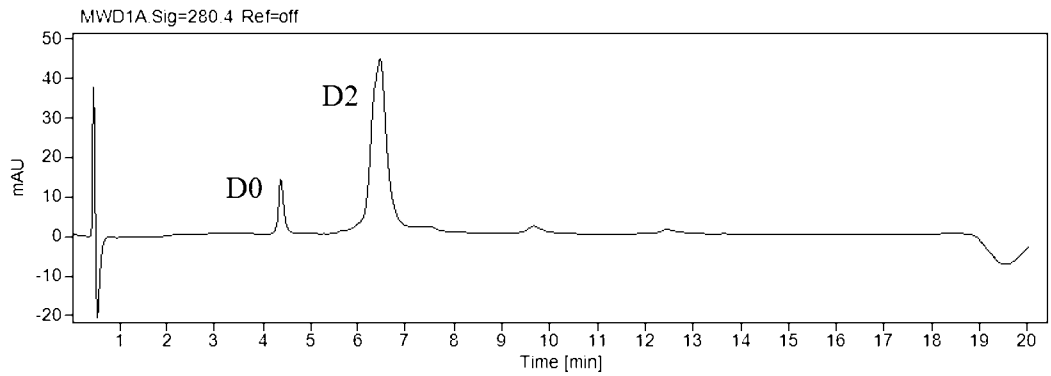
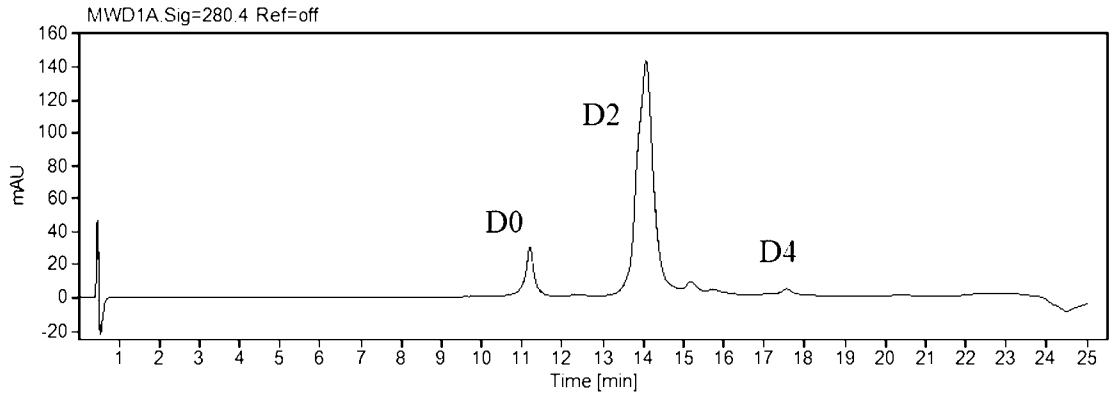
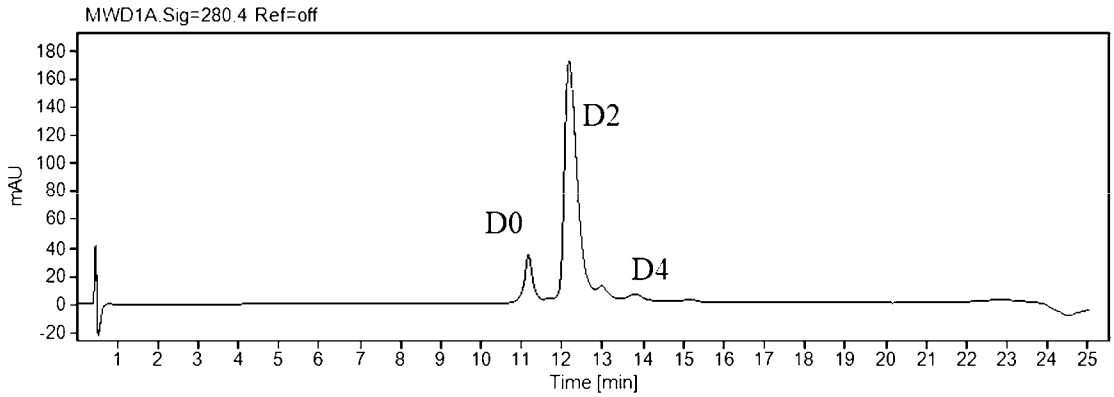


Fig. 2

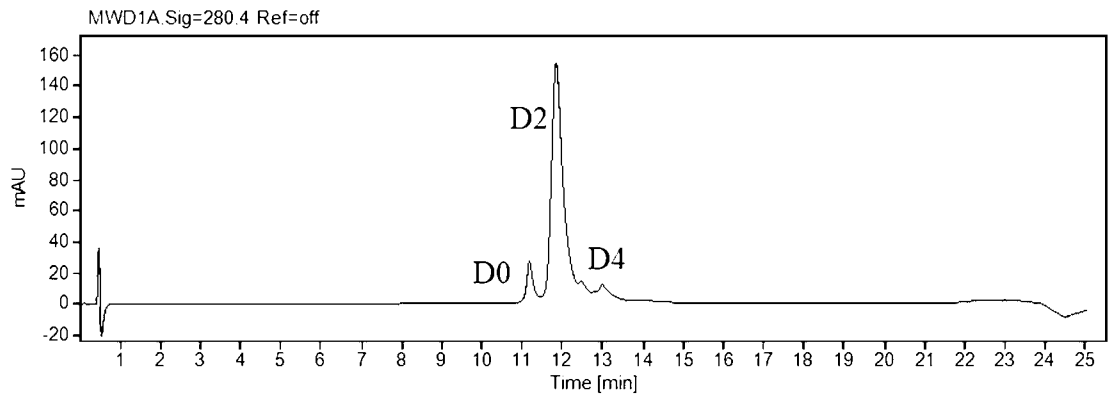
A mAb1 DAR2 conjugation（Mc-VC-MMAE）



B mAb1 DAR2 conjugation（Mc-MMAF）



C mAb1 DAR2 conjugation（GGFG-Dxd）



D mAb1 DAR2 conjugation（PBD）

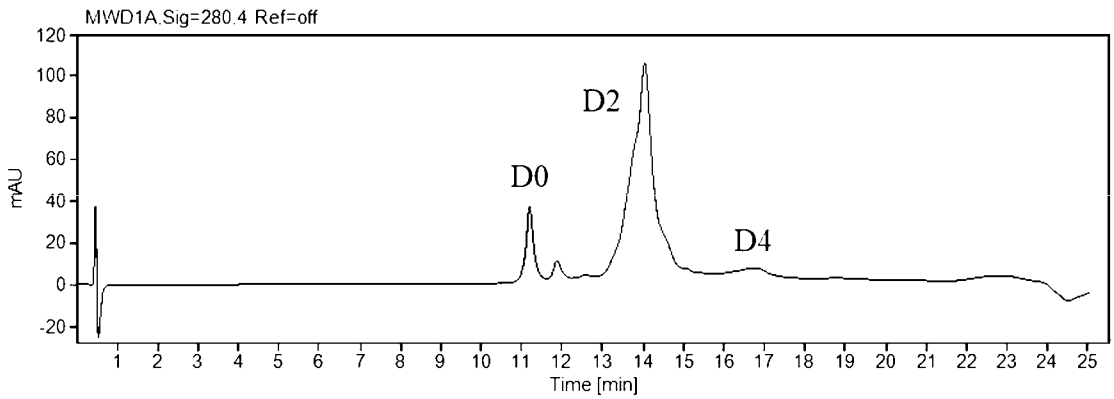
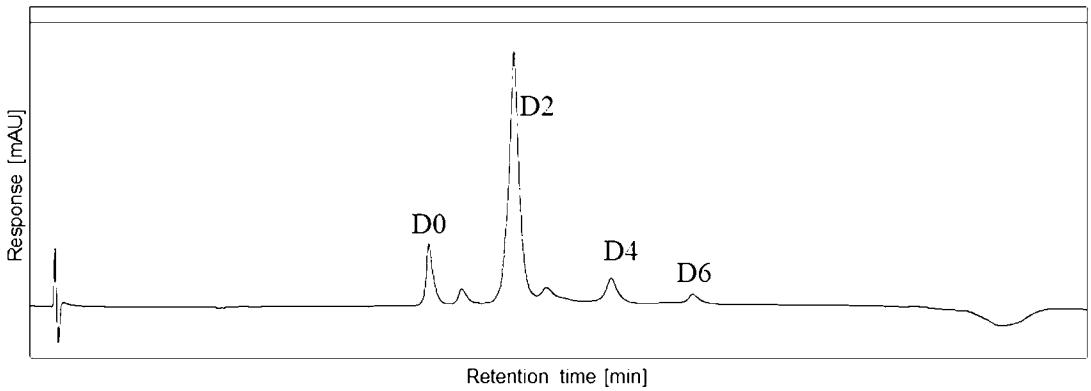


Fig. 3

E **mAb1 DAR2 conjugation（DL1-VC-PAB -MMAE）**



F **mAb1 DAR2 conjugation（DM21）**

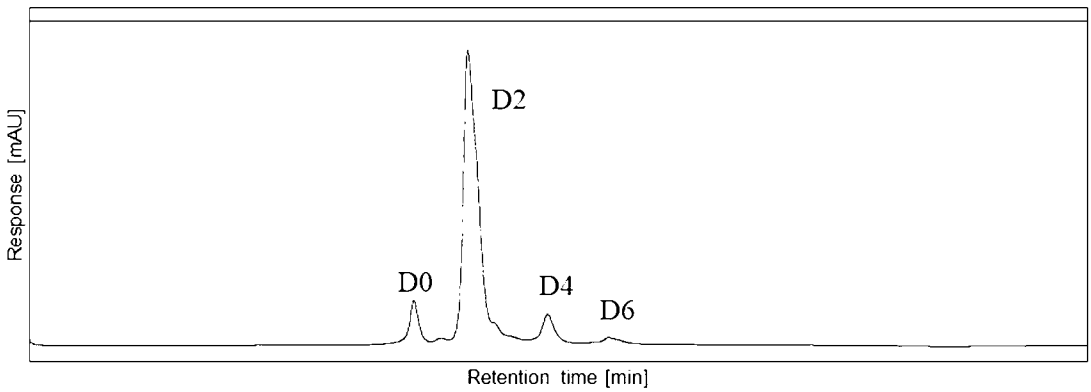
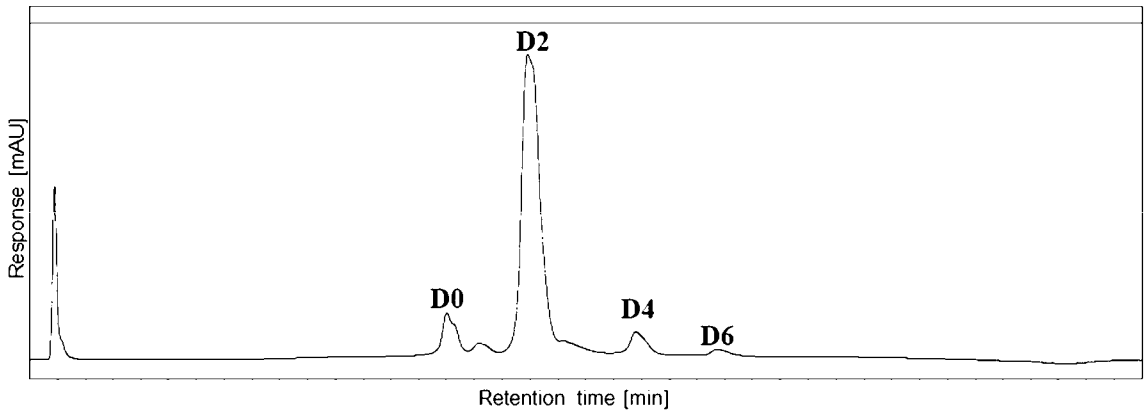
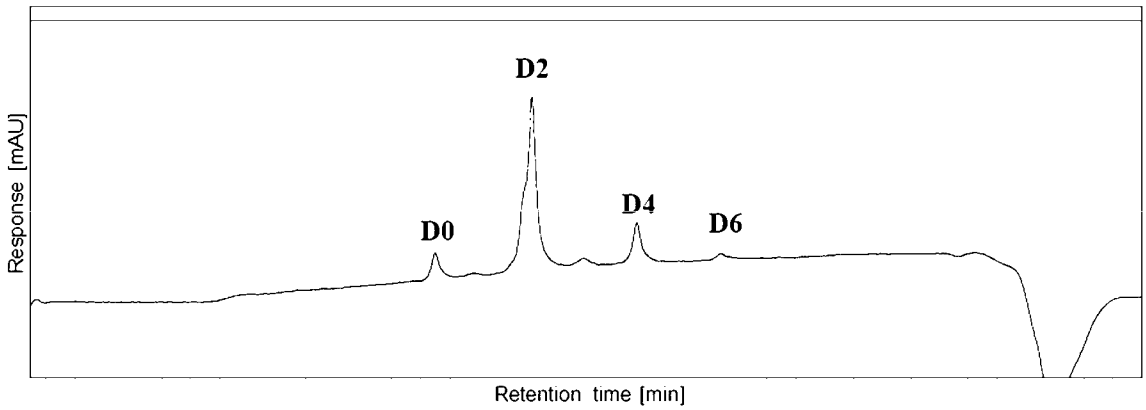


Fig. 3 (continued)

A mAb1 3.25eq TCEP



B mAb1 35eq diPPBS1



C mAb1 10.00eq DPAA

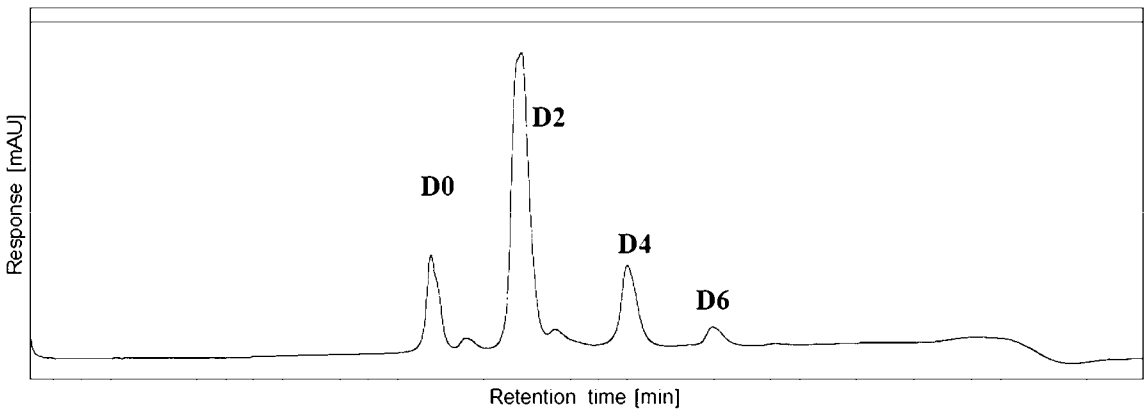
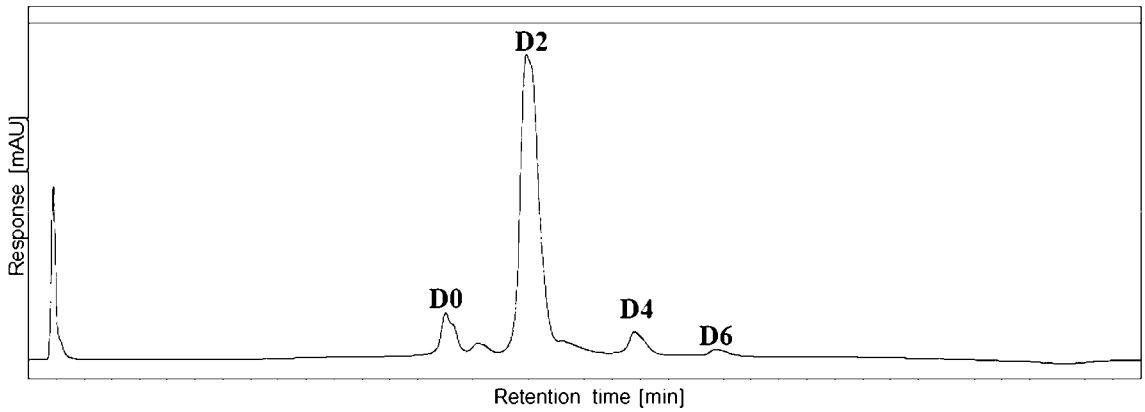
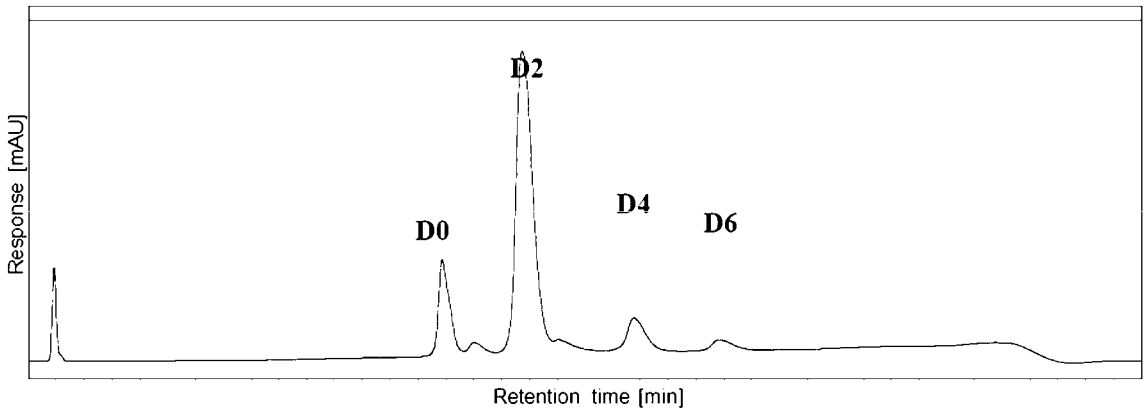


Fig. 4

D mAb1 3.25eq TCEP



E mAb1 10eq TCEP



F mAb1 16eq TCEP

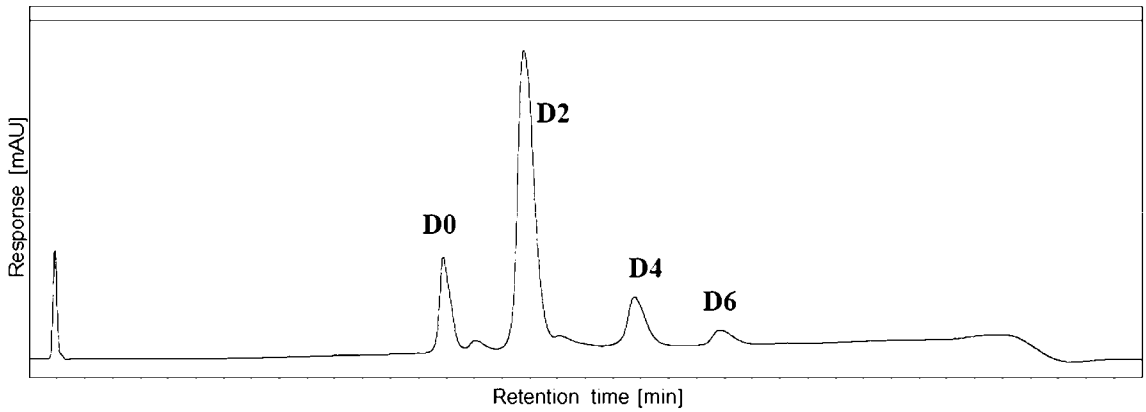
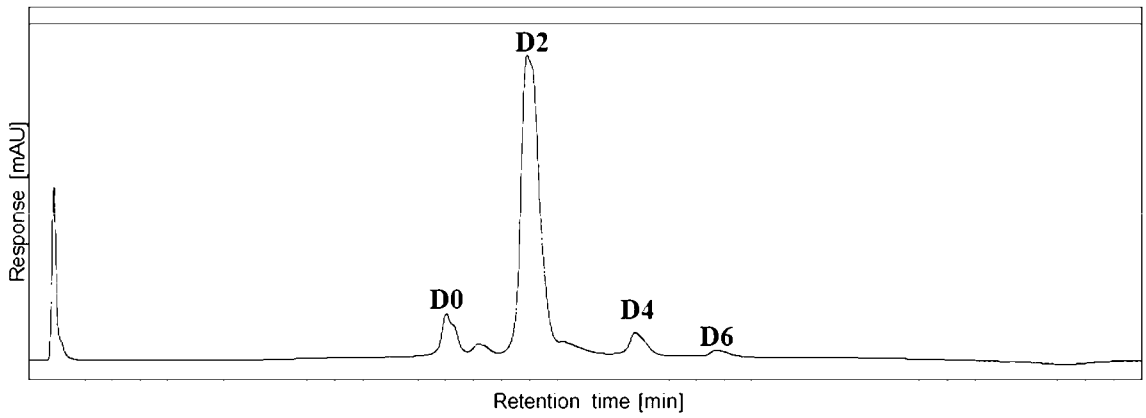
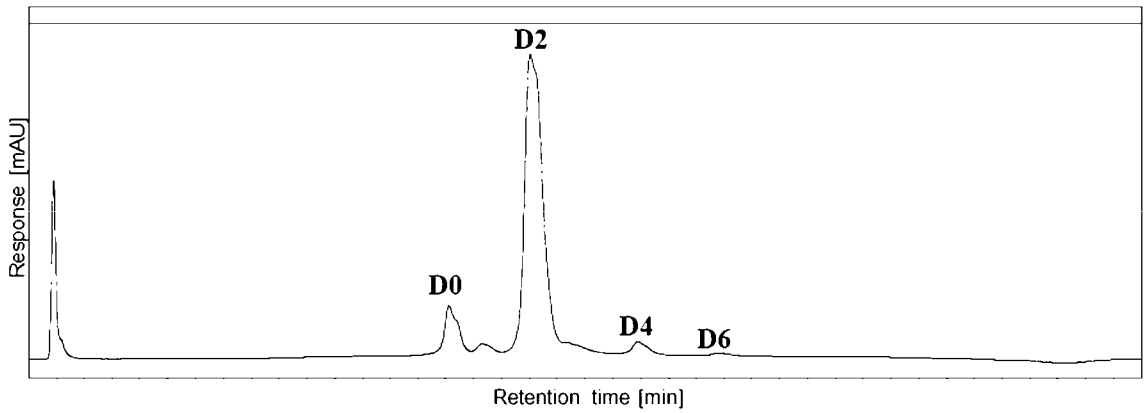


Fig. 4 (continued)

A mAb1 HEPES



B mAb1 MES



C mAb1 Tris

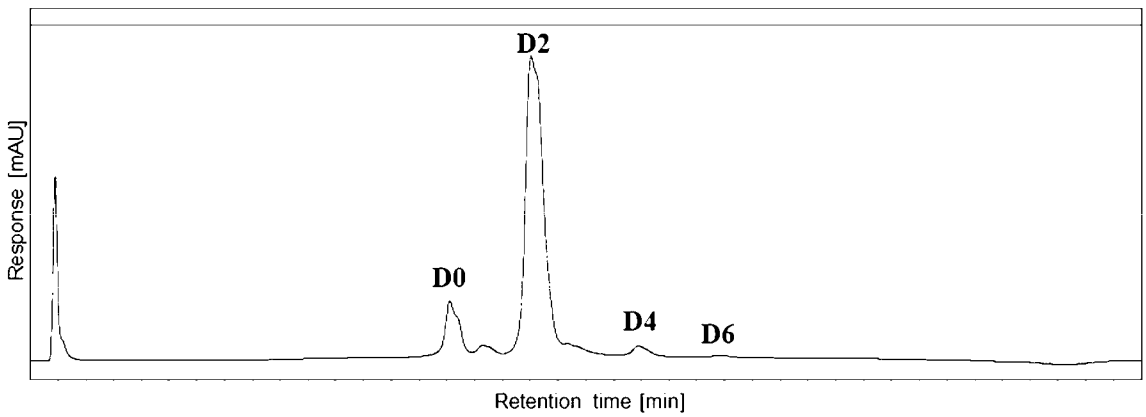
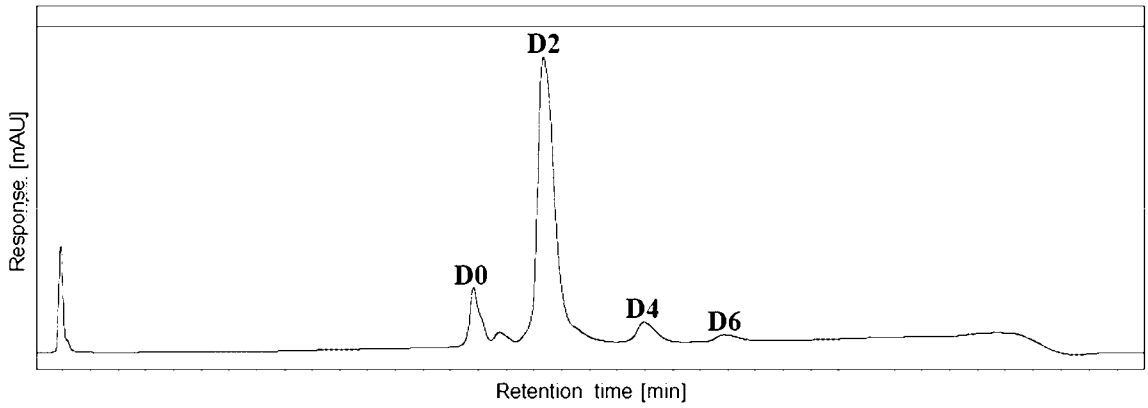
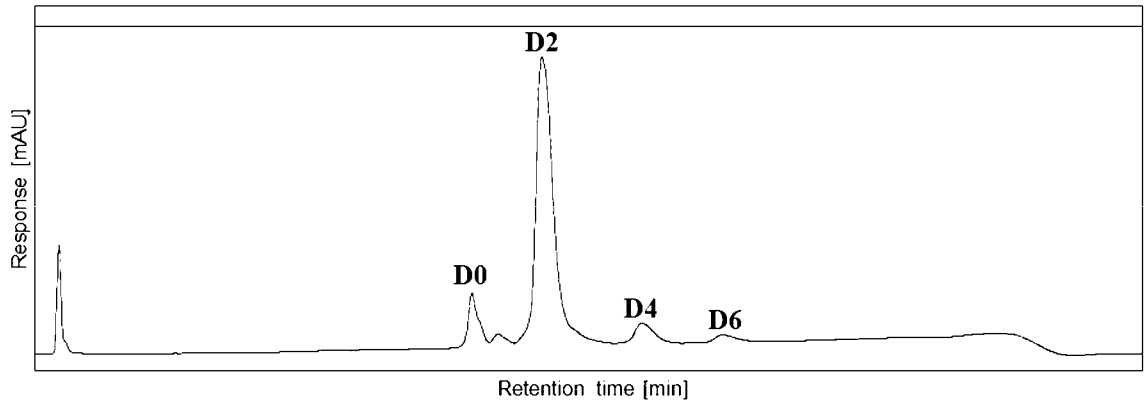


Fig. 5

A mAb3 HEPES



B mAb3 MES



C mAb3 Tris

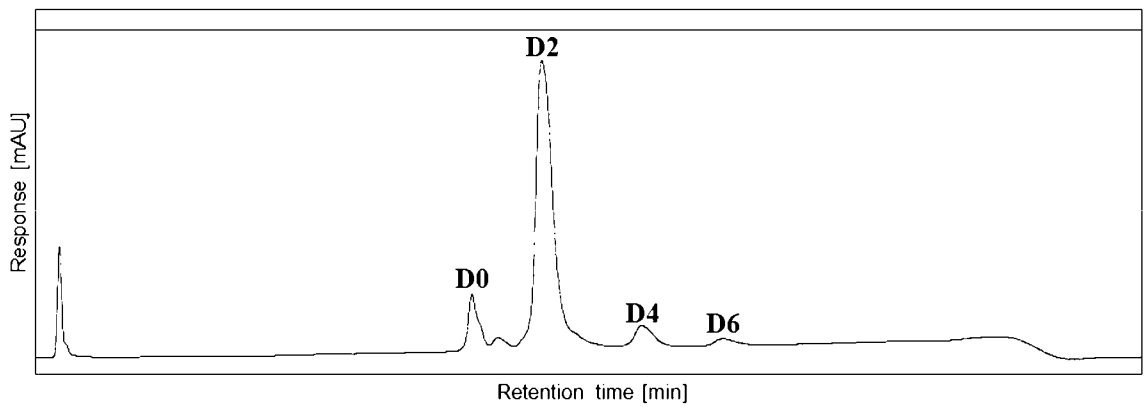
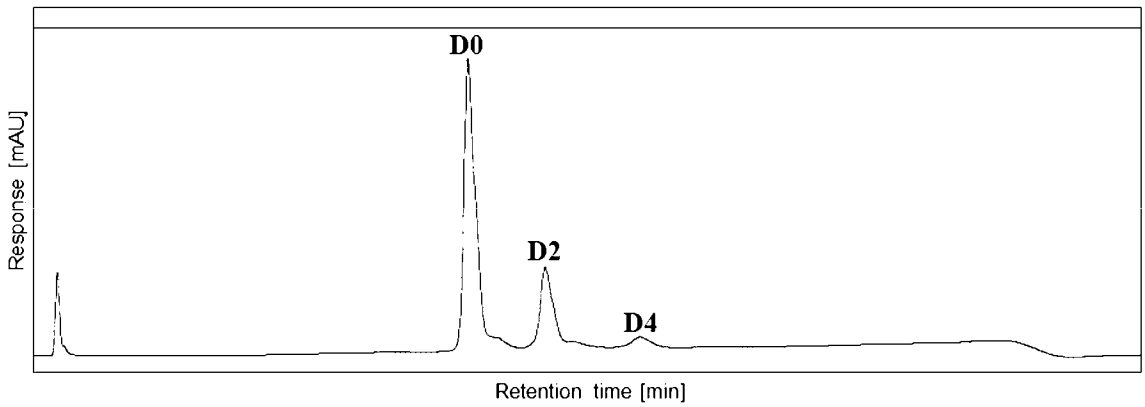
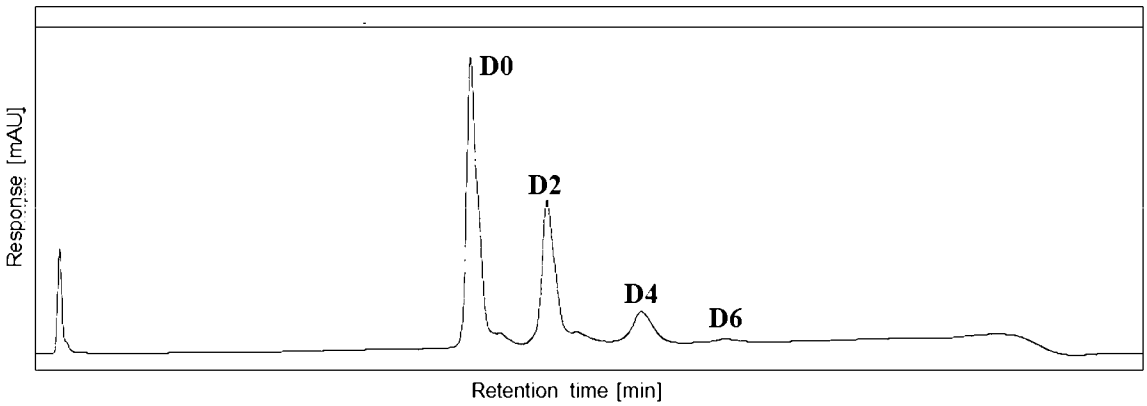


Fig. 6

A mAb3 0Zn^{2+} HEPES



B mAb3 0Zn^{2+} MES



C mAb3 0Zn^{2+} Tris

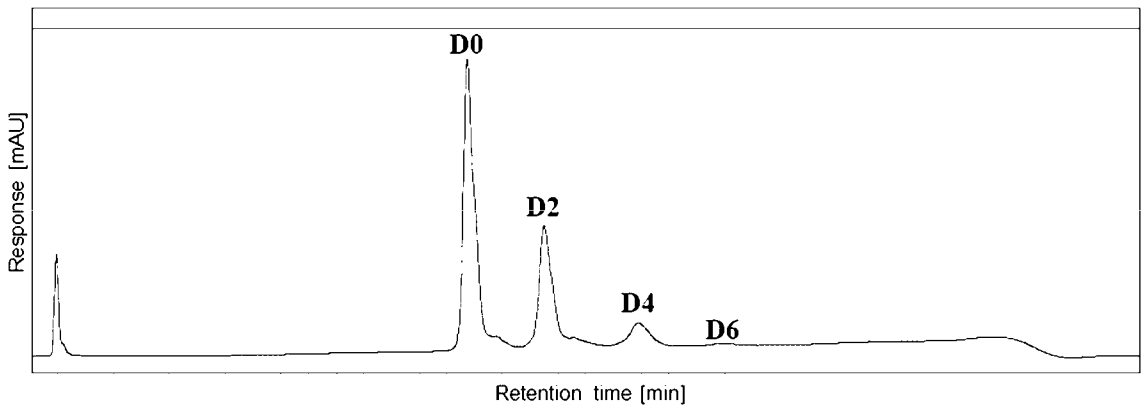


Fig. 7

mAb4（wuxibody) conjugation （ Mc-VC-MMAE ）

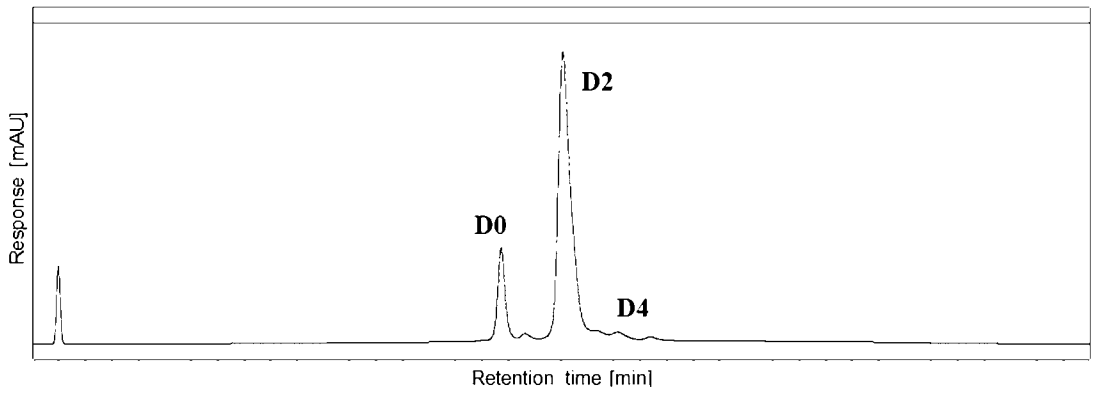
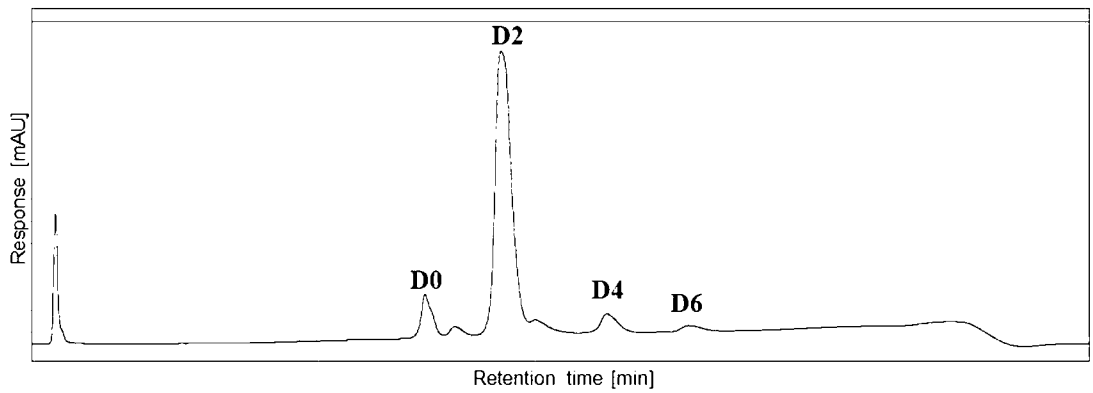
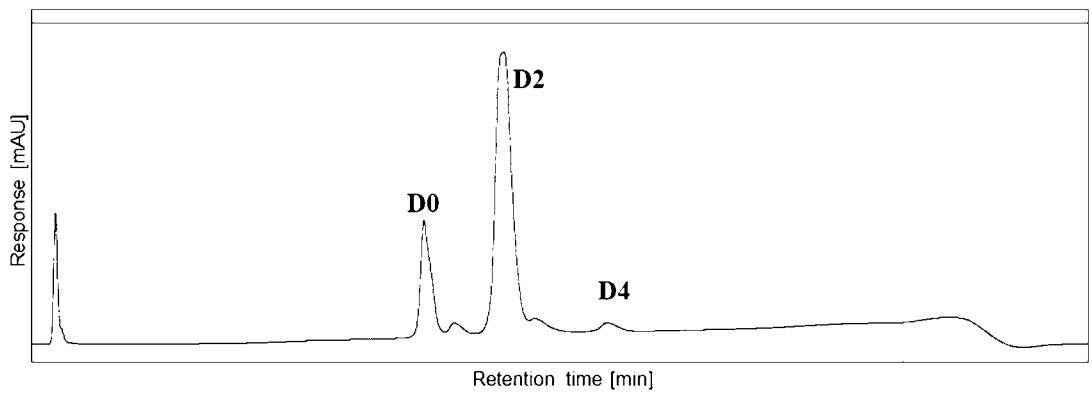


Fig. 8

A mAb1 4°C/19h



B mAb1 22°C/19h



C mAb1 37°C/2h

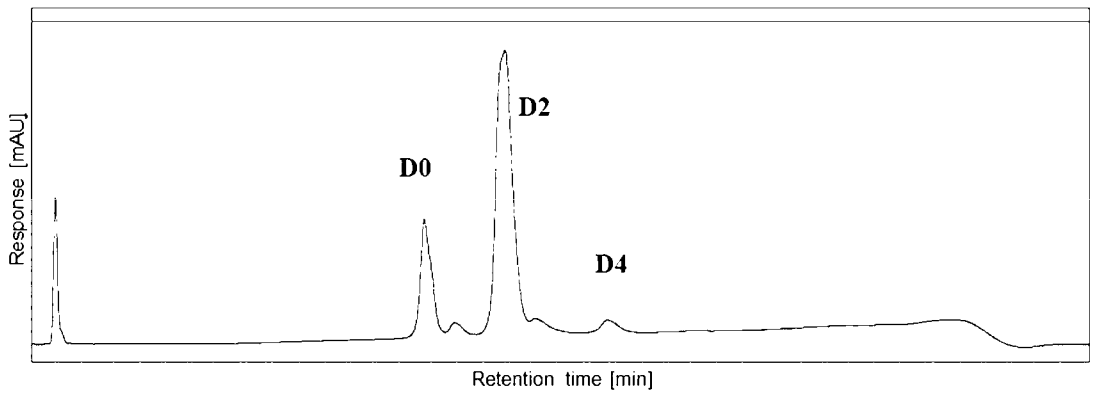
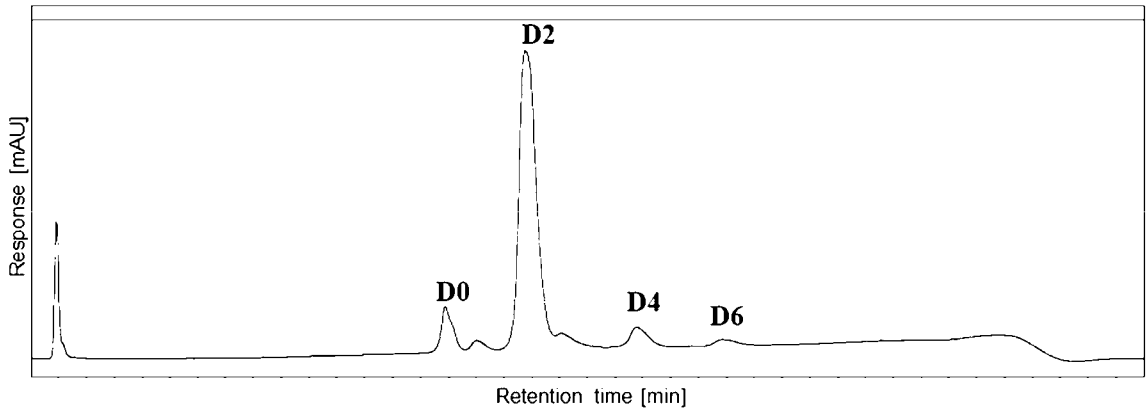
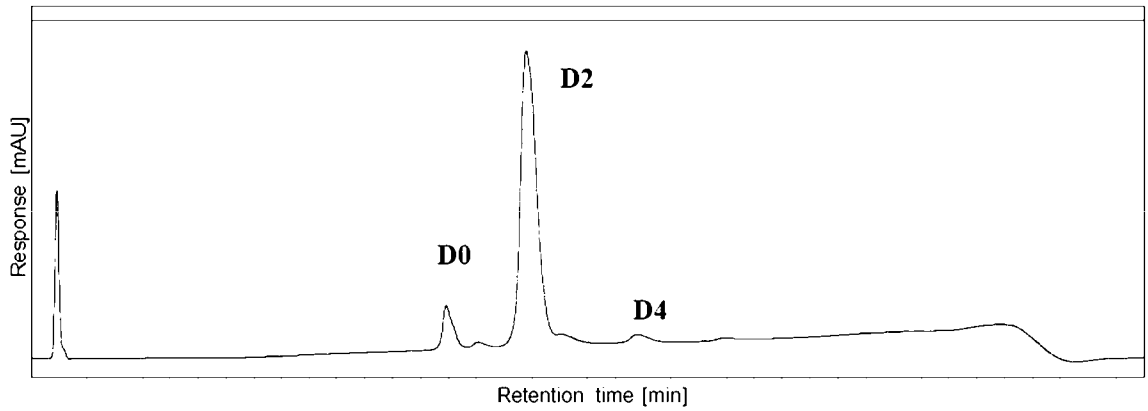


Fig. 9

D mAb1 4°C/19h



E mAb1 4°C/45h



F mAb1 4°C/92h

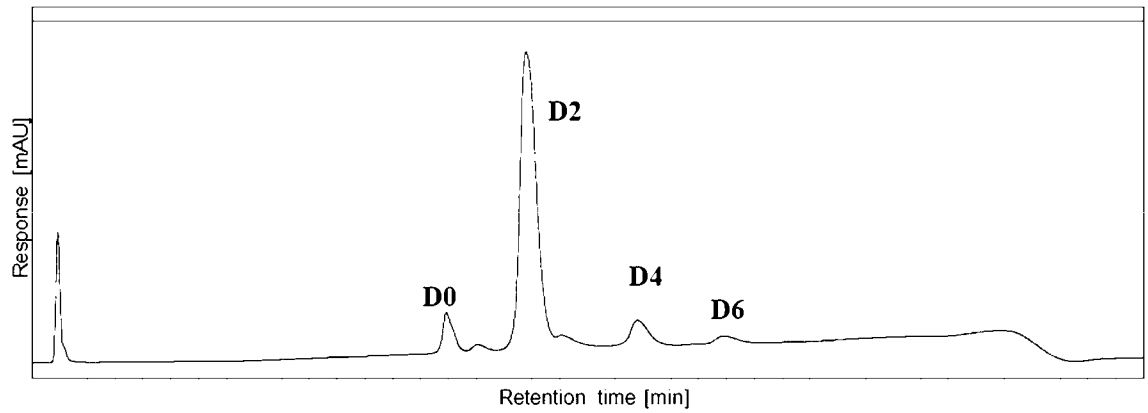
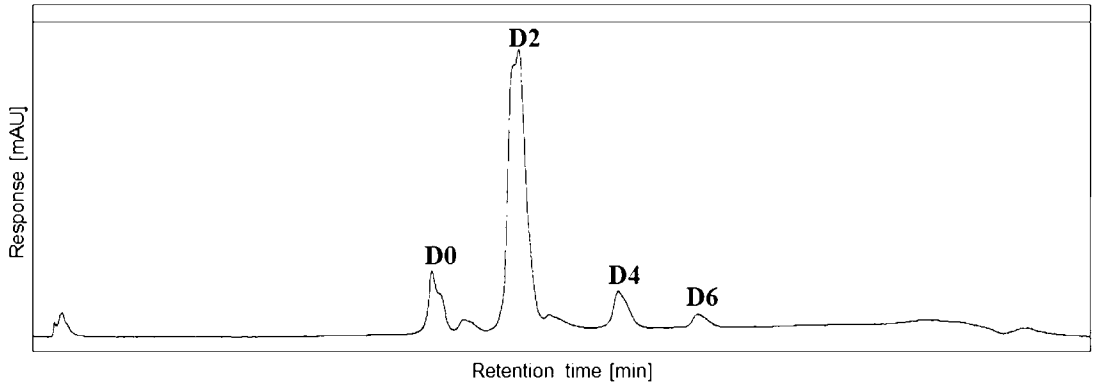
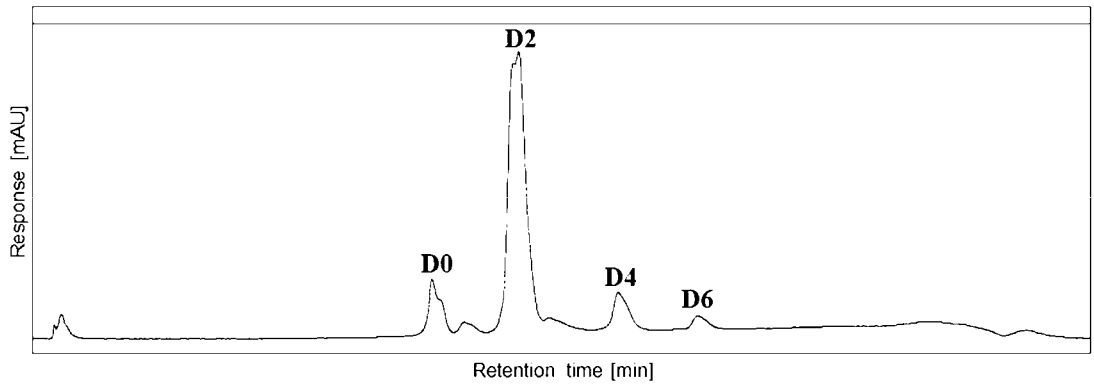


Fig. 9 (continued)

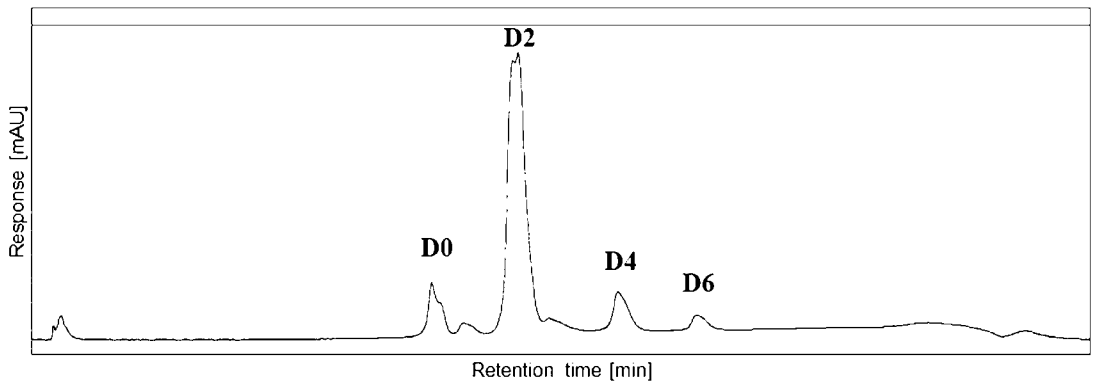
A mAb1 $ZnSO_4 \cdot 7H_2O$



B mAb1 $ZnBr_2 \cdot 2H_2O$



C mAb1 $Zn(ClO_4)_2 \cdot 6H_2O$



D mAb1 $Zn(OAc)_2 \cdot 2H_2O$

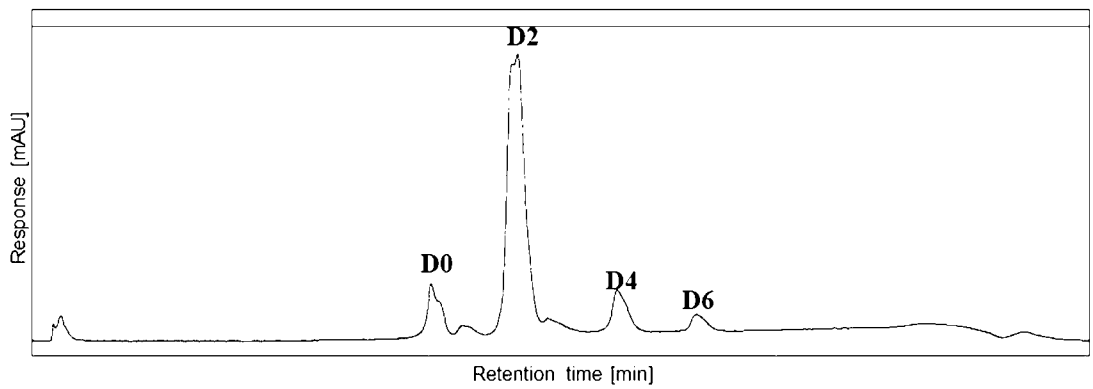
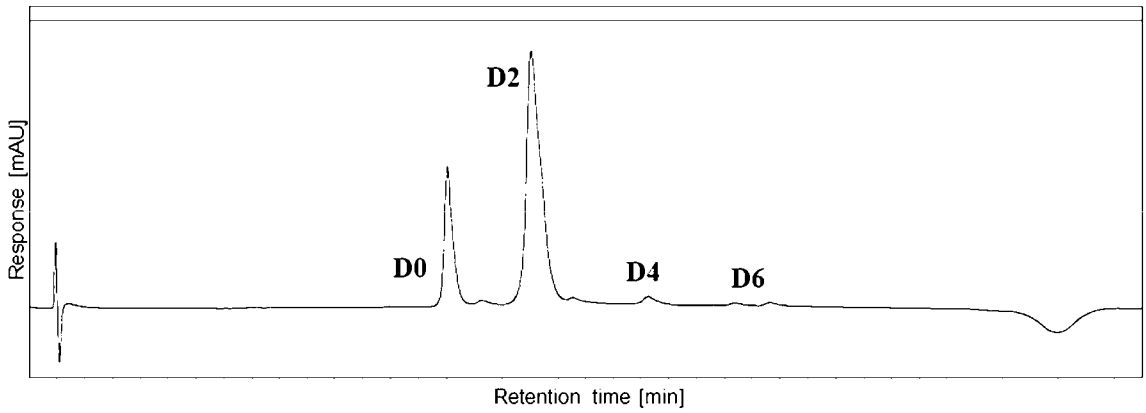
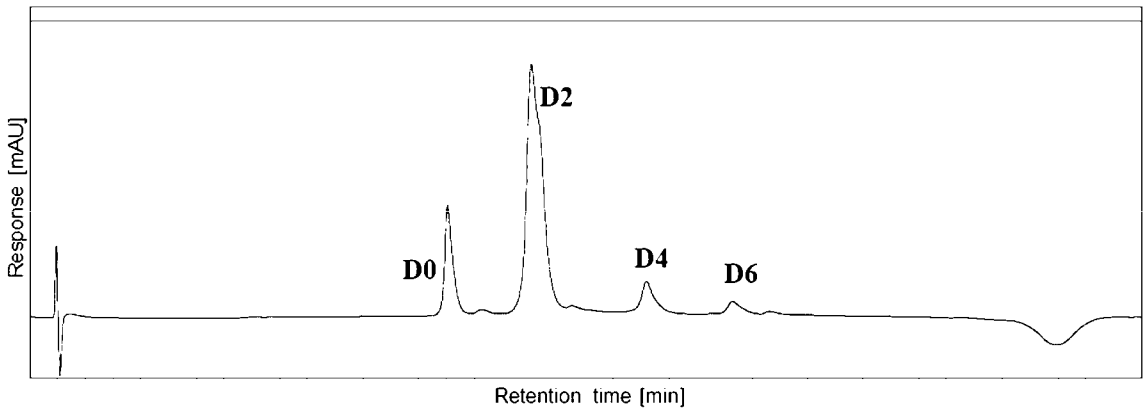


Fig. 10

A mAb1 2eq Zn^{2+}



B mAb1 8eq Zn^{2+}



C mAb1 16eq Zn^{2+}

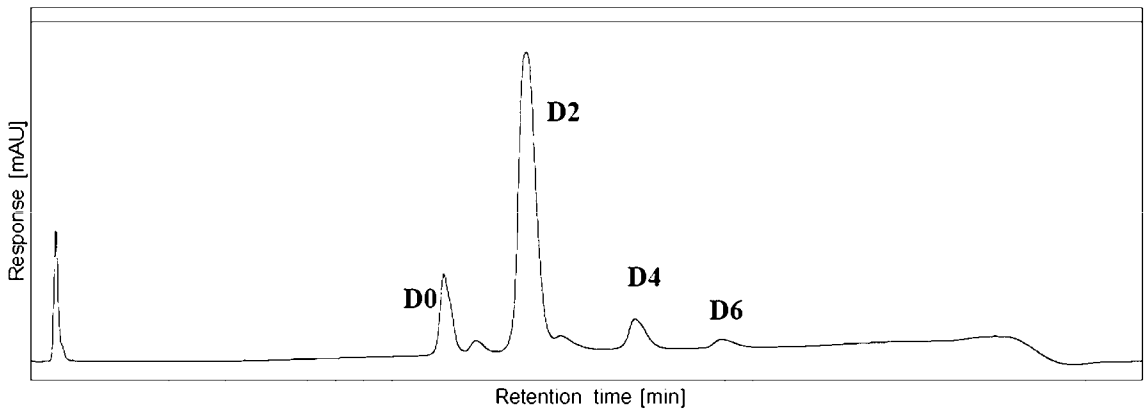


Fig. 11

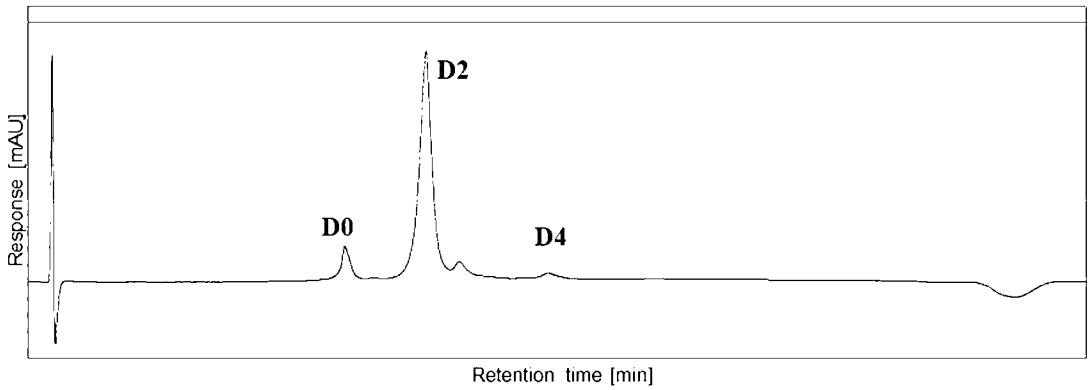
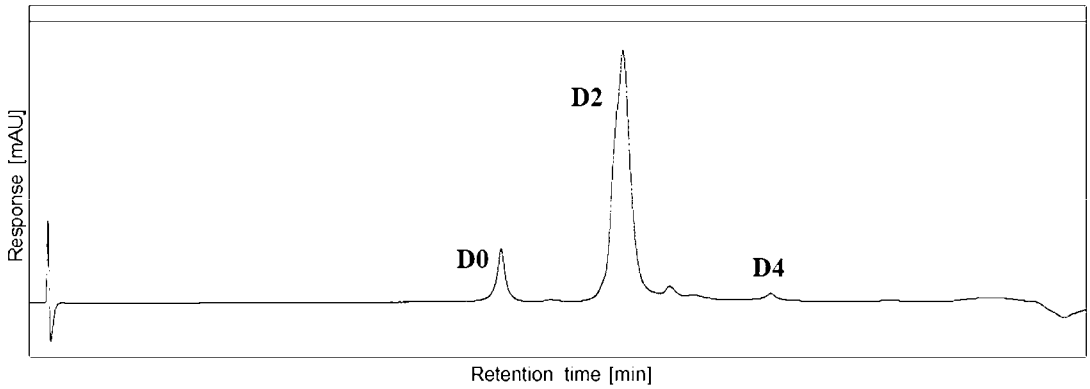
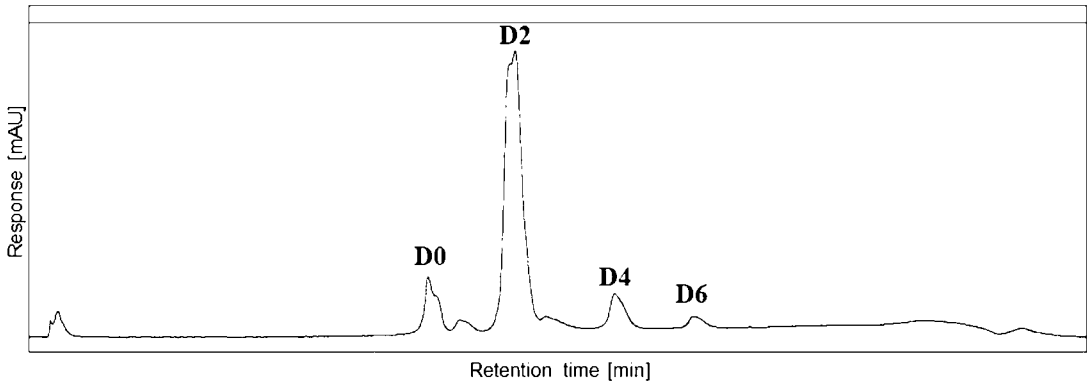


Fig. 12

A mAb1 12eq DHAA



B mAb1 2.2eq DTNB



C mAb1 1.82eq DDD

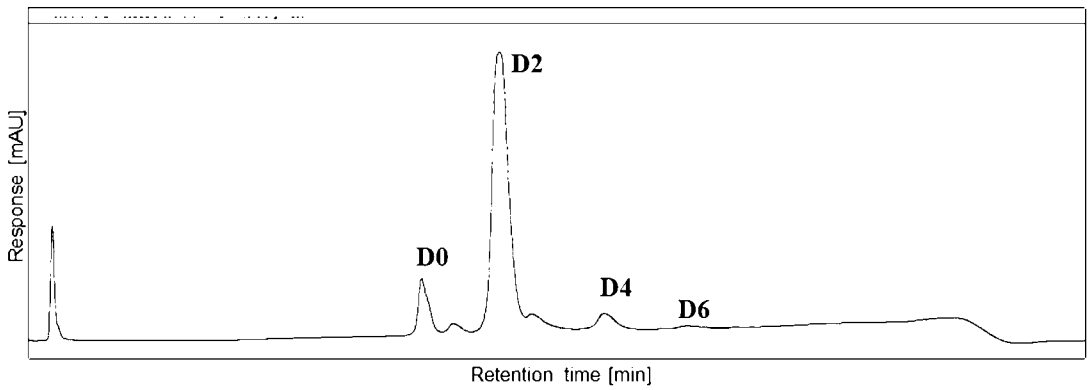
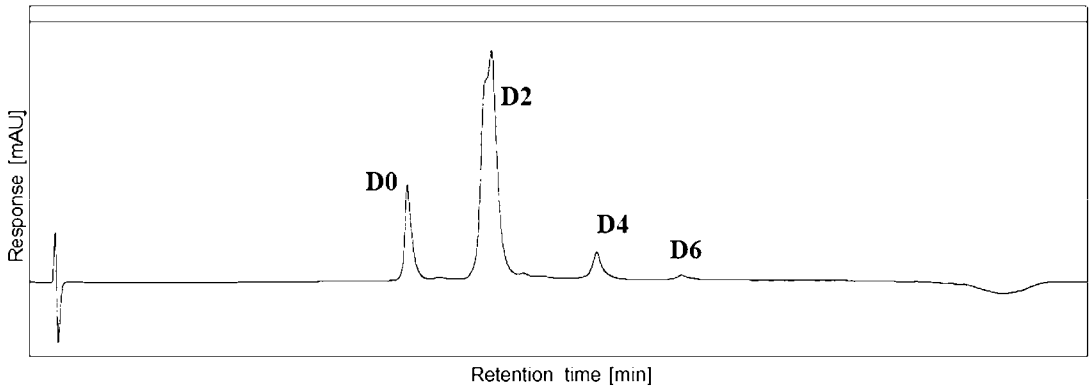
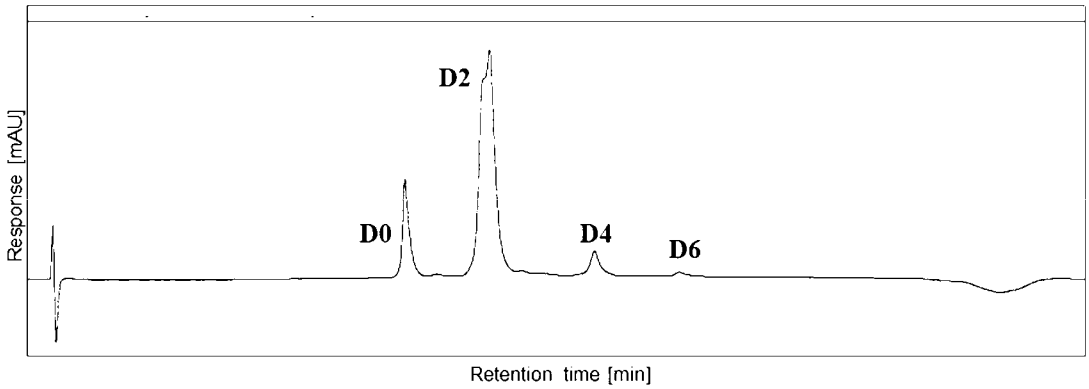


Fig. 13

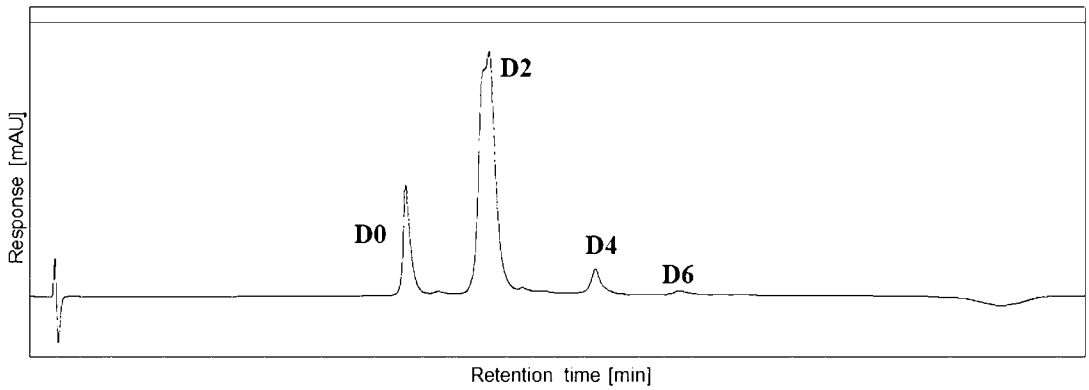
A mAb1 oxidation 0℃



B mAb1 oxidation 12℃



C mAb1 oxidation 22℃



D mAb1 oxidation 37℃

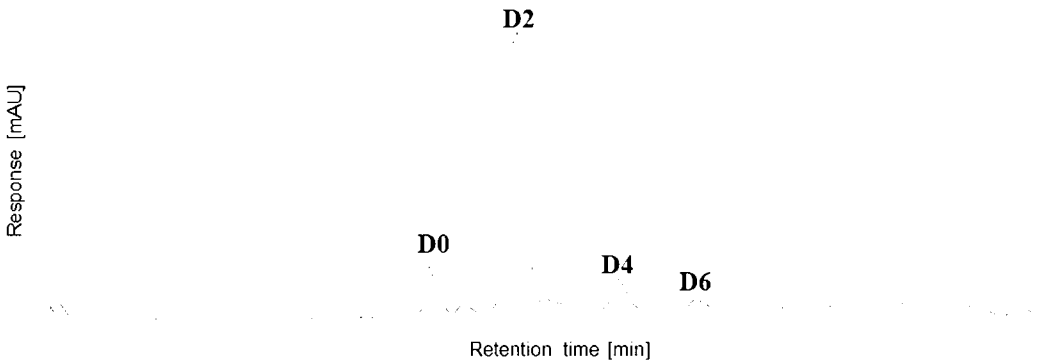
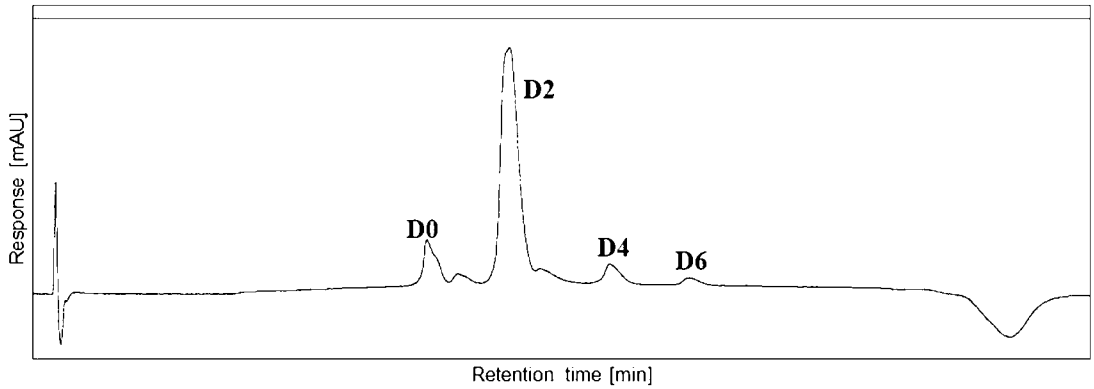
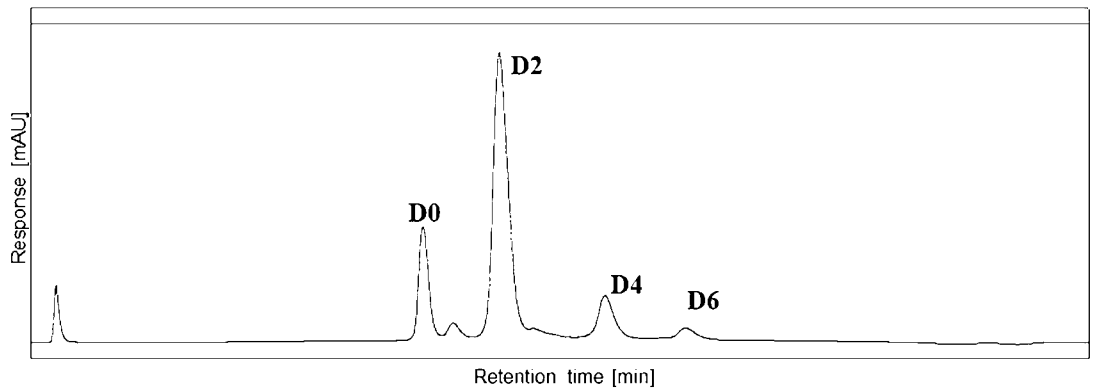


Fig. 14

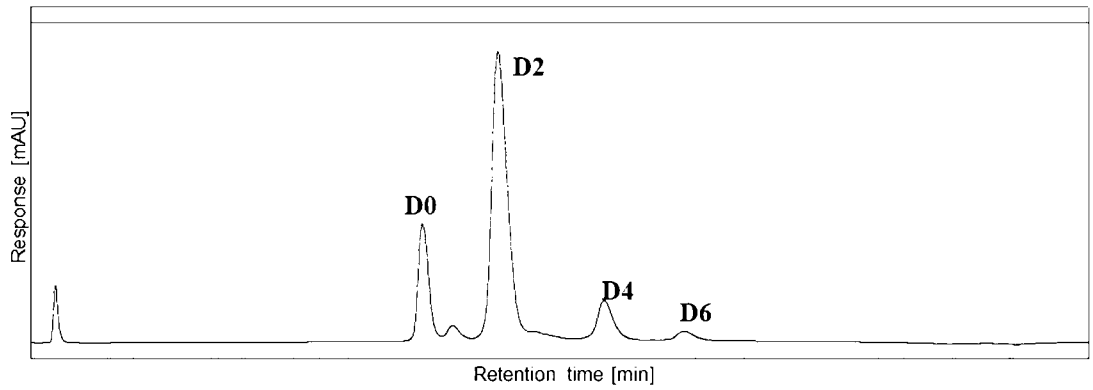
E mAb1 re-oxidation at 12 °C for 0.5h



F mAb1 re-oxidation at 12 °C for 2h



G mAb1 re-oxidation at 12 °C for 4h



H mAb1 re-oxidation at 12 °C for 24h

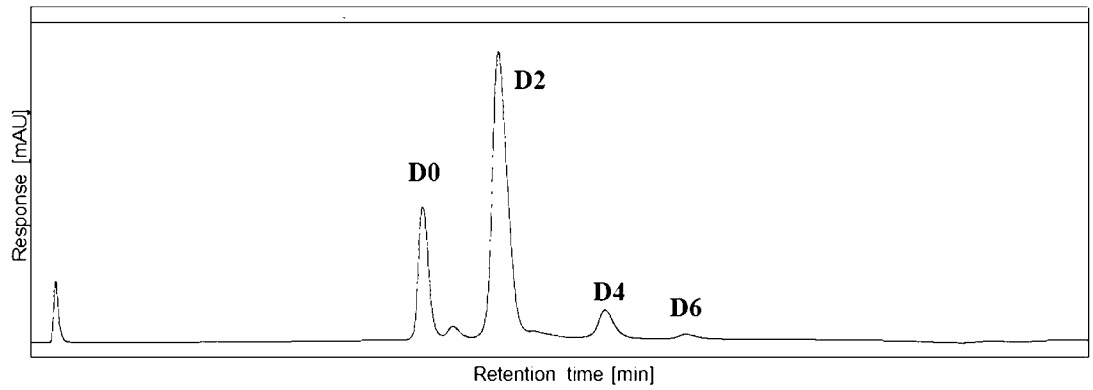
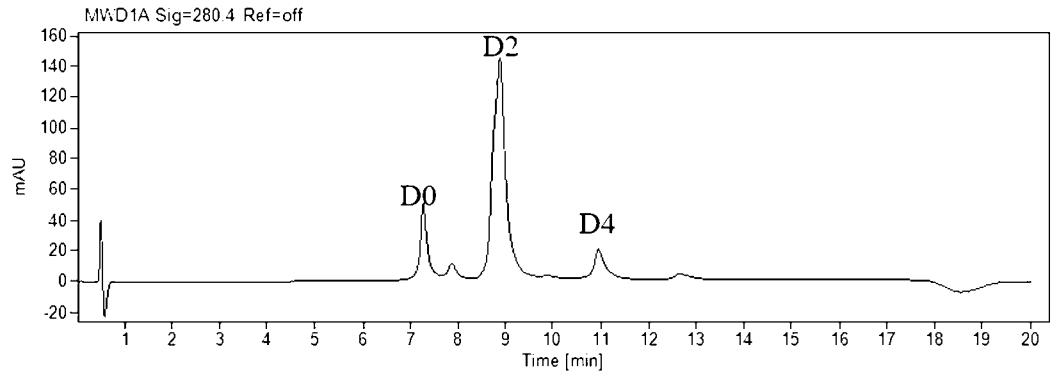


Fig. 14 (continued)

A **mAb1 conjugation at 0℃**



B **mAb1 conjugation at 12℃**

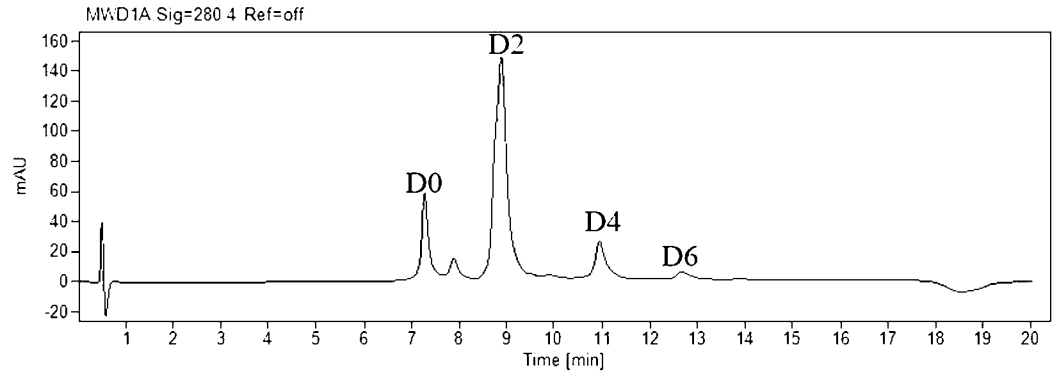


Fig. 15

mAb1 re-oxidation at 22°C for 47.5h

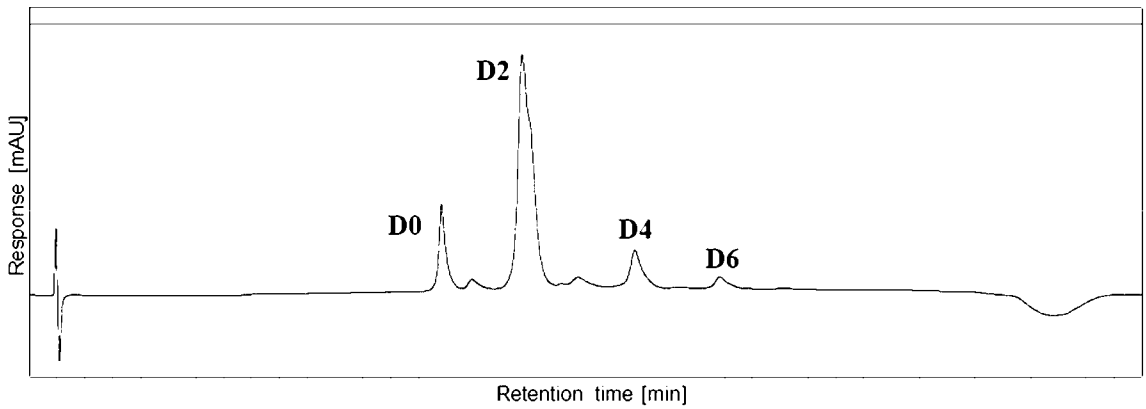
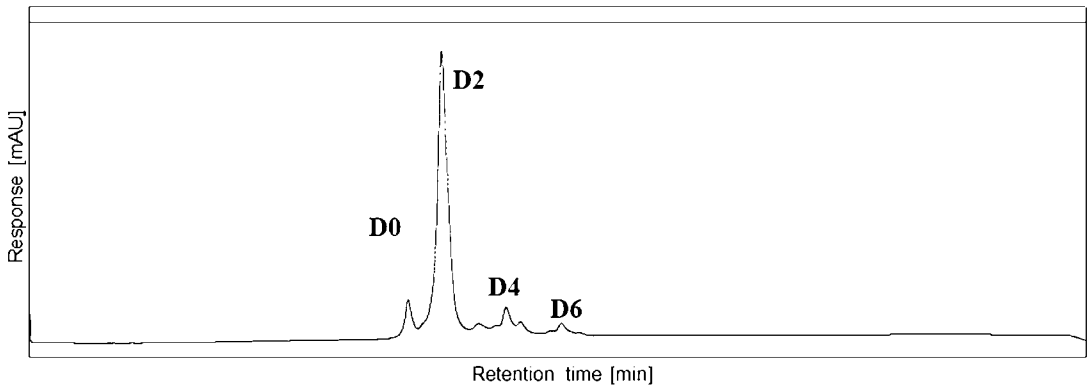
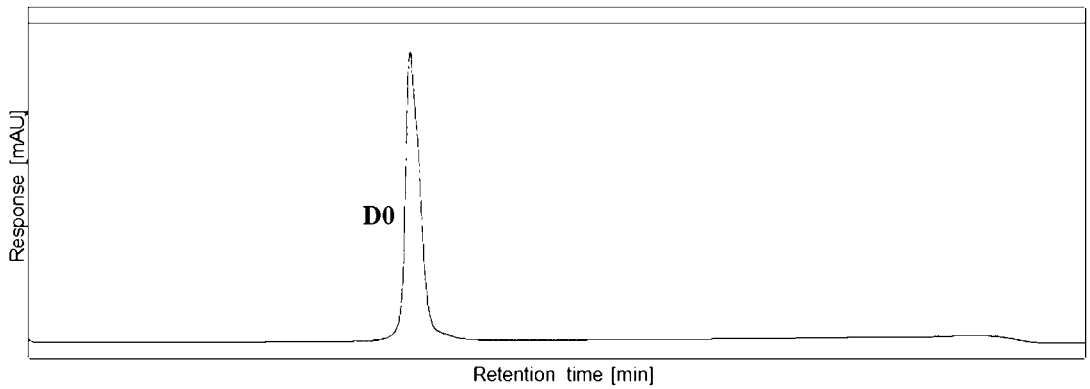


Fig. 16

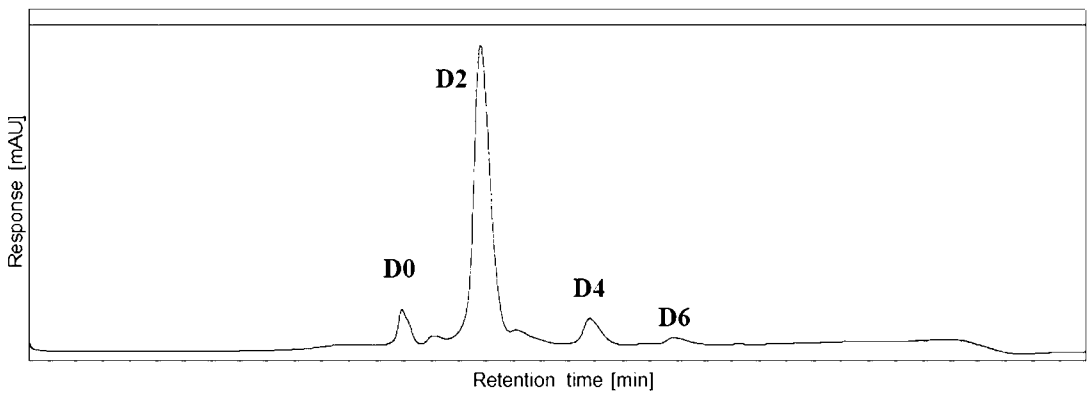
A mAb1 re-oxidation PB/4Zn^{2+} 170h/12°C



B mAb1 re-oxidation PB/0Zn^{2+} 24h/12°C



C mAb1 re-oxidation HEPES+PB/4Zn^{2+} 170h/12°C



D mAb1 re-oxidation HEPES/0Zn^{2+} 24h/12°C

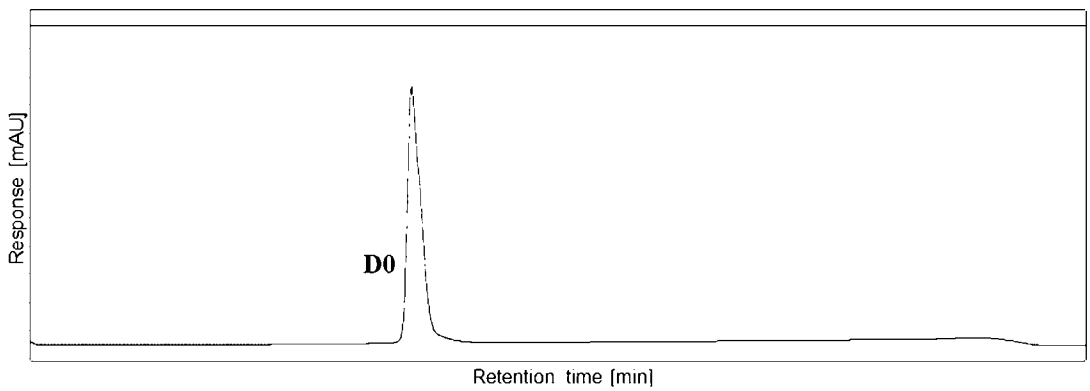
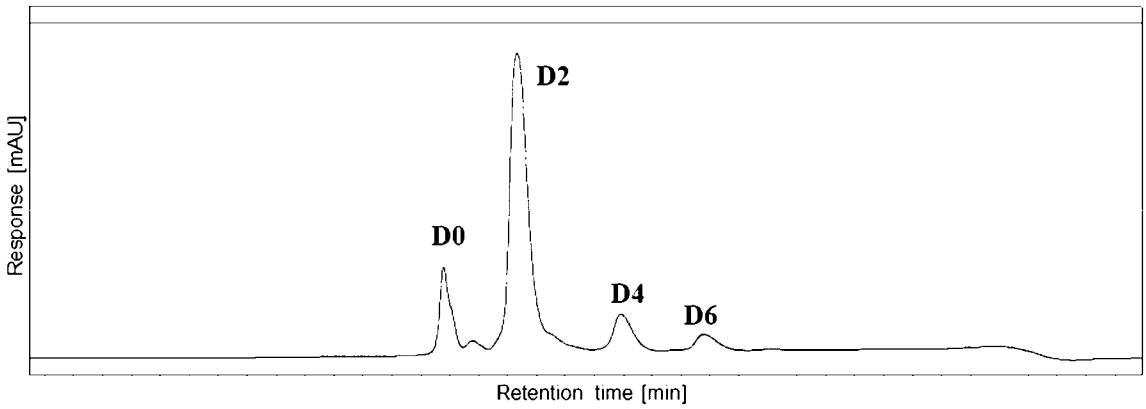


Fig. 17

A **mAb3 re-oxidation PB/4Zn^{2+} 170h/12°C**



B **mAb1 re-oxidation PB/0Zn^{2+} 24h/12°C**

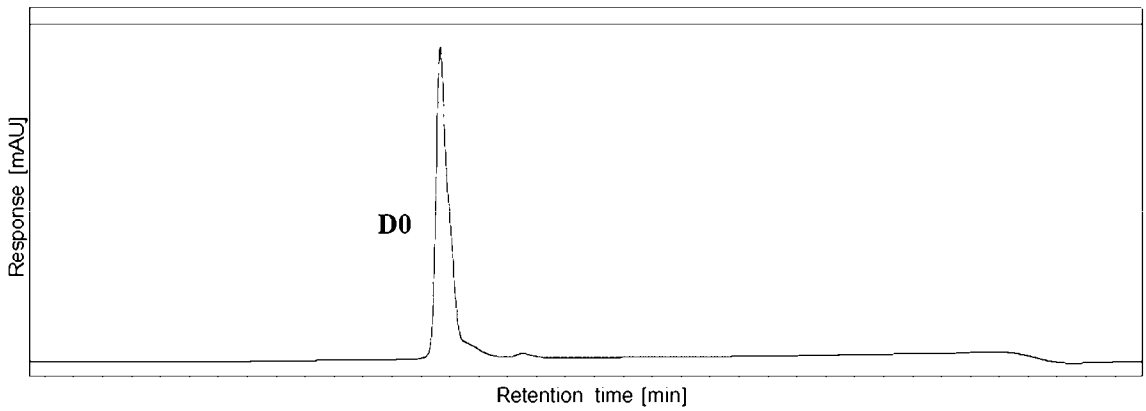
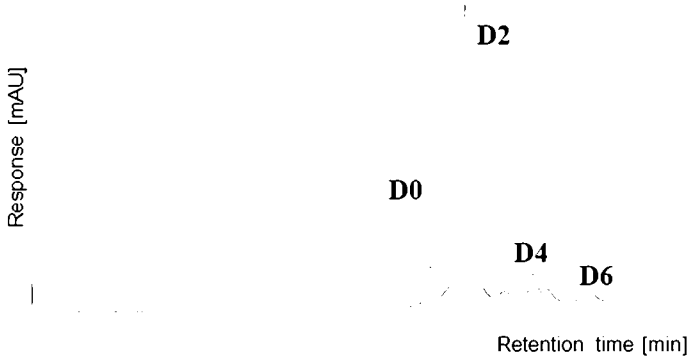
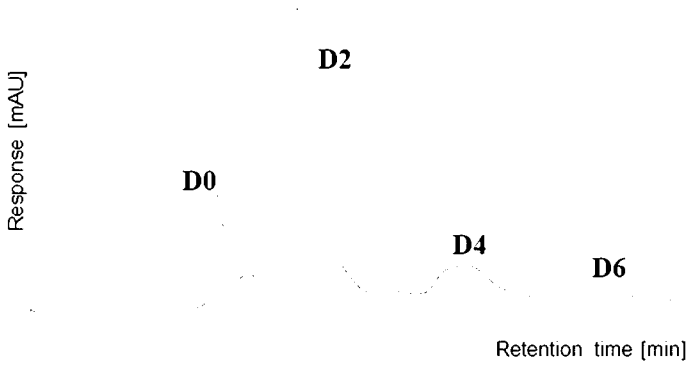


Fig. 18

A mAb1 GGFG-Dxd PB/4Zn^{2+} 170H/12°C



B mAb1 PBD PB/4Zn^{2+} 170H/12°C



C mAb1 MC-VC-MMAE HEPES+PB/4Zn^{2+} 170H/12°C

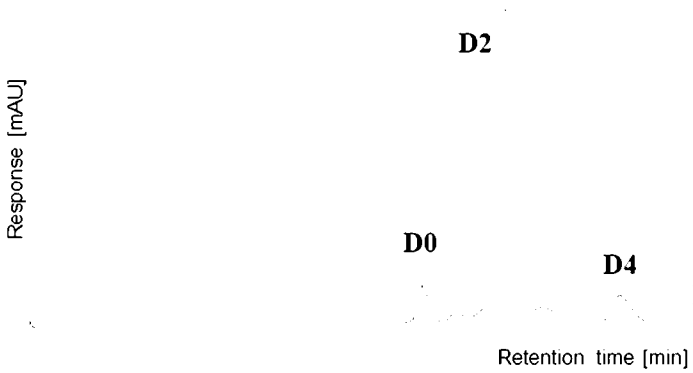
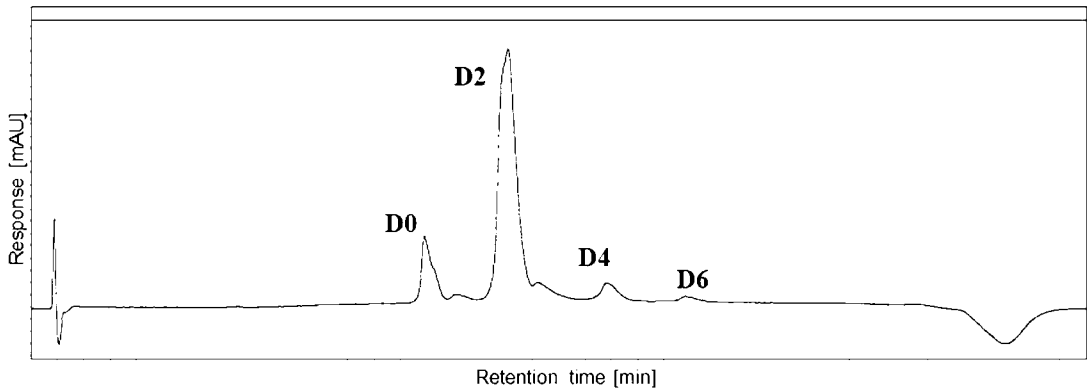


Fig. 19

A mAb1 5mg/ml



B mAb1 10mg/ml

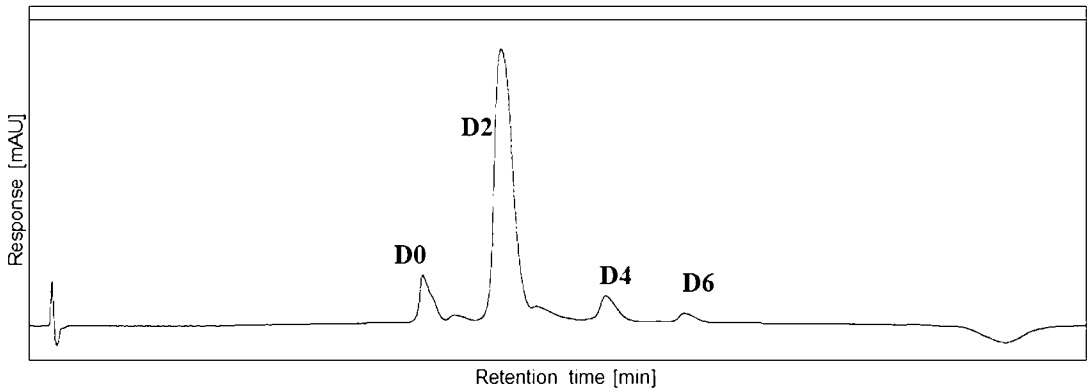
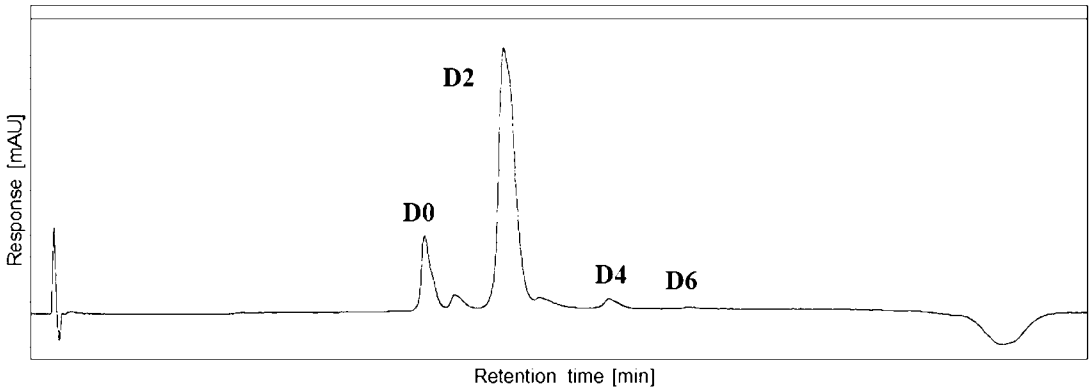


Fig. 20

A mAb1 40mM HEPES



B mAb1 20mM HEPES

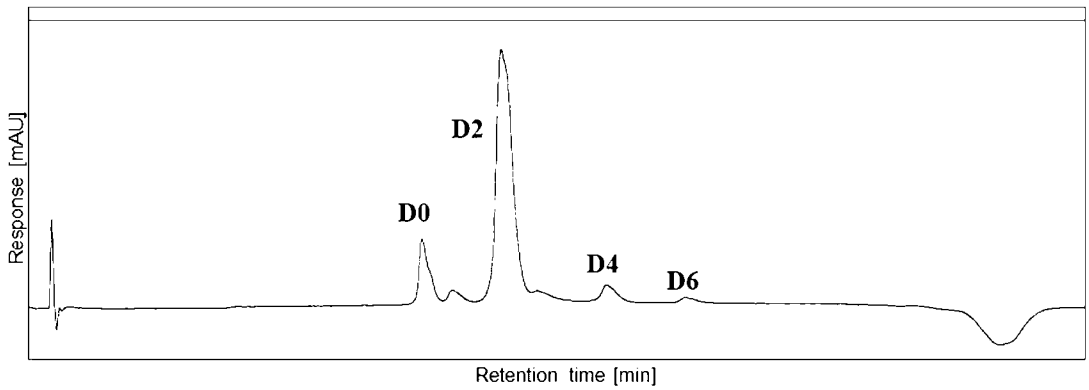


Fig. 21

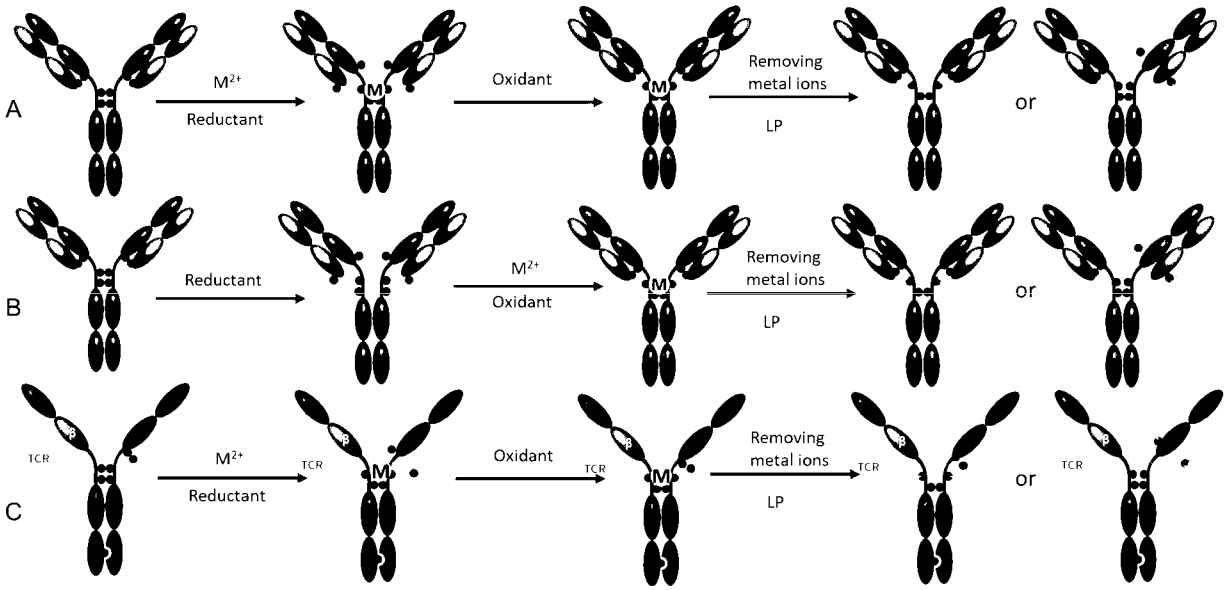

A
M2+
Reductant
M
Oxidant
M
Removing
metal ions
LP
or
B
Reductant
M2+
Oxidant
Removing
metal ions
LP
or
C
TCR
M2+
Reductant
TCR
Oxidant
TCR
Removing
metal ions
LP
TCR
or
TCR

Fig. 22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2024/097745

A. CLASSIFICATION OF SUBJECT MATTER

A61K47/68(2017.01)i; C07K16/00(2006.01)i; C07K16/28(2006.01)i; A61K39/395(2006.01)i; A61P35/00(2006.01)i; A61P31/00(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC:A61K; C07K; A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNTXT, WPABS, WPABSC, ENTXT, ENTXTC, VCN, VEN, CJFD, CNKI, ISI-WEB OF SCIENCE, PUBMED, BING: antibody-drug conjugates, ADC, reductant, TCEP, oxidation, DHAA. buffer, tris, PBS, transition metal ions, Zn2+, Ni2+, metal chelating agent, EDTA, antibody, mAb, trastuzumab, cetuximab, rituximab, MC-VC-PAB-MMA, coupling

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	WO 2024041542 A1 (SUZHOU BIOREINNO BIOTECHNOLOGY LIMITED COMPANY, et al.) 29 February 2024 (2024-02-29) description, page 145, Example Ш.1	1-21
PX	WO 2024041545 A1 (SUZHOU BIOREINNO BIOTECHNOLOGY LIMITED COMPANY) 29 February 2024 (2024-02-29) description, page 94, Example 125	1-21
PX	WO 2024041544 A1 (SUZHOU BIOREINNO BIOTECHNOLOGY LIMITED COMPANY) 29 February 2024 (2024-02-29) description, page 75, Example 61	1-21
A	CN 111670051 A (YAFEI SHANGHAI BIOLOG MEDICINE SCIENCE & TECHNOLOG CO., LED.) 15 September 2020 (2020-09-15) description,paragraph [0280]	1-21
A	WO 2005084390 A2 (SEATTLE GENETICS, INC.) 15 September 2005 (2005-09-15) claim 64	1-21

☑ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 August 2024	**20 August 2024**

Name and mailing address of the ISA/CN	Authorized officer
CHINA NATIONAL INTELLECTUAL PROPERTY ADMINISTRATION **6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088, China**	**KANG,PengCheng** Telephone No. **(+86) 010-53961875**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2024/097745

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☑ Claims Nos.: **19-21**
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 19-21 relate to a method for the treatment a condition or disorder, and thus do not warrant aninternational search according to the criteria set out in PCT Rule 39.1(iv). The search report is based on the subject-matter of the manufacture of a medicament correspondingly.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2024/097745

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2023036137 A1 (WUXI BIOLOGICS IRELAND LIMITED) 16 March 2023 (2023-03-16) claims 1-36	1-21

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2024/097745

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
WO	2024041542	A1	29 February 2024	None			
WO	2024041545	A1	29 February 2024	None			
WO	2024041544	A1	29 February 2024	WO	2024041544	A9	11 April 2024
CN	111670051	A	15 September 2020	CA	3081539	A1	16 May 2019
				WO	2019091384	A1	16 May 2019
				EP	3706803	A1	16 September 2020
				EP	3706803	A4	04 August 2021
				KR	20200086312	A	16 July 2020
				US	2023158141	A1	25 May 2023
				JP	2021502406	A	28 January 2021
				JP	7404252	B2	25 December 2023
				AU	2018364114	A1	21 May 2020
WO	2005084390	A2	15 September 2005	AU	2005218642	A1	15 September 2005
				AU	2005218642	B2	28 April 2011
				EP	1725586	A2	29 November 2006
				EP	1725586	A4	01 June 2011
				EP	1725586	B1	14 January 2015
				ES	2533695	T3	14 April 2015
				US	2009010945	A1	08 January 2009
				US	7837980	B2	23 November 2010
				US	2011097322	A1	28 April 2011
				JP	2008500961	A	17 January 2008
				JP	4942643	B2	30 May 2012
				CA	2558399	A1	15 September 2005
				CA	2558399	C	19 May 2015
				WO	2005084390	A3	05 October 2006
WO	2023036137	A1	16 March 2023	None			