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WO-A1-2009/012256
WO-A1-2015/086853
WO-A1-2015/123265
WO-A1-2016/067013
WO-A1-2017/055582
WO-A1-2017/137628
WO-A1-2018/136440
WO-A2-2005/084390
WO-A2-2017/025897
JAGATH R JUNUTULA ET AL: "Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index", NATURE BIOTECHNOLOGY,, vol. 26, no. 8, 1 August 2008 (2008-08-01), pages 925 - 932, XP002727747, ISSN: 1546-1696, [retrieved on 20080720], DOI: 10.1038/NBT.1480
CHRISTOPHER R BEHRENS ET AL: "Methods for site-specific drug conjugation to antibodies", MABS, vol. 6,

Fortsættes ...

no. 1, 20 January 2014 (2014-01-20), US, pages 46 - 53, XP055293357, ISSN: 1942-0870, DOI: 10.4161/mabs.26632

DATABASE Geneseq [online] 20 September 2018 (2018-09-20), "Human GLP-1 agonist peptide, SEQ 3293.", retrieved from EBI accession no. GSP:BFM63200 Database accession no. BFM63200

LYON ROBERT P ET AL: "Chapter 6: CONJUGATION OF ANTICANCER DRUGS THROUGH ENDOGENOUS MONOCLONAL ANTIBODY CYSTEINE RESIDUES", METHODS IN ENZYMO, ACADEMIC PRESS, US, vol. 502, 1 January 2012 (2012-01-01), pages 123 - 138, XP008157545, ISSN: 0076-6879, DOI: 10.1016/B978-0-12-416039-2.00006-9

DESCRIPTION

Description

FIELD OF THE INVENTION

[0001] The present disclosure relates to a method of capping, reducing, and oxidizing cys-mAbs in order to provide homogenous material for subsequent conjugation reactions.

BACKGROUND OF THE INVENTION

[0002] Conjugated biomolecules are a diverse array of substances that comprise multiple precursor molecules, at least one of which is derived from a biological system. Most often, the biologically derived component(s) are produced using recombinant DNA techniques. In the pharmaceutical industry, conjugated biomolecules have been investigated as treatments for a variety of medical conditions. In these cases, the conjugate can provide a number of therapeutic benefits by combining useful properties of two or more precursor molecules into a single entity.

[0003] One particularly successful class of pharmaceutical bioconjugates are antibody conjugates, also called antibody-drug conjugates. These molecules consist of an antibody, typically derived from mammalian cell culture, and a synthetic molecule with biologic or pharmacologic activity. Several antibody-drug conjugates have been approved as cancer therapies, and many more are in clinical and pre-clinical development. All of the antibody-drug conjugates that have been approved to date are manufactured using non-specific chemistry that produces a mixture of conjugated molecules.

[0004] Pharmaceuticals based on site-specific antibody conjugates, where a synthetic molecule is attached at defined sites in the antibody molecule, can provide therapeutic benefits as well as improved quality control and/or shelf life. Numerous efforts have therefore been undertaken to develop methods for producing site-specific conjugates of antibodies. A common approach to site-specific conjugation is the use of a cysteine mutant antibody (Cys-mAb or Thiomab), in which a new cysteine amino acid is introduced into the antibody primary structure. This engineered cysteine can be used as a site for conjugating a synthetic molecule.

Jagath R Junutula et al., "Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index", Nature Biotechnology, Vol.26, No.8 (2008) concerns the engineering of cysteine substitutions at positions on light and heavy chains of antibodies that provide reactive thiol groups and do not perturb immunoglobulin folding and assembly, or alter antigen binding.

Christopher R Behrens et al., "Methods for site-specific drug conjugation to antibodies", MABS, Vol.6, No.1 (2014) relates to methods for site-specific drug conjugation to antibodies, and compares in vivo results with their non-specifically conjugated counterparts.

WO 2016/067013 A1 concerns a solid phase method of synthesising biomolecule-drug-conjugates.

WO 2009/012256 A1 relates to humanized and conjugated antibodies against CD79b useful for the treatment of hematopoietic tumor in mammals.

WO 2005/084390 A2 concerns protein-drug conjugates and methods of making protein-drug and protein-labeled conjugates, as well as proteins having points of conjugation for receiving a drug or label.

WO 2018/136440 A1 relates to methods of treating metabolic diseases and disorders using a composition comprising an antigen binding protein specific for GIPR polypeptide conjugated to a GLP-1 receptor agonist.

WO 2017/137628 A1 concerns a process for the selective reduction of cysteine-engineered antibodies. WO 2015/086853 A1 relates to methods for obtaining a protein conjugate wherein a cysteine residue of the protein serves as attachment point for the chemical moiety conjugated to the protein.

WO 2017/055582 A1 concerns protein conjugates and in particular conjugates of more than two protein or polypeptides.

[0005] Site-specific conjugation with a Cys-mAb protein requires the engineered cysteine side chain in the reduced thiol form. However, when antibodies are isolated from mammalian cell culture, the engineered cysteine is generally "capped" as a mixed disulfide with a cytoplasmic thiol such as glutathione. Thus directly adding the reactive synthetic molecule will not produce the conjugate since the side chain of the engineered cysteine is not available to react.

[0006] A single step selective reduction to remove the cap would be highly desirable, but is currently not feasible due to the chemical similarity between the mixed disulfide and the structural disulfides in the antibody. When a reducing agent is added to the Cys-mAb to remove the caps, some portion of the other disulfides in the antibody will also be reduced, and the resulting thiols can react to form conjugates at undesired locations.

[0007] Accordingly, there remains a need for efficient, robust ways to manufacture conjugates of cysteine-engineered antibodies that offer high yield and consistent product quality.

SUMMARY OF THE INVENTION

[0008] The invention is defined in the appended claims.

[0009] In one aspect, the present disclosure provides a method for preparing an antibody conjugate or antibody fragment conjugate, the method comprising the steps of: a) obtaining a composition comprising an antibody or antibody fragment; b) exposing the antibody or antibody fragment to a cysteine blocking agent, wherein the cysteine blocking agent is

cysteamine and the cysteine blocking agent forms a stable mixed-disulfide with at least one cysteine residue of the antibody or antibody fragment; c) adding a reducing agent to the composition to form a reduction mix and allowing a reduction reaction to occur such that the reduction mix comprises a reduced antibody or reduced antibody fragment, wherein the reducing agent is triphenylphosphine-3, 3',3''-trisulfonate

[0010] ("TPPTS"); d) adding an oxidizing agent to the reduction mix to form an oxidized mix and allowing an oxidizing reaction to occur such that the oxidizing mix comprises an oxidized antibody or oxidized antibody fragment; and e) adding an activated chemical moiety to the oxidized mix to form a conjugation mix and allowing a conjugation reaction to occur such that an antibody conjugate or antibody fragment conjugate is formed.

[0011] In one aspect, following step b) and before step c) cation exchange chromatography is performed to remove excess cysteine blocking agent. In one aspect, following step c) and before step d) a buffer exchange step is performed to remove the reducing agent. In one embodiment, the buffer exchange step is ultrafiltration/diafiltration. In one aspect, following step e), a purification step is performed to remove the activated chemical moiety. In one embodiment, the purification step includes hydrophobic interaction chromatography ("HIC"), ultrafiltration/diafiltration, or hydrophobic interaction chromatography ("HIC") followed by ultrafiltration/diafiltration.

[0012] In another aspect, the present disclosure provides a method for preparing an antibody conjugate or antibody fragment conjugate, the method comprising the steps of: a) obtaining a composition comprising a mixed disulfide comprising an antibody or antibody fragment with a capped free cysteine that comprises a cysteamine cap; b) adding a reducing agent to the composition to form a reduction mix and allowing a reduction reaction to occur such that the reduction mix comprises a reduced antibody or reduced antibody fragment, wherein the reducing agent is triphenylphosphine-3, 3',3''-trisulfonate ("TPPTS"); c) adding an oxidizing agent to the reduction mix to form an oxidized mix and allowing an oxidizing reaction to occur such that the oxidizing mix comprises an oxidized antibody or oxidized antibody fragment; and d) adding an activated chemical moiety to the oxidized mix to form a conjugation mix and allowing a conjugation reaction to occur such that an antibody conjugate or antibody fragment conjugate is formed.

[0013] In one aspect, following step a) and before step b) cation exchange chromatography is performed to remove excess cysteine blocking agent. In one aspect, following step b) and before step c) a buffer exchange step is performed to remove the reducing agent. In one embodiment, the buffer exchange step is ultrafiltration/diafiltration. In one aspect, following step d), a purification step is performed to remove the activated chemical moiety. In one embodiment, the purification step includes hydrophobic interaction chromatography ("HIC"), ultrafiltration/diafiltration, or hydrophobic interaction chromatography ("HIC") followed by ultrafiltration/diafiltration.

[0014] In another aspect, the present disclosure provides a method for preparing an antibody

conjugate or antibody fragment conjugate, the method comprising the steps of: a) obtaining a composition comprising a mixed disulfide comprising an antibody or antibody fragment with a capped free cysteine that comprises a cysteamine cap; b) adding a reducing agent to the composition to form a reduction mix and allowing a reduction reaction to occur such that the reduction mix comprises a reduced antibody or reduced antibody fragment, wherein the reducing agent is triphenylphosphine-3, 3',3''-trisulfonate ("TPPTS"); c) adding an oxidizing agent to the reduction mix to form an oxidized mix and allowing an oxidizing reaction to occur such that the oxidizing mix comprises an oxidized antibody or oxidized antibody fragment; and d) adding an activated chemical moiety to the oxidized mix to form a conjugation mix and allowing a conjugation reaction to occur such that an antibody conjugate or antibody fragment conjugate is formed.

[0015] In one aspect, following step a) and before step b) a buffer exchange step is performed to remove the reducing agent. In one aspect, following step c), a purification step is performed to remove the activated chemical moiety. In one embodiment, the buffer exchange step is ultrafiltration/diafiltration. In one embodiment, the purification step includes hydrophobic interaction chromatography ("HIC"), ultrafiltration/diafiltration, or hydrophobic interaction chromatography ("HIC") followed by ultrafiltration/diafiltration.

[0016] In one embodiment, the mixed disulfide is an antibody or antibody fragment with a capped free cysteine. In the method of the invention, the antibody or antibody fragment with a capped free cysteine comprises a cap, wherein the cap is cysteamine. In the method of the invention, the reducing agent is triphenylphosphine-3,3',3''-trisulfonate ("TPPTS"). In one embodiment, the ratio of reducing agent to antibody or antibody fragment is 2 to 4:1 (mole/mole)). In one embodiment, the oxidizing agent is dehydroascorbic acid ("DHAA"). In one embodiment, the ratio of oxidizing agent to antibody or antibody fragment is 3 to 6: 1 (mole/mole)). In one embodiment, the activated chemical moiety is a peptide comprising a halogen, wherein the halogen is selected from the group consisting of Br, I, and Cl). In one embodiment, the ratio of activated chemical moiety to antibody or antibody fragment is 2 to 3:1 (mole/mole).

[0017] In one embodiment, the antibody or antibody fragment comprises a cysteine residue at a position selected from the group consisting of D70 of the antibody light chain relative to reference sequence SEQ ID NO: 7; E276 of the antibody heavy chain relative to reference sequence SEQ ID NO: 8; and T363 of the antibody heavy chain relative to reference sequence SEQ ID NO: 8.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018]

Fig. 1. Typical Cys mAb (IgG1) consisting of four polypeptide chains (two Light Chains, two Heavy Chains) held together by 16 native disulfide bonds (shown in green). Additional

cysteines engineered into the antibody carry two additional disulfides (shown in orange). Selective reduction of the engineered disulfides in the presence of other 16 native disulfides would be highly desirable, but is not feasible (in a single step).

Fig. 2. Net Selective Reduction can be carried-out in two steps. (1) Reduction step ensures complete "un-capping" of the engineered disulfides - some native disulfide bonds are also cleaved. (2) Oxidation step restores the native structure of IgG1. UF/DF clearance of the thiol liberated in the Reduction step (R-SH) is required prior to the oxidation to prevent "re-capping".

Fig. 3. "Un-capped" Cys mAb readily undergoes site-selective conjugation with alkylating agents (e.g. peptide bromoacetamide derivatives).

Fig. 4. Head-to-head comparison of the reduction performance of a matched pair (CA + TCEP) and mismatched pair (MES + TCEP).

Fig. 5. Formation of "Un-capped" Cys mAb over time. Reaction Conditions: 10 g/L of Cysteamine-capped Cys mAb in the specified buffer at pH 5.0, 3.5 equiv. of phosphine (TPPTS, TPPDS, or TCEP), RT. Reaction mixture monitored by Cation Exchange Chromatography and quantified at 280 nm. Only "Un-capped" Cys mAb plotted in this figure.

Fig. 6. Cysteamine-based conjugation process allows nearly stoichiometric amounts of reagents for the reduction, oxidation, and alkylation to afford mAb-peptide conjugate with uniform PAR2 content (e.g. $\geq 95\%$).

DETAILED DESCRIPTION OF THE INVENTION

[0019] The present disclosure provides a method of capping, reducing, and oxidizing cys-mAbs in order to provide homogenous material for subsequent conjugation reactions. The present method demonstrates robust ways to manufacture conjugates of cysteine-engineered antibodies that offer high yield and consistent product quality.

[0020] "Free cysteines" have been found to be suitable attachment points for conjugations of various property modifying groups. A free cysteine herein refers to a cysteine residue that is not engaged in an ordinary di-sulfide bond between two cysteine's of one or two polypeptides. Usually a free cysteine will be a cysteine that has been introduced in a polypeptide sequence of interest by site-selective mutagenesis, but some proteins may alternatively include a cysteine in a suitable position. As described in the background, an added cysteine may be a suitable attachment point for a property modifying group to a protein. By introducing a cysteine residue a free cysteine is usually obtained as no partner for forming a di-sulfide bond is present in the protein.

[0021] In one aspect, the present disclosure provides a method for preparing an antibody conjugate or antibody fragment conjugate, the method comprising the steps of: a) obtaining a

composition comprising an antibody or antibody fragment; b) exposing the antibody or antibody fragment to a cysteine blocking agent, wherein the cysteine blocking agent is cysteamine and the cysteine blocking agent forms a stable mixed-disulfide with at least one cysteine residue of the antibody or antibody fragment; c) adding a reducing agent to the composition to form a reduction mix and allowing a reduction reaction to occur such that the reduction mix comprises a reduced antibody or reduced antibody fragment, wherein the reducing agent is triphenylphosphine-3, 3',3"-trisulfonate ("TPPTS"); d) adding an oxidizing agent to the reduction mix to form an oxidized mix and allowing an oxidizing reaction to occur such that the oxidizing mix comprises an oxidized antibody or oxidized antibody fragment; and e) adding an activated chemical moiety to the oxidized mix to form a conjugation mix and allowing a conjugation reaction to occur such that an antibody conjugate or antibody fragment conjugate is formed.

[0022] In one aspect, following step b) and before step c) cation exchange chromatography is performed to upgrade antibody homogeneity and to remove excess cysteine blocking agent. In one aspect, following step c) and before step d) a buffer exchange step is performed to remove the released caps (thiols) and to remove the reducing agent. In one embodiment, the buffer exchange step is ultrafiltration/diafiltration. In one aspect, following step e), a purification step is performed to remove any excess of the activated chemical moiety and upgrade purity of the antibody or antibody fragment conjugate. In one embodiment, the purification step includes hydrophobic interaction chromatography ("HIC"), ultrafiltration/diafiltration, or hydrophobic interaction chromatography ("HIC") followed by ultrafiltration/diafiltration.

[0023] In another aspect, the present disclosure provides a method for preparing an antibody conjugate or antibody fragment conjugate, the method comprising the steps of: a) obtaining a composition comprising a mixed disulfide comprising an antibody or antibody fragment with a capped free cysteine that comprises a cysteamine cap; b) adding a reducing agent to the composition to form a reduction mix and allowing a reduction reaction to occur such that the reduction mix comprises a reduced antibody or reduced antibody fragment, wherein the reducing agent is triphenylphosphine-3, 3',3 "-trisulfonate ("TPPTS"); c) adding an oxidizing agent to the reduction mix to form an oxidized mix and allowing an oxidizing reaction to occur such that the oxidizing mix comprises an oxidized antibody or oxidized antibody fragment; and d) adding an activated chemical moiety to the oxidized mix to form a conjugation mix and allowing a conjugation reaction to occur such that an antibody conjugate or antibody fragment conjugate is formed.

[0024] In one aspect, following step a) and before step b) cation exchange chromatography is performed to remove excess cysteine blocking agent. In one aspect, following step b) and before step c) a buffer exchange step is performed to remove caps and to remove excess of the reducing agent.. In one embodiment, the buffer exchange step is ultrafiltration/diafiltration. In one aspect, following step d), a purification step is performed to remove the activated chemical moiety. In one embodiment, the purification step includes hydrophobic interaction chromatography ("HIC"), ultrafiltration/diafiltration, or hydrophobic interaction chromatography ("HIC") followed by ultrafiltration/diafiltration.

[0025] In another aspect, the present disclosure provides a method for preparing an antibody conjugate or antibody fragment conjugate, the method comprising the steps of: a) obtaining a composition comprising a mixed disulfide comprising an antibody or antibody fragment with a capped free cysteine that comprises a cysteamine cap; b) adding a reducing agent to the composition to form a reduction mix and allowing a reduction reaction to occur such that the reduction mix comprises a reduced antibody or reduced antibody fragment, wherein the reducing agent is triphenylphosphine-3, 3',3"-trisulfonate ("TPPTS"); c) adding an oxidizing agent to the reduction mix to form an oxidized mix and allowing an oxidizing reaction to occur such that the oxidizing mix comprises an oxidized antibody or oxidized antibody fragment; and d) adding an activated chemical moiety to the oxidized mix to form a conjugation mix and allowing a conjugation reaction to occur such that an antibody conjugate or antibody fragment conjugate is formed.

[0026] In one aspect, following step a) and before step b) a buffer exchange step is performed to remove the reducing agent. In one aspect, following step c), a purification step is performed to remove the activated chemical moiety. In one embodiment, the buffer exchange step is ultrafiltration/diafiltration. In one embodiment, the purification step includes hydrophobic interaction chromatography ("HIC"), ultrafiltration/diafiltration, or hydrophobic interaction chromatography ("HIC") followed by ultrafiltration/diafiltration.

[0027] In one embodiment, the mixed disulfide is an antibody or antibody fragment with a capped free cysteine. In the method of the invention, the antibody or antibody fragment with a capped free cysteine comprises a cap, wherein the cap is cysteamine. In the method of the invention, the reducing agent is triphenylphosphine-3,3',3"-trisulfonate ("TPPTS"). In one embodiment, the ratio of reducing agent to antibody or antibody fragment is 2 to 4:1 (mole/mole)). In one embodiment, the oxidizing agent is dehydroascorbic acid ("DHAA")). In one embodiment, the ratio of oxidizing agent to antibody or antibody fragment is 3 to 6:1 (mole/mole)). In one embodiment, the activated chemical moiety is a peptide comprising a halogen, wherein the halogen is selected from the group consisting of Br, I, and Cl). In one embodiment, the ratio of activated chemical moiety to antibody or antibody fragment is 2 to 3:1 (mole/mole).

[0028] In one embodiment, the antibody or antibody fragment comprises a cysteine residue at a position selected from the group consisting of D70 of the antibody light chain relative to reference sequence SEQ ID NO: 7; E276 of the antibody heavy chain relative to reference sequence SEQ ID NO: 8; and T363 of the antibody heavy chain relative to reference sequence SEQ ID NO: 8.

[0029] A conjugation site being "amenable to conjugation" means that the side chain of the amino acid residue at the selected conjugation site will react with the additional functional moiety of interest (or with a linker covalently attached to the additional functional moiety), under the defined chemical conditions, resulting in covalent binding of the additional functional moiety (directly or via the linker) to the side chain as a major reaction product.

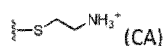
[0030] Disulfides are covalent bindings of two sulfur atoms which may be present in different (or the same) molecules. In proteins, cysteine residues may be linked via a disulfide bond also called a cystine.

[0031] In order to be an effective target of a conjugation reaction the free cysteine must be in the reduced form. A protein with a free cysteine, may for the same reason, be difficult to produce, and is thus frequently obtained as a mixed disulfide including a small organic moiety. Mixed disulfides are molecules including a di-sulfide, similar to the di-sulfide bond between two cysteine amino acid residues, each included in a polypeptides sequence (which may be the same or not). The small organic moiety is herein referred to as a Cap and the mixed disulfide is thus a protein-S-S-Cap molecule. In the present application the term "mixed di-sulfides" is used for molecules which comprise a disulfide bond linking two different entities which are not both polypeptides, although the molecules may additionally include "ordinary" disulfides bonds in addition to the mixed disulfide.

[0032] In one embodiment the method of the invention includes a step of reduction of a protein-S-S-Cap molecule as the protein subject to conjugation is obtained in the form of a composition of protein-S-S-Cap molecule.

[0033] As described above, the Cap is usually derived from a small organic moiety, including at least one sulfur atom that is part of the disulfide bond of the mixed di-sulfide. Such organic moieties can exist as monomers in the reduced form or as dimers in the oxidised form. In the mixed disulfide, -S-Cap is thus the oxidised form of the monomer or half a dimer. The -S-Cap is derived from cysteamine/cystamine (which is a decarboxylated cystine), and the mixed disulfide is thus Protein-S-S-cyst, where cyst refers to half of cystamine. The Cap of protein-S-S-Cap is derived from cysteamine.

[0034] The cap is



[0035] As described above the aim of the reduction is to obtain a molecule with a free reduced cysteine (-SH) that is reactive in a conjugation reaction.

[0036] In one embodiment the mixed disulfide is a protein-S-S-Cap molecule wherein the protein-S is derived from a protein comprising a free cysteine.

[0037] In order to obtain a protein with a reactive sulfur atom a reducing agent is added to the mixed disulfide composition, and the mixture is incubated to allow the reduction to occur to obtain a protein with a reactive sulfur atom e.g. a reduced protein of the format: protein-S- H.

The steps described are a) obtaining a composition of a mixed di-sulfide comprising the protein, b) adding a reducing agent to said protein composition, c) allowing reduction to occur and obtaining a solution comprising a reduced protein (P-SH).

[0038] In one embodiment the reducing agent is trisodium triphenylphosphine-3,3',3"-trisulfonate (TPPTS).

[0039] Once the mixed di-sulfide has been reduced a solution comprising a reduced protein (P-SH) has been obtained. Before the subsequent conjugation it may be beneficial to remove the reducing agent and/or the released Cap molecule. In one embodiment, an optional step of removing small molecules, such as molecules with a molecular weight below 10 kDa from the solution comprising the reduced protein (P-SH) may be included. In one embodiment molecules with a molecular weight below 10 kDa are removed from the solution comprising the reduced protein by diafiltration.

[0040] In a conjugation reaction a chemical moiety is covalently bonded to the sulfur atom of the free cysteine of the reduced protein (protein-SH). The chemical moiety may be any moiety suitable for conjugation to a protein, such as a property modifying moiety. The property modifying moiety may be a chemical moiety capable of altering one or more features of the protein of interest. In one embodiment the chemical moiety is a property-modifying group, such as a chemical moiety capable of stabilizing the protein, increasing the circulatory half-life or increasing potency. In one embodiment the chemical moiety is a protracting agent. In order for the conjugation to occur effectively, the chemical moiety may be used in an activated form. In the method according to the invention as described herein above, an activated chemical moiety is added to the solution comprising the reduced protein and the conjugation of the chemical moiety to the reduced protein results in preparation of a conjugated protein. Thus, the method according to the invention includes the further steps of: adding an activated chemical moiety to the solution comprising the reduced protein, allowing conjugation reaction to occur and obtaining a preparation of said conjugated protein.

[0041] The chemical moiety may be any moiety suitable for conjugation to a protein, such as a property modifying moiety. The property modifying moiety may be a chemical moiety capable of altering one or more features of the protein of interest. In one embodiment, the chemical moiety is a peptide and/or ligand with or without a linker sequence. In one embodiment the chemical moiety is a property-modifying group, such as a chemical moiety capable of stabilizing the protein, increasing the circulatory half-life or increasing potency. In one embodiment the chemical moiety is an albumin binder. In order for the conjugation to occur effectively, the chemical moiety may be used in an activated form. In the method according to the invention as described herein above, an activated chemical moiety is combined with the reduced protein and the conjugation of the chemical moiety to the reduced protein results in preparation of a conjugated protein via a sulfur atom.

[0042] The chemical moiety is preferably an activated chemical moiety, which means a moiety which is capable of reacting with the protein-SH forming a protein-S-chemical moiety molecule.

Such activated chemical moieties may include soft electrophilic alkylation reagents including a maleimide or haloacetyl groups, which are known in the art.

[0043] In one embodiment the activated chemical moiety is a halogenated chemical moiety, such as a halogenated peptide ligand. The halogenated chemical moiety may include Br, I or Cl.

[0044] In one embodiment the activated chemical moiety is a halogenated albumin binder (AB-halo).

[0045] In order to have an effective reduction of the mixed di-sulfide an excess of the reducing agent in molar concentrations is usually applied. By addition of the reducing agent to the composition of the mixed disulfide a reduction mix is obtained. The amount of reducing agent may be expressed in equivalents of the amount of the mixed di-sulfide, such that in the case where the amount of reducing agent is 1 equivalent of the amount of the mixed di-sulfide, the molar concentrations of the mixed di-sulfide and the reducing agent in the mixture are equal.

[0046] In one embodiment the amount of the reducing agent added from about 2 molar equivalents to about 4 molar equivalents of the molar amount of the mixed di-sulfide.

[0047] In order to reduce the amount of over-reduction of the antibody or antibody fragment, it is advantageous to reduce the amount required, which as described herein is possible if the process steps are optimized. An effective reduction reaction using lower amounts of the reducing agent requires that remaining reaction conditions are carefully selected as is provided by the present invention.

[0048] The reduction of the mixed di-sulfide may, depending on the conditions, take minutes or hours. The skilled person will know that different conditions will result in different efficacy and thus the time and conditions needed to obtain complete or almost complete reduction of the mixed-di-sulfide are described in more detailed in the Examples below.

[0049] The reducing agent may be added as concentrate or simply by adding the agent as a solid powder to the mixed di-sulfide composition. The reducing agent is mixed with the mixed di-sulfide composition to initiate reduction. The mix maybe termed the reduction mix.

[0050] In order to have a sufficiently effective process the reduction should result in at least an 80 % reduction, such as at least 90 % reduction of the total amount of mixed disulfide. In such cases the reduction is considered satisfactory when the amount of the mixed di-sulfide is at most 20%, such as at most 10 % of the amount of the mixed di-sulfide in the reduction mix. In preferred embodiments a reduction of around 95% of the mixed di-sulfide may be obtained leaving around 5 % non-reduced mixed di-sulfide in the solution comprising the reduced protein. In a further embodiment an efficient process leaves at most 2 % mixed disulfide within a suitable time.

[0051] The reduction may occur during a period of at least 15 minutes, such as at least 30 minutes or such as at least 1 hour. In one embodiment the reduction mix is left for 2-10 hours, such as 3-6 hours or around 3-4 hours after addition of the reducing agent.

[0052] In one embodiment the reduction is performed for up to 24 hours, such as for up to 12 hours, such as for up to 8 hours, such as for up to 6 hours such as for up to 4 hours.

[0053] The reduction may in one embodiment take place at 1 -50 °C, such as at room temperature, such as at 18-25 °C. In alternative embodiments the reduction may be performed at a colder temperature, such as below 10 °C, such as around 4-6 °C.

[0054] Before proceeding with the conjugation step the reduced protein may be separated from the reduction mix, such as from excess reducing agent and/or the small organic molecule of the mixed di-sulfide e.g. the H-S-Cap of the protein with a capped free cysteine. This optional step may be a step of removing molecules with a low molecular weight, such as molecules with a molecular weight below 10 kDa.

[0055] The skilled person will know of various methods for removing small molecular weight compounds, such as by filtration using a suitable membrane. In one embodiment the method comprises a step of buffer exchange (ultrafiltration/diafiltration).

[0056] The efficacy of a diafiltration step e.g. the amount of small molecules and excipients which are removed, is related to the filtrate volume generated, relative to the retentate volume. It is also noted that the word "remove" in this context should be read as "reducing the concentration of" as residual amounts of low molecular weight molecules and excipients will usually be present after a diafiltration step (or an alternative process steps) "removing" molecules with a low molecular weight.

[0057] Before proceeding with the conjugation step the reduced protein is oxidized in order to decrease the amount of over-reduced antibody or antibody fragment.

[0058] In order to have an effective oxidation of the over-reduced antibody or antibody fragment an excess of the oxidizing agent in molar concentrations is usually applied. By addition of the oxidizing agent to the composition of the over-reduced antibody or antibody fragment an oxidized mix is obtained. The amount of oxidizing agent may be expressed in equivalents of the amount of the over-reduced antibody or antibody fragment, such that in the case where the amount of oxidizing agent is 1 equivalent of the amount of the mixed di-sulfide, the molar concentrations of the mixed di-sulfide and the reducing agent in the mixture are equal.

[0059] In one embodiment the amount of the oxidizing agent added from about 3 molar equivalents to about 6 molar equivalents of the molar amount of the over-reduced antibody or antibody fragment.

[0060] The oxidation of the over-reduced antibody or antibody fragment may, depending on the conditions, take minutes or hours. The skilled person will know that different conditions will result in different efficacy and thus the time and conditions needed to obtain complete or almost complete oxidation of the over-reduced antibody or antibody fragment are described in more detailed in the Examples below.

[0061] The oxidizing agent may be added as concentrate or simply by adding the agent as a solid powder to the over-reduced antibody or antibody fragment composition. The oxidizing agent is mixed with the over-reduced antibody or antibody fragment composition to initiate oxidation. The mix may be termed the oxidation mix.

[0062] In order to have a sufficiently effective process the oxidation should result in at least an 80 % oxidation, such as at least 90 % oxidation of the total amount of over-reduced antibody or antibody fragment. In such cases the oxidation is considered satisfactory when the amount of the over-reduced antibody or antibody fragment is at most 20%, such as at most 10 % of the amount of the over-reduced antibody or antibody fragment in the oxidation mix. In preferred embodiments an oxidation of around 95% of the over-reduced antibody or antibody fragment may be obtained leaving around 5 % over-reduced antibody or antibody fragment in the solution comprising the oxidized protein. In a further embodiment an efficient process leaves at most 2 % over-reduced antibody or antibody fragment within a suitable time.

[0063] The oxidation may occur during a period of at least 15 minutes, such as at least 30 minutes or such as at least 1 hour. In one embodiment the oxidation mix is left for 2-10 hours, such as 3-6 hours or around 3-4 hours after addition of the oxidation agent.

[0064] In one embodiment the oxidation is performed for up to 24 hours, such as for up to 12 hours, such as for up to 8 hours, such as for up to 6 hours such as for up to 4 hours.

[0065] The oxidation may in one embodiment take place at 1 -50 °C, such as at room temperature, such as at 18-25 °C. In alternative embodiments the reduction may be performed at a colder temperature, such as below 10 °C, such as around 2-8 °C. In one embodiment the oxidizing agent is dehydroascorbic acid.

[0066] As described above the conjugation is according to the method performed by adding an activated chemical moiety to the solution comprising the reduced protein.

[0067] If the prior reduction is not complete the ratio of reduced protein to mixed di-sulfide may prevent a high yielding conjugation reaction. Furthermore the presence of excess reduction agent and released Cap molecules may interfere with the conjugation reaction.

[0068] Again the relative ratio of the reactants e.g. the reduced protein and the activated chemical moiety influences the effectiveness of the reaction.

[0069] In one embodiment the molar concentration of the activated chemical moiety is at least

equal or maybe twice the molar concentration of the protein to be conjugated. This may also be expressed in equivalents e.g. at least 10, such as 8, such as 6, such as 4, such as 2 or such as at least one (1) equivalent(s) of the activated chemical moiety relative to the protein to be conjugated may be used. As the activated chemical moiety may be a costly resource it is advantageous to reduce the amount required, which as described herein is possible if the previous steps are optimized. An effective conjugation reaction using reduced amounts of the activated chemical moiety requires that remaining reaction conditions are carefully selected as is provided by the present invention. In one embodiment the amount of activated chemical moiety is at most 8 equivalents of the protein, such as at most 6, such as at most 4, such as at most 3, such as at most 2.5, such as at most 2, such as at most 1.5 equivalents of the protein to be conjugated.

[0070] The conjugation of the oxidized protein with the chemical moiety may, depending on the conditions, take minutes or hours. The skilled person will know that different conditions will result in different efficacy and thus the time needed to obtain complete or almost complete conjugation will vary based on the conditions as will be described more detailed herein below. According to the present method the conjugation reaction is considered satisfactory when the amount of the starting material e.g. the reduced protein reached 10 %, such as 5 % or preferably 2 % or less.

[0071] The activated chemical moiety may be added as concentrate or simply by adding the agent as a solid powder to the solution comprising the oxidized protein. In one embodiment the activated chemical moiety is dissolved in a suitable solution prior to adding the activated chemical moiety to the solution comprising the oxidized protein. It may also be that the chemical moiety is activated in a solution prior to the conjugation reaction.

[0072] The term "half-life extending moiety" refers to a pharmaceutically acceptable moiety, domain, or "vehicle" covalently linked or conjugated to the Fc domain and/or a pharmaceutically active moiety, that prevents or mitigates in vivo proteolytic degradation or other activity-diminishing chemical modification of the pharmaceutically active moiety, increases half-life or other pharmacokinetic properties such as but not limited to increasing the rate of absorption, reduces toxicity, improves solubility, increases biological activity and/or target selectivity of the pharmaceutically active moiety with respect to a target of interest, increases manufacturability, and/or reduces immunogenicity of the pharmaceutically active moiety (e.g., a peptide or non-peptide moiety), compared to an unconjugated form of the pharmaceutically active moiety. Polyethylene glycol (PEG) is an example of a useful half-life extending moiety. Other examples of the half-life extending moiety, in accordance with the invention, include a copolymer of ethylene glycol, a copolymer of propylene glycol, a carboxymethylcellulose, a polyvinyl pyrrolidone, a poly-1,3-dioxolane, a poly-1,3,6-trioxane, an ethylene maleic anhydride copolymer, a polyaminoacid (e.g., polylysine), a dextran n-vinyl pyrrolidone, a poly n-vinyl pyrrolidone, a propylene glycol homopolymer, a propylene oxide polymer, an ethylene oxide polymer, a polyoxyethylated polyol, a polyvinyl alcohol, a linear or branched glycosylated chain, a polyacetal, a long chain fatty acid, a long chain hydrophobic aliphatic group, an immunoglobulin F_c domain (see, e.g., Feige et al., Modified peptides as

therapeutic agents, US Patent No. 6,660,843), an albumin (e.g., human serum albumin; see, e.g., Rosen et al., Albumin fusion proteins, US Patent No. 6,926,898 and US 2005/0054051; Bridon et al., Protection of endogenous therapeutic peptides from peptidase activity through conjugation to blood components, US 6,887,470), a transthyretin (TTR; see, e.g., Walker et al., Use of transthyretin peptide/protein fusions to increase the serum half-life of pharmacologically active peptides/proteins, US 2003/0195154 A1; 2003/0191056 A1), or a thyroxine-binding globulin (TBG).

[0073] Other embodiments of the useful half-life extending moiety, in accordance with the invention, include peptide ligands or small (non-peptide organic) molecule ligands that have binding affinity for a long half-life serum protein under physiological conditions of temperature, pH, and ionic strength. Examples include an albumin-binding peptide or small molecule ligand, a transthyretin-binding peptide or small molecule ligand, a thyroxine-binding globulin-binding peptide or small molecule ligand, an antibody-binding peptide or small molecule ligand, or another peptide or small molecule that has an affinity for a long half-life serum protein. (See, e.g., Blaney et al., Method and compositions for increasing the serum half-life of pharmacologically active agents by binding to transthyretin-selective ligands, US Patent No. 5,714,142; Sato et al., Serum albumin binding moieties, US 2003/0069395 A1; Jones et al., Pharmaceutical active conjugates, US Patent No. 6,342,225). A "long half-life serum protein" is one of the hundreds of different proteins dissolved in mammalian blood plasma, including so-called "carrier proteins" (such as albumin, transferrin and haptoglobin), fibrinogen and other blood coagulation factors, complement components, immunoglobulins, enzyme inhibitors, precursors of substances such as angiotensin and bradykinin and many other types of proteins. The invention encompasses the use of any single species of pharmaceutically acceptable half-life extending moiety, such as, but not limited to, those described herein, or the use of a combination of two or more different half-life extending moieties.

[0074] Recombinant polypeptide and nucleic acid methods used herein, including in the Examples, are generally those set forth in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 1989) or *Current Protocols in Molecular Biology* (Ausubel et al., eds., Green Publishers Inc. and Wiley and Sons 1994).

[0075] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

[0076] Unless otherwise defined herein, scientific and technical terms used in connection with the present application 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.

[0077] Generally, nomenclatures 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 application 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., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2001), Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates (1992), and Harlow and Lane *Antibodies: A Laboratory Manual* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1990). Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein. The terminology 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. Standard techniques can be used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

[0078] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used in connection with percentages may mean $\pm 1\%$.

[0079] Following convention, as used herein "a" and "an" mean "one or more" unless specifically indicated otherwise.

[0080] As used herein, the terms "amino acid" and "residue" are interchangeable and, when used in the context of a peptide or polypeptide, refer to both naturally occurring and synthetic amino acids, as well as amino acid analogs, amino acid mimetics and non-naturally occurring amino acids that are chemically similar to the naturally occurring amino acids.

[0081] A "naturally occurring amino acid" is an amino acid that is encoded by the genetic code, as well as those amino acids that are encoded by the genetic code that are modified after synthesis, e.g., hydroxyproline, γ -carboxyglutamate, and O-phosphoserine. An amino acid analog is a compound that has the same basic chemical structure as a naturally occurring amino acid, i.e., an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs can have modified R groups (e.g., norleucine) or modified peptide backbones, but will retain the same basic chemical structure as a naturally occurring amino acid.

[0082] An "amino acid mimetic" is a chemical compound that has a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid. Examples include a methacryloyl or acryloyl derivative of an amide, β -, γ -, δ -imino acids (such as piperidine-4-carboxylic acid) and the like.

[0083] A "non-naturally occurring amino acid" is a compound that has the same basic chemical structure as a naturally occurring amino acid, but is not incorporated into a growing polypeptide

chain by the translation complex. "Non-naturally occurring amino acid" also includes, but is not limited to, amino acids that occur by modification (e.g., posttranslational modifications) of a naturally encoded amino acid (including but not limited to, the 20 common amino acids) but are not themselves naturally incorporated into a growing polypeptide chain by the translation complex. A non-limiting lists of examples of non-naturally occurring amino acids that can be inserted into a polypeptide sequence or substituted for a wild-type residue in polypeptide sequence include β -amino acids, homoamino acids, cyclic amino acids and amino acids with derivatized side chains. Examples include (in the L-form or D-form; abbreviated as in parentheses): citrulline (Cit), homocitrulline (hCit), N α -methylcitrulline (NMeCit), N α -methylhomocitrulline (N α -MeHoCit), ornithine (Orn), N α -Methylornithine (N α -MeOrn or NMeOrn), sarcosine (Sar), homolysine (hLys or hK), homoarginine (hArg or hR), homoglutamine (hQ), N α -methylarginine (NMeR), N α -methyllleucine (N α -MeL or NMeL), N-methylhomolysine (NMeHoK), N α -methylglutamine (NMeQ), norleucine (Nle), norvaline (Nva), 1,2,3,4-tetrahydroisoquinoline (Tic), Octahydroindole-2-carboxylic acid (Oic), 3-(1-naphthyl)alanine (1-Nal), 3-(2-naphthyl)alanine (2-Nal), 1,2,3,4-tetrahydroisoquinoline (Tic), 2-indanylglycine (Igl), para-iodophenylalanine (pi-Phe), para-aminophenylalanine (4AmP or 4-Amino-Phe), 4-guanidino phenylalanine (Guf), glycyllsine (abbreviated "K(N ϵ -glycyl)" or "K(glycyl)" or "K(gly)"), nitrophenylalanine (nitrophe), aminophenylalanine (aminophe or Amino-Phe), benzylphenylalanine (benzylphe), γ -carboxyglutamic acid (γ -carboxyglu), hydroxyproline (hydroxypro), p-carboxyl-phenylalanine (Cpa), α -amino adipic acid (Aad), N α -methyl valine (NMeVal), N- α -methyl leucine (NMeLeu), N α -methylnorleucine (NMeNle), cyclopentylglycine (Cpg), cyclohexylglycine (Chg), acetylarginine (acetylarg), α , β -diaminopropionic acid (Dpr), α , γ -diaminobutyric acid (Dab), diaminopropionic acid (Dap), cyclohexylalanine (Cha), 4-methylphenylalanine (MePhe), β , β -diphenyl-alanine (BiPhA), aminobutyric acid (Abu), 4-phenyl-phenylalanine (or biphenylalanine; 4Bip), α -amino-isobutyric acid (Aib), beta-alanine, beta-aminopropionic acid, piperidinic acid, aminocaproic acid, aminoheptanoic acid, aminopimelic acid, desmosine, diaminopimelic acid, N-ethylglycine, N-ethylasparagine, hydroxylysine, allo-hydroxylysine, isodesmosine, allo-isoleucine, N-methylglycine, N-methylisoleucine, N-methylvaline, 4-hydroxyproline (Hyp), γ -carboxyglutamate, ϵ -N,N,N-trimethyllysine, ϵ -N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω -methylarginine, 4-Amino-O-Phthalic Acid (4APA), and other similar amino acids, and derivatized forms of any of those specifically listed.

[0084] The term "isolated nucleic acid molecule" refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end, or an analog thereof, that has been separated from at least about 50 percent of polypeptides, peptides, lipids, carbohydrates, polynucleotides or other materials with which the nucleic acid is naturally found when total nucleic acid is isolated from the source cells. Preferably, an isolated nucleic acid molecule is substantially free from any other contaminating nucleic acid molecules or other molecules that are found in the natural environment of the nucleic acid that would interfere with its use in polypeptide production or its therapeutic, diagnostic, prophylactic or research use.

[0085] The term "isolated polypeptide" refers to a polypeptide that has been separated from at

least about 50 percent of polypeptides, peptides, lipids, carbohydrates, polynucleotides, or other materials with which the polypeptide is naturally found when isolated from a source cell. Preferably, the isolated polypeptide is substantially free from any other contaminating polypeptides or other contaminants that are found in its natural environment that would interfere with its therapeutic, diagnostic, prophylactic or research use.

[0086] A composition of the present invention that includes a drug or peptide linked, attached, or bound, either directly or indirectly through a linker moiety, to another an antibody or antibody fragment is a "conjugate" or "conjugated" molecule.

[0087] The term "encoding" refers to a polynucleotide sequence encoding one or more amino acids. The term does not require a start or stop codon.

[0088] The terms "identical" and percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same. "Percent identity" means the percent of identical residues between the amino acids or nucleotides in the compared molecules and is calculated based on the size of the smallest of the molecules being compared. For these calculations, gaps in alignments (if any) can be addressed by a particular mathematical model or computer program (i.e., an "algorithm"). Methods that can be used to calculate the identity of the aligned nucleic acids or polypeptides include those described in Computational Molecular Biology, (Lesk, A. M., ed.), (1988) New York: Oxford University Press; Biocomputing Informatics and Genome Projects, (Smith, D. W., ed.), 1993, New York: Academic Press; Computer Analysis of Sequence Data, Part I, (Griffin, A. M., and Griffin, H. G., eds.), 1994, New Jersey: Humana Press; von Heinje, G., (1987) Sequence Analysis in Molecular Biology, New York: Academic Press; Sequence Analysis Primer, (Gribskov, M. and Devereux, J., eds.), 1991, New York: M. Stockton Press; and Carillo et al., (1988) SIAM J. Applied Math. 48:1073.

[0089] In calculating percent identity, the sequences being compared are aligned in a way that gives the largest match between the sequences. The computer program used to determine percent identity is the GCG program package, which includes GAP (Devereux et al., (1984) Nucl. Acid Res. 12:387; Genetics Computer Group, University of Wisconsin, Madison, WI). The computer algorithm GAP is used to align the two polypeptides or polynucleotides for which the percent sequence identity is to be determined. The sequences are aligned for optimal matching of their respective amino acid or nucleotide (the "matched span", as determined by the algorithm). A gap opening penalty (which is calculated as 3x the average diagonal, wherein the "average diagonal" is the average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually 1/10 times the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. In certain embodiments, a standard comparison matrix (see, Dayhoff et al., (1978) Atlas of Protein Sequence and Structure 5:345-352 for the PAM 250 comparison matrix; Henikoff et al., (1992) Proc. Natl. Acad. Sci. U.S.A. 89:10915-10919 for the BLOSUM 62 comparison matrix) is also used by the algorithm.

[0090] Recommended parameters for determining percent identity for polypeptides or nucleotide sequences using the GAP program are the following:

Algorithm: Needleman et al., 1970, J. Mol. Biol. 48:443-453;

Comparison matrix: BLOSUM 62 from Henikoff et al., 1992, *supra*;

Gap Penalty: 12 (but with no penalty for end gaps)

Gap Length Penalty: 4

Threshold of Similarity: 0

[0091] Certain alignment schemes for aligning two amino acid sequences can result in matching of only a short region of the two sequences, and this small aligned region can have very high sequence identity even though there is no significant relationship between the two full-length sequences. Accordingly, the selected alignment method (e.g., the GAP program) can be adjusted if so desired to result in an alignment that spans at least 50 contiguous amino acids of the target polypeptide.

[0092] An "antigen binding protein" as used herein means any protein that specifically binds a specified target antigen. The term encompasses intact antibodies that comprise at least two full-length heavy chains and two full-length light chains, as well as derivatives, variants, fragments, and mutations thereof. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments. An antigen binding protein also includes domain antibodies such as nanobodies and scFvs as described further below.

[0093] In general, an antigen binding protein is said to "specifically bind" its target antigen when the antigen binding protein exhibits essentially background binding to non-target molecules. An antigen binding protein that specifically binds to a target may, however, cross-react with target antigens from different species. Typically, an antigen binding protein specifically binds to target when the dissociation constant (KD) is $\leq 10^{-7}$ M as measured via a surface plasma resonance technique (e.g., BIAcore, GE-Healthcare Uppsala, Sweden) or Kinetic Exclusion Assay (KinExA, Sapidyne, Boise, Idaho). An antigen binding protein specifically binds target with "high affinity" when the KD is $\leq 5 \times 10^{-9}$ M, and with "very high affinity" when the KD is $\leq 5 \times 10^{-10}$ M, as measured using methods described.

[0094] "Antigen binding region" means a protein, or a portion of a protein, that specifically binds a specified antigen. For example, that portion of an antigen binding protein that contains the amino acid residues that interact with an antigen and confer on the antigen binding protein its specificity and affinity for the antigen is referred to as "antigen binding region." An antigen binding region typically includes one or more "complementary binding regions" ("CDRs") of an

immunoglobulin, single-chain immunoglobulin, or camelid antibody. Certain antigen binding regions also include one or more "framework" regions. A "CDR" is an amino acid sequence that contributes to antigen binding specificity and affinity. "Framework" regions can aid in maintaining the proper conformation of the CDRs to promote binding between the antigen binding region and an antigen.

[0095] A "recombinant protein" is a protein made using recombinant techniques, i.e., through the expression of a recombinant nucleic acid as described herein. Methods and techniques for the production of recombinant proteins are well known in the art.

[0096] The term "antibody" refers to an intact immunoglobulin of any isotype, or a fragment thereof that can compete with the intact antibody for specific binding to the target antigen, and includes, for instance, chimeric, humanized, fully human, and bispecific antibodies. An "antibody" as such is a species of an antigen binding protein. An intact antibody generally will comprise at least two full-length heavy chains and two full-length light chains. Antibodies may be derived solely from a single source, or may be "chimeric," that is, different portions of the antibody may be derived from two different antibodies as described further below. The antigen binding proteins, antibodies, or binding fragments may be produced in hybridomas, by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact antibodies.

[0097] The term "light chain" as used with respect to an antibody or fragments thereof includes a full-length light chain and fragments thereof having sufficient variable region sequence to confer binding specificity. A full-length light chain includes a variable region domain, VL, and a constant region domain, CL. The variable region domain of the light chain is at the amino-terminus of the polypeptide. Light chains include kappa chains and lambda chains.

[0098] The term "heavy chain" as used with respect to an antibody or fragment thereof includes a full-length heavy chain and fragments thereof having sufficient variable region sequence to confer binding specificity. A full-length heavy chain includes a variable region domain, VH, and three constant region domains, CH1, CH2, and CH3. The VH domain is at the amino-terminus of the polypeptide, and the CH domains are at the carboxyl-terminus, with the CH3 being closest to the carboxy-terminus of the polypeptide. Heavy chains may be of any isotype, including IgG (including IgG1, IgG2, IgG3 and IgG4 subtypes), IgA (including IgA1 and IgA2 subtypes), IgM and IgE.

[0099] The term "immunologically functional fragment" (or simply "fragment") of an antibody or immunoglobulin chain (heavy or light chain), as used herein, is an antigen binding protein comprising a portion (regardless of how that portion is obtained or synthesized) of an antibody that lacks at least some of the amino acids present in a full-length chain but which is capable of specifically binding to an antigen. Such fragments are biologically active in that they bind specifically to the target antigen and can compete with other antigen binding proteins, including intact antibodies, for specific binding to a given epitope.

[0100] These biologically active fragments may be produced by recombinant DNA techniques,

or may be produced by enzymatic or chemical cleavage of antigen binding proteins, including intact antibodies. Immunologically functional immunoglobulin fragments include, but are not limited to, Fab, Fab', and F(ab')₂ fragments.

[0101] In another embodiment, Fvs, domain antibodies and scFvs, and may be derived from an antibody of the present invention.

[0102] It is contemplated further that a functional portion of the antigen binding proteins disclosed herein, for example, one or more CDRs, could be covalently bound to a second protein or to a small molecule to create a therapeutic agent directed to a particular target in the body, possessing bifunctional therapeutic properties, or having a prolonged serum half-life.

[0103] A "Fab fragment" is comprised of one light chain and the CH1 and variable regions of one heavy chain. The heavy chain of a Fab molecule cannot form a disulfide bond with another heavy chain molecule.

[0104] An "Fc" region contains two heavy chain fragments comprising the CH2 and CH3 domains of an antibody. The two heavy chain fragments are held together by two or more disulfide bonds and by hydrophobic interactions of the CH3 domains.

[0105] An "Fab' fragment" contains one light chain and a portion of one heavy chain that contains the VH domain and the CH1 domain and also the region between the CH1 and CH2 domains, such that an interchain disulfide bond can be formed between the two heavy chains of two Fab' fragments to form an F(ab')₂ molecule.

[0106] An "F(ab')₂ fragment" contains two light chains and two heavy chains containing a portion of the constant region between the CH1 and CH2 domains, such that an interchain disulfide bond is formed between the two heavy chains. A F(ab')₂ fragment thus is composed of two Fab' fragments that are held together by a disulfide bond between the two heavy chains.

[0107] The "Fv region" comprises the variable regions from both the heavy and light chains, but lacks the constant regions.

[0108] "Single chain antibodies" or "scFvs" are Fv molecules in which the heavy and light chain variable regions have been connected by a flexible linker to form a single polypeptide chain, which forms an antigen-binding region. scFvs are discussed in detail in International Patent Application Publication No. WO 88/01649 and United States Patent Nos. 4,946,778 and No. 5,260,203.

[0109] A "domain antibody" or "single chain immunoglobulin" is an immunologically functional immunoglobulin fragment containing only the variable region of a heavy chain or the variable region of a light chain. Examples of domain antibodies include Nanobodies[®]. In some instances, two or more VH regions are covalently joined with a peptide linker to create a bivalent domain antibody. The two VH regions of a bivalent domain antibody may target the

same or different antigens.

[0110] A "bivalent antigen binding protein" or "bivalent antibody" comprises two antigen binding regions. In some instances, the two binding regions have the same antigen specificities. Bivalent antigen binding proteins and bivalent antibodies may be bispecific, see, *infra*.

[0111] A multispecific antigen binding protein" or "multispecific antibody" is one that targets more than one antigen or epitope.

[0112] A "bispecific," "dual-specific" or "bifunctional" antigen binding protein or antibody is a hybrid antigen binding protein or antibody, respectively, having two different antigen binding sites. Bispecific antigen binding proteins and antibodies are a species of multispecific antigen binding protein or multispecific antibody and may be produced by a variety of methods including, but not limited to, fusion of hybridomas or linking of Fab' fragments. See, e.g., Songsivilai and Lachmann, 1990, Clin. Exp. Immunol. 79:315-321; Kostelny et al., 1992, J. Immunol. 148:1547-1553. The two binding sites of a bispecific antigen binding protein or antibody will bind to two different epitopes, which may reside on the same or different protein targets.

[0113] The term "compete" when used in the context of antigen binding proteins (e.g., antibodies) means competition between antigen binding proteins is determined by an assay in which the antigen binding protein (e.g., antibody or immunologically functional fragment thereof) under test prevents or inhibits specific binding of a reference antigen binding protein to a common antigen. Numerous types of competitive binding assays can be used, for example: solid phase direct or indirect radioimmunoassay (RIA), solid phase direct or indirect enzyme immunoassay (EIA), sandwich competition assay (see, e.g., Stahli et al., 1983, Methods in Enzymology 9:242-253); solid phase direct biotin-avidin EIA (see, e.g., Kirkland et al., 1986, J. Immunol. 137:3614-3619) solid phase direct labeled assay, solid phase direct labeled sandwich assay (see, e.g., Harlow and Lane, 1988, Antibodies, A Laboratory Manual, Cold Spring Harbor Press); solid phase direct label RIA using I-125 label (see, e.g., Morel et al., 1988, Molec. Immunol. 25:7-15); solid phase direct biotin-avidin EIA (see, e.g., Cheung, et al., 1990, Virology 176:546-552); and direct labeled RIA (Moldenhauer et al., 1990, Scand. J. Immunol. 32:77-82). Typically, such an assay involves the use of purified antigen bound to a solid surface or cells bearing either of these, an unlabeled test antigen binding protein and a labeled reference antigen binding protein. Competitive inhibition is measured by determining the amount of label bound to the solid surface or cells in the presence of the test antigen binding protein. Usually the test antigen binding protein is present in excess. Additional details regarding methods for determining competitive binding are provided in the examples herein. Usually, when a competing antigen binding protein is present in excess, it will inhibit specific binding of a reference antigen binding protein to a common antigen by at least 40%, 45%, 50%, 55%, 60%, 65%, 70% or 75%. In some instances, binding is inhibited by at least 80%, 85%, 90%, 95%, or 97% or more.

[0114] The term "antigen" refers to a molecule or a portion of a molecule capable of being

bound by a selective binding agent, such as an antigen binding protein (including, e.g., an antibody), and additionally capable of being used in an animal to produce antibodies capable of binding to that antigen. An antigen may possess one or more epitopes that are capable of interacting with different antigen binding proteins, e.g., antibodies.

[0115] The term "epitope" is the portion of a molecule that is bound by an antigen binding protein (for example, an antibody). The term includes any determinant capable of specifically binding to an antigen binding protein, such as an antibody. An epitope can be contiguous or non-contiguous (discontinuous) (e.g., in a polypeptide, amino acid residues that are not contiguous to one another in the polypeptide sequence but that within in context of the molecule are bound by the antigen binding protein). A conformational epitope is an epitope that exists within the conformation of an active protein but is not present in a denatured protein. In certain embodiments, epitopes may be mimetic in that they comprise a three-dimensional structure that is similar to an epitope used to generate the antigen binding protein, yet comprise none or only some of the amino acid residues found in that epitope used to generate the antigen binding protein. Most often, epitopes reside on proteins, but in some instances may reside on other kinds of molecules, such as nucleic acids. Epitope determinants may include chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl or sulfonyl groups, and may have specific three-dimensional structural characteristics, and/or specific charge characteristics. Generally, antigen binding proteins specific for a particular target antigen will preferentially recognize an epitope on the target antigen in a complex mixture of proteins and/or macromolecules.

[0116] As used herein, "substantially pure" means that the described species of molecule is the predominant species present, that is, on a molar basis it is more abundant than any other individual species in the same mixture. In certain embodiments, a substantially pure molecule is a composition wherein the object species comprises at least 50% (on a molar basis) of all macromolecular species present. In other embodiments, a substantially pure composition will comprise at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of all macromolecular species present in the composition. In other embodiments, the object species is purified to essential homogeneity wherein contaminating species cannot be detected in the composition by conventional detection methods and thus the composition consists of a single detectable macromolecular species.

[0117] The term "polynucleotide" or "nucleic acid" includes both single-stranded and double-stranded nucleotide polymers. The nucleotides comprising the polynucleotide can be ribonucleotides or deoxyribonucleotides or a modified form of either type of nucleotide. The modifications include base modifications such as bromouridine and inosine derivatives, ribose modifications such as 2',3'-dideoxyribose, and internucleotide linkage modifications such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoraniladate and phosphoroamidate.

[0118] The term "oligonucleotide" means a polynucleotide comprising 200 or fewer nucleotides. In some embodiments, oligonucleotides are 10 to 60 bases in length. In other

embodiments, oligonucleotides are 12, 13, 14, 15, 16, 17, 18, 19, or 20 to 40 nucleotides in length. Oligonucleotides may be single stranded or double stranded, e.g., for use in the construction of a mutant gene. Oligonucleotides may be sense or antisense oligonucleotides. An oligonucleotide can include a label, including a radiolabel, a fluorescent label, a hapten or an antigenic label, for detection assays. Oligonucleotides may be used, for example, as PCR primers, cloning primers or hybridization probes.

[0119] An "isolated nucleic acid molecule" means a DNA or RNA of genomic, mRNA, cDNA, or synthetic origin or some combination thereof which is not associated with all or a portion of a polynucleotide in which the isolated polynucleotide is found in nature, or is linked to a polynucleotide to which it is not linked in nature. For purposes of this disclosure, it should be understood that "a nucleic acid molecule comprising" a particular nucleotide sequence does not encompass intact chromosomes. Isolated nucleic acid molecules "comprising" specified nucleic acid sequences may include, in addition to the specified sequences, coding sequences for up to ten or even up to twenty other proteins or portions thereof, or may include operably linked regulatory sequences that control expression of the coding region of the recited nucleic acid sequences, and/or may include vector sequences.

[0120] Unless specified otherwise, the left-hand end of any single-stranded polynucleotide sequence discussed herein is the 5' end; the left-hand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA transcript that are 5' to the 5' end of the RNA transcript are referred to as "upstream sequences;" sequence regions on the DNA strand having the same sequence as the RNA transcript that are 3' to the 3' end of the RNA transcript are referred to as "downstream sequences."

[0121] The term "control sequence" refers to a polynucleotide sequence that can affect the expression and processing of coding sequences to which it is ligated. The nature of such control sequences may depend upon the host organism. In particular embodiments, control sequences for prokaryotes may include a promoter, a ribosomal binding site, and a transcription termination sequence. For example, control sequences for eukaryotes may include promoters comprising one or a plurality of recognition sites for transcription factors, transcription enhancer sequences, and transcription termination sequences. "Control sequences" can include leader sequences and/or fusion partner sequences.

[0122] The term "vector" means any molecule or entity (e.g., nucleic acid, plasmid, bacteriophage or virus) used to transfer protein coding information into a host cell.

[0123] The term "expression vector" or "expression construct" refers to a vector that is suitable for transformation of a host cell and contains nucleic acid sequences that direct and/or control (in conjunction with the host cell) expression of one or more heterologous coding regions operatively linked thereto. An expression construct may include, but is not limited to, sequences that affect or control transcription, translation, and, if introns are present, affect

RNA splicing of a coding region operably linked thereto.

[0124] As used herein, "operably linked" means that the components to which the term is applied are in a relationship that allows them to carry out their inherent functions under suitable conditions. For example, a control sequence in a vector that is "operably linked" to a protein coding sequence is ligated thereto so that expression of the protein coding sequence is achieved under conditions compatible with the transcriptional activity of the control sequences.

[0125] The term "host cell" means a cell that has been transformed with a nucleic acid sequence and thereby expresses a gene of interest. The term includes the progeny of the parent cell, whether or not the progeny is identical in morphology or in genetic make-up to the original parent cell, so long as the gene of interest is present.

[0126] The terms "polypeptide" or "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms also apply to amino acid polymers in which one or more amino acid residues is an analog or mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The terms can also encompass amino acid polymers that have been modified, e.g., by the addition of carbohydrate residues to form glycoproteins, or phosphorylated. Polypeptides and proteins can be produced by a naturally-occurring and non-recombinant cell; or it is produced by a genetically-engineered or recombinant cell, and comprise molecules having the amino acid sequence of the native protein, or molecules having deletions from, additions to, and/or substitutions of one or more amino acids of the native sequence. The terms "polypeptide" and "protein" specifically encompass antigen binding proteins, antibodies, or sequences that have deletions from, additions to, and/or substitutions of one or more amino acids of an antigen-binding protein. The term "polypeptide fragment" refers to a polypeptide that has an amino-terminal deletion, a carboxyl-terminal deletion, and/or an internal deletion as compared with the full-length protein. Such fragments may also contain modified amino acids as compared with the full-length protein. In certain embodiments, fragments are about five to 500 amino acids long. For example, fragments may be at least 5, 6, 8, 10, 14, 20, 50, 70, 100, 110, 150, 200, 250, 300, 350, 400, or 450 amino acids long. Useful polypeptide fragments include immunologically functional fragments of antibodies, including binding domains.

[0127] The term "isolated protein" means that a subject protein (1) is free of at least some other proteins with which it would normally be found, (2) is essentially free of other proteins from the same source, e.g., from the same species, (3) is expressed by a cell from a different species, (4) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates, or other materials with which it is associated in nature, (5) is operably associated (by covalent or noncovalent interaction) with a polypeptide with which it is not associated in nature, or (6) does not occur in nature. Typically, an "isolated protein" constitutes at least about 5%, at least about 10%, at least about 25%, or at least about 50% of a given sample. Genomic DNA, cDNA, mRNA or other RNA, of synthetic origin, or any combination thereof may encode such an isolated protein. Preferably, the isolated protein is substantially free from proteins or polypeptides or other contaminants that are found in its natural

environment that would interfere with its therapeutic, diagnostic, prophylactic, research or other use.

[0128] A "variant" of a polypeptide (e.g., an antigen binding protein such as an antibody) comprises an amino acid sequence wherein one or more amino acid residues are inserted into, deleted from and/or substituted into the amino acid sequence relative to another polypeptide sequence. Variants include fusion proteins.

[0129] A "derivative" of a polypeptide is a polypeptide (e.g., an antigen binding protein such as an antibody) that has been chemically modified in some manner distinct from insertion, deletion, or substitution variants, e.g., via conjugation to another chemical moiety.

[0130] The term "naturally occurring" as used throughout the specification in connection with biological materials such as polypeptides, nucleic acids, host cells, and the like, refers to materials which are found in nature.

[0131] A "subject" or "patient" as used herein can be any mammal. In a typical embodiment, the subject or patient is a human.

[0132] A "conservative amino acid substitution" can involve a substitution of a native amino acid residue (i.e., a residue found in a given position of the wild-type polypeptide sequence) with a nonnative residue (i.e., a residue that is not found in a given position of the wild-type polypeptide sequence) such that there is little or no effect on the polarity or charge of the amino acid residue at that position. Conservative amino acid substitutions also encompass non-naturally occurring amino acid residues that are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include peptidomimetics, and other reversed or inverted forms of amino acid moieties.

[0133] Naturally occurring residues can be divided into classes based on common side chain properties:

1. (1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;
2. (2) neutral hydrophilic: Cys, Ser, Thr;
3. (3) acidic: Asp, Glu;
4. (4) basic: Asn, Gln, His, Lys, Arg;
5. (5) residues that influence chain orientation: Gly, Pro; and
6. (6) aromatic: Trp, Tyr, Phe.

[0134] Additional groups of amino acids can also be formulated using the principles described in, e.g., Creighton (1984) *PROTEINS: STRUCTURE AND MOLECULAR PROPERTIES* (2d Ed. 1993), W.H. Freeman and Company. In some instances it can be useful to further characterize substitutions based on two or more of such features (e.g., substitution with a "small polar" residue, such as a Thr residue, can represent a highly conservative substitution in an

appropriate context).

[0135] Conservative substitutions can involve the exchange of a member of one of these classes for another member of the same class. Non-conservative substitutions can involve the exchange of a member of one of these classes for a member from another class.

[0136] Synthetic, rare, or modified amino acid residues having known similar physiochemical properties to those of an above-described grouping can be used as a "conservative" substitute for a particular amino acid residue in a sequence. For example, a D-Arg residue may serve as a substitute for a typical L-Arg residue. It also can be the case that a particular substitution can be described in terms of two or more of the above described classes (e.g., a substitution with a small and hydrophobic residue means substituting one amino acid with a residue(s) that is found in both of the above-described classes or other synthetic, rare, or modified residues that are known in the art to have similar physiochemical properties to such residues meeting both definitions).

[0137] A "vector" refers to a delivery vehicle that (a) promotes the expression of a polypeptide-encoding nucleic acid sequence; (b) promotes the production of the polypeptide therefrom; (c) promotes the transfection/transformation of target cells therewith; (d) promotes the replication of the nucleic acid sequence; (e) promotes stability of the nucleic acid; (f) promotes detection of the nucleic acid and/or transformed/transfected cells; and/or (g) otherwise imparts advantageous biological and/or physiochemical function to the polypeptide-encoding nucleic acid. A vector can be any suitable vector, including chromosomal, non-chromosomal, and synthetic nucleic acid vectors (a nucleic acid sequence comprising a suitable set of expression control elements). Examples of such vectors include derivatives of SV40, bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors derived from combinations of plasmids and phage DNA, and viral nucleic acid (RNA or DNA) vectors.

[0138] A recombinant expression vector can be designed for expression of a protein in prokaryotic (e.g., *E. coli*) or eukaryotic cells (e.g., insect cells, using baculovirus expression vectors, yeast cells, or mammalian cells). In one embodiment the host cell is a mammalian, non-human host cell. Representative host cells include those hosts typically used for cloning and expression, including *Escherichia coli* strains TOP10F', TOP10, DH10B, DH5a, HB101, W3110, BL21(DE3) and BL21 (DE3)pLysS, BLUESCRIPT (Stratagene), mammalian cell lines CHO, CHO-K1, HEK293, 293-EBNA pIN vectors (Van Heeke & Schuster, *J. Biol. Chem.* 264: 5503-5509 (1989); pET vectors (Novagen, Madison Wis.). Alternatively, the recombinant expression vector can be transcribed and translated in vitro, for example using T7 promoter regulatory sequences and T7 polymerase and an in vitro translation system. Preferably, the vector contains a promoter upstream of the cloning site containing the nucleic acid sequence encoding the polypeptide. Examples of promoters, which can be switched on and off, include the lac promoter, the T7 promoter, the trc promoter, the tac promoter and the trp promoter.

[0139] In various embodiments, the vectors comprise an operably linked nucleotide sequence which regulates the expression of a target polypeptide. A vector can comprise or be associated

with any suitable promoter, enhancer, and other expression-facilitating elements. Examples of such elements include strong expression promoters (e.g., a human CMV IE promoter/enhancer, an RSV promoter, SV40 promoter, SL3-3 promoter, MMTV promoter, or HIV LTR promoter, EF1alpha promoter, CAG promoter), effective poly (A) termination sequences, an origin of replication for plasmid product in *E. coli*, an antibiotic resistance gene as a selectable marker, and/or a convenient cloning site (e.g., a polylinker). Vectors also can comprise an inducible promoter as opposed to a constitutive promoter such as CMV IE. In one aspect, a nucleic acid comprising a sequence encoding a target polypeptide which is operatively linked to a tissue specific promoter which promotes expression of the sequence in a metabolically-relevant tissue, such as liver or pancreatic tissue is provided.

[0140] In another aspect of the instant disclosure, host cells comprising the nucleic acids and vectors disclosed herein are provided. In various embodiments, the vector or nucleic acid is integrated into the host cell genome, which in other embodiments the vector or nucleic acid is extra-chromosomal.

[0141] Recombinant cells, such as yeast, bacterial (e.g., *E. coli*), and mammalian cells (e.g., immortalized mammalian cells) comprising such a nucleic acid, vector, or combinations of either or both thereof are provided. In various embodiments cells comprising a non-integrated nucleic acid, such as a plasmid, cosmid, phagemid, or linear expression element, which comprises a sequence coding for expression of a target polypeptide, are provided.

[0142] A vector comprising a nucleic acid sequence encoding a target polypeptide provided herein can be introduced into a host cell by transformation or by transfection. Methods of transforming a cell with an expression vector are well known.

[0143] A target-encoding nucleic acid can be positioned in and/or delivered to a host cell or host animal via a viral vector. Any suitable viral vector can be used in this capacity. A viral vector can comprise any number of viral polynucleotides, alone or in combination with one or more viral proteins, which facilitate delivery, replication, and/or expression of the nucleic acid of the invention in a desired host cell. The viral vector can be a polynucleotide comprising all or part of a viral genome, a viral protein/nucleic acid conjugate, a virus-like particle (VLP), or an intact virus particle comprising viral nucleic acids and a polypeptide-encoding nucleic acid. A viral particle viral vector can comprise a wild-type viral particle or a modified viral particle. The viral vector can be a vector which requires the presence of another vector or wild-type virus for replication and/or expression (e.g., a viral vector can be a helper-dependent virus), such as an adenoviral vector amplicon. Typically, such viral vectors consist of a wild-type viral particle, or a viral particle modified in its protein and/or nucleic acid content to increase transgene capacity or aid in transfection and/or expression of the nucleic acid (examples of such vectors include the herpes virus/AAV amplicons). Typically, a viral vector is similar to and/or derived from a virus that normally infects humans. Suitable viral vector particles in this respect, include, for example, adenoviral vector particles (including any virus of or derived from a virus of the adenoviridae), adeno-associated viral vector particles (AAV vector particles) or other parvoviruses and parvoviral vector particles, papillomaviral vector particles, flaviviral vectors,

alphaviral vectors, herpes viral vectors, pox virus vectors, retroviral vectors, including lentiviral vectors.

[0144] A target polypeptide expressed as described herein can be isolated using standard protein purification methods. A target polypeptide can be isolated from a cell in which it naturally expressed or it can be isolated from a cell that has been engineered to express target polypeptide, for example a cell that does not naturally express target polypeptide.

[0145] Protein purification methods that can be employed to isolate a target polypeptide, as well as associated materials and reagents, are known in the art. Additional purification methods that may be useful for isolating a target polypeptide can be found in references such as Bootcov MR, 1997, Proc. Natl. Acad. Sci. USA 94:11514-9, Fairlie WD, 2000, Gene 254: 67-76.

[0146] The antigen binding proteins provided are polypeptides into which one or more complementary determining regions (CDRs), as described herein, are embedded and/or joined. In some antigen binding proteins, the CDRs are embedded into a "framework" region, which orients the CDR(s) such that the proper antigen binding properties of the CDR(s) are achieved. Certain antigen binding proteins described herein are antibodies or are derived from antibodies. In other antigen binding proteins, the CDR sequences are embedded in a different type of protein scaffold.

[0147] In general the antigen binding proteins that are provided typically comprise one or more CDRs as described herein (e.g., 1, 2, 3, 4, 5 or 6). In some instances, the antigen binding protein comprises (a) a polypeptide structure and (b) one or more CDRs that are inserted into and/or joined to the polypeptide structure. The polypeptide structure can take a variety of different forms. For example, it can be, or comprise, the framework of a naturally occurring antibody, or fragment or variant thereof, or may be completely synthetic in nature. Examples of various polypeptide structures are further described below.

[0148] In certain embodiments, the polypeptide structure of the antigen binding proteins is an antibody or is derived from an antibody. Accordingly, examples of certain antigen binding proteins that are provided include, but are not limited to, monoclonal antibodies, bispecific antibodies, minibodies, domain antibodies such as Nanobodies[®], synthetic antibodies (sometimes referred to herein as "antibody mimetics"), chimeric antibodies, humanized antibodies, human antibodies, antibody fusions, and portions or fragments of each, respectively. In some instances, the antigen binding protein is an immunological fragment of a complete antibody (e.g., a Fab, a Fab', a F(ab')₂). In other instances the antigen binding protein is a scFv that uses CDRs from an antibody of the present invention.

[0149] In another aspect, an antigen-binding protein is provided having a half-life of at least one day in vitro or in vivo (e.g., when administered to a human subject). In one embodiment, the antigen binding protein has a half-life of at least three days. In various other embodiments, the antigen binding protein has a half-life of 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, or 60 days or longer. In another embodiment, the antigen binding protein is derivatized or modified

such that it has a longer half-life as compared to the underivatized or unmodified antibody. In another embodiment, the antigen binding protein contains point mutations to increase serum half-life. Further details regarding such mutant and derivatized forms are provided below.

[0150] Some of the antigen binding proteins that are provided have the structure typically associated with naturally occurring antibodies. The structural units of these antibodies typically comprise one or more tetramers, each composed of two identical couplets of polypeptide chains, though some species of mammals also produce antibodies having only a single heavy chain. In a typical antibody, each pair or couplet includes one full-length "light" chain (in certain embodiments, about 25 kDa) and one full-length "heavy" chain (in certain embodiments, about 50-70 kDa). Each individual immunoglobulin chain is composed of several "immunoglobulin domains", each consisting of roughly 90 to 110 amino acids and expressing a characteristic folding pattern. These domains are the basic units of which antibody polypeptides are composed. The amino-terminal portion of each chain typically includes a variable domain that is responsible for antigen recognition. The carboxy-terminal portion is more conserved evolutionarily than the other end of the chain and is referred to as the "constant region" or "C region". Human light chains generally are classified as kappa and lambda light chains, and each of these contains one variable domain and one constant domain. Heavy chains are typically classified as mu, delta, gamma, alpha, or epsilon chains, and these define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. IgG has several subtypes, including, but not limited to, IgG1, IgG2, IgG3, and IgG4. IgM subtypes include IgM, and IgM2. IgA subtypes include IgA1 and IgA2. In humans, the IgA and IgD isotypes contain four heavy chains and four light chains; the IgG and IgE isotypes contain two heavy chains and two light chains; and the IgM isotype contains five heavy chains and five light chains. The heavy chain C region typically comprises one or more domains that may be responsible for effector function. The number of heavy chain constant region domains will depend on the isotype. IgG heavy chains, for example, each contain three C region domains known as CH1, CH2 and CH3. The antibodies that are provided can have any of these isotypes and subtypes. In certain embodiments, the antibody is of the IgG1, IgG2, or IgG4 subtype.

[0151] In full-length light and heavy chains, the variable and constant regions are joined by a "J" region of about twelve or more amino acids, with the heavy chain also including a "D" region of about ten more amino acids. See, e.g. *Fundamental Immunology*, 2nd ed., Ch. 7 (Paul, W., ed.) 1989, New York: Raven Press. The variable regions of each light/heavy chain pair typically form the antigen binding site.

[0152] For the antibodies provided herein, the variable regions of immunoglobulin chains generally exhibit the same overall structure, comprising relatively conserved framework regions (FR) joined by three hypervariable regions, more often called "complementarity determining regions" or CDRs. The CDRs from the two chains of each heavy chain/light chain pair mentioned above typically are aligned by the framework regions to form a structure that binds specifically with a specific epitope on the antigen. From N-terminal to C-terminal, naturally-occurring light and heavy chain variable regions both typically conform with the following order of these elements: FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. A numbering system has

been devised for assigning numbers to amino acids that occupy positions in each of these domains. This numbering system is defined in Kabat Sequences of Proteins of Immunological Interest (1987 and 1991, NIH, Bethesda, Md.), or Chothia & Lesk, 1987, J. Mol. Biol. 196:901-917; Chothia et al., 1989, Nature 342:878-883.

[0153] The present invention relates to a composition comprising an antigen binding protein having at least one internal conjugation site. The conjugation site must be amenable to conjugation of an additional functional moiety (e.g., a drug, ligand, or peptide) by a defined conjugation chemistry through the side chain of an amino acid residue at the conjugation site. Achieving highly selective, site-specific conjugation to the antigen binding protein, in accordance with the present invention, requires consideration of a diverse variety of design criteria. First, a preferred conjugation or coupling chemistry must be defined or predetermined. Functional moieties can be conjugated or coupled to the selected conjugation site of the antigen binding protein through an assortment of different conjugation chemistries known in the art. For example, a maleimide-activated conjugation partner targeting an accessible cysteine thiol on the antigen binding protein is one embodiment, but numerous conjugation or coupling chemistries targeting the side chains of either canonical or non-canonical, e.g., unnatural amino acids in the antigen binding protein sequence, can be employed in accordance with the present invention.

[0154] Chemistries for the chemoselective conjugation include: copper(I)-catalyzed azide-alkyne [3+2] dipolar cycloadditions, Staudinger ligation, other acyl transfers processes (S→N; X→N), oximations, hydrazone bonding formation and other suitable organic chemistry reactions such as cross-couplings using water-soluble palladium catalysts. (E.g., Bong et al., Chemoselective Pd(0)-catalyzed peptide coupling in water, Organic Letters 3(16):2509-11 (2001); Dibowski et al., Bioconjugation of peptides by palladium-catalyzed C-C cross-coupling in water, Angew. Chem. Int. Ed. 37(4):476-78 (1998); DeVasher et al., Aqueous-phase, palladium-catalyzed cross-coupling of aryl bromides under mild conditions, using water-soluble, sterically demanding alkylphosphines, J. Org. Chem. 69:7919-27 (2004); Shaugnessy et al., J. Org. Chem, 2003, 68, 6767-6774; Prescher, JA and Bertozzi CR, Chemistry in living system, Nature Chemical Biology 1(1); 13-21 (2005)).

[0155] As mentioned above, the conjugation (or covalent binding) to the antigen binding protein is through the side chain of an amino acid residue at the conjugation site, for example, but not limited to, a cysteinyl residue. The amino acid residue, for example, a cysteinyl residue, at the internal conjugation site that is selected can be one that occupies the same amino acid residue position in a native Fc domain sequence, or the amino acid residue can be engineered into the Fc domain sequence by substitution or insertion.

[0156] The selection of the placement of the conjugation site in the overall antigen binding protein is another important facet of selecting an internal conjugation site in accordance with the present invention. Any of the exposed amino acid residues on the antigen binding protein can be potentially useful conjugation sites and can be mutated to cysteine or some other reactive amino acid for site-selective coupling, if not already present at the selected

conjugation site of the antigen binding protein sequence. However, this approach does not take into account potential steric constraints that may perturb the activity of the conjugated partner or limit the reactivity of the engineered mutation.

[0157] In one embodiment, the antigen binding protein is an antibody or functional fragment thereof. In one embodiment, the antibody or functional fragment thereof comprises a cysteine or non-canonical amino acid amino acid substitution at one or more conjugation site(s) selected from the group consisting of D70 of the antibody light chain relative to reference sequence SEQ ID NO: 7; E276 of the antibody heavy chain relative to reference sequence SEQ ID NO: 8; and T363 of the antibody heavy chain relative to reference sequence SEQ ID NO: 8. For sake of clarity, "D70 of the antibody light chain relative to reference sequence SEQ ID NO: 7" is the same substitution site as AHo position D88 of the light chain of antibody 5G12.006 and Kabat position D70 of the light chain of antibody 5G12.006; "E276 of the antibody heavy chain relative to reference sequence SEQ ID NO: 8" is the same substitution site as AHo position E384 of the heavy chain of antibody 5G12.006 and Kabat position E285 of the heavy chain of antibody 5G12.006; and "T363 of the antibody heavy chain relative to reference sequence SEQ ID NO: 8" is the same substitution site as AHo position T487 of the heavy chain of antibody 5G12.006 and Kabat position T382 of the heavy chain of antibody 5G12.006.

[0158] 8" is the same substitution site as AHo position E384 of the heavy chain of antibody 5G12.006 and Kabat position E285 of the heavy chain of antibody 5G12.006; and "T363 of the antibody heavy chain relative to reference sequence SEQ ID NO: 8" is the same substitution site as AHo position T487 of the heavy chain of antibody 5G12.006 and Kabat position T382 of the heavy chain of antibody 5G12.006.

Table 1. Amino acid SEQ ID NOs.

Identifier	Construct	VL	VH	LC	HC	CDR L1	CDR L2	CDR L3	CDR H1	CDR H2	CDR H3
iPS:336067	5G12.006	3	4	7	8	12	13	14	18	19	20

Table 2. Nucleic acid SEQ ID NOs.

Identifier	Construct	VL	VH	LC	HC	CDR L1	CDR L2	CDR L3	CDR H1	CDR H2	CDR H3
iPS:336067	5G12.006	1	2	5	6	9	10	11	15	16	17

Table 3. Variable Light and Variable Heavy Regions: Nucleic Acid ("NA") and Amino Acid ("AA") Sequences

iPS	Ab	Type	VL	VH
#				

iPS:336067	5G12.006	NA	GACATCCAGCTGACCCAGTCTCC ATCCTCCCTGTCTGCATCTGTAGG AGACAGAGTCACTATCACTTGCC GGGCAAGTCAGACCATTAGCAGG TTTTTAAATTGGTATCAGCAGAA ACCTGGGAAAGCCCCTGAGCTCC TGATCTATGTTGCATCCAGTTTGC AAAGTGGGGTCCCATCAAGATTG AGTGGCAGTGGTTCTGGGACAGA TTTCACTCTCACCATCAGCAGTCT GCAACCTGAAGATTTTGCAACTT ACTACTGTCAACAGAGTTACAGT ACCTTGATCAGTTTGGCCAGGG GACCAAGCTGGAGATCACACGA	CAGGTGCAGTTGGTGGAGTCTGG GGGAGGCGTGGTCCAGCCTGGGA GGTCCCTGAGACTCTCCTGTACAG CGTCTGGATTACCTTCAGTAGCT ATGGCATACTGGGTCCGCCAG GCTCCAGGCAAGGGGCTGGAGTG GGTGGCAGTTATATGGTATGATG GAAGTAATAAGTTCCATGCAGAC TCCGTGAAGGGCCGATTACCCAT CTCCAGAGACAATTCCAAGAACA CGCTGTATCTGCAAATGAACAGC CTGAGAGCCGAGGACTCGGCTAT GTACTTCTGTGCGAGAGGAAAAG TGGCTGGTATGCCTGAAGCTTTTG AAATCTGGGGCCAAGGGACAAAG GTCACCGTCTCTTCA
			SEQ ID NO: 1	SEQ ID NO: 2
		AA	DIQLTQSPSSLSASVGDRTITCRAS QTISRFLNWWYQKPGKAPELLIYV ASSLQSGVPSRFSGSGSGTDFLTIS SLQPEDFATYYCQQSYSTLISFGQG TKLEITR	QVQLVESGGGVVQPGRSLRLSCTA SGFTFSSYGIHWVRQAPGKGLEWV AVIWDGSKNFHADS VKGRFTISR DNSKNTLYLQMNSLRAEDSAMFY CARGKVAGMPEAFEIWGQGTKVT VSS
			SEQ ID NO: 3	SEQ ID NO: 4

Table 4A. CDRL1, CDRL2, and CDRL3 Nucleic Acid ("NA") and Amino Acid ("AA") Sequences

iPS#	Ab	Type	CDRL1	CDRL2	CDRL3
iPS:336067	5G12.006	NA	CGGGCAAGTCAGAC CATTAGCAGGTTTT AAAT	GTTGCATCCAGTTG CAAAGT	CAACAGAGTTACAG TACCCTGATCAGT
			SEQ ID NO: 9	SEQ ID NO: 10	SEQ ID NO: 11
		AA	RASQTISRFLN	VASSLQS	QQSYSTLIS
			SEQ ID NO: 12	SEQ ID NO: 13	SEQ ID NO: 14

Table 4B. CDRH1, CDRH2, and CDRH3 Nucleotide and Amino Acid Sequences

iPS#	Ab	Type	CDRH1	CDRH2	CDRH3
		NA	AGCTATGGCATACA C	GTTATATGGTATGAT GGAAGTAATAAGTTC CATGCAGACTCCGTG	GGAAAAGTGGCTGG TATGCCTGAAGCTTT TGAAATC

iPS:336067	5G12.006	AA		AAGGGC	
			SEQ ID NO: 15	SEQ ID NO: 16	SEQ ID NO: 17
			SYGIH	VIWYDGSNKFHADSV	GKVAGMPEAFEI
			SEQ ID NO: 18	SEQ ID NO: 19	SEQ ID NO: 20

Table 5. Light and Heavy Chain Nucleic Acid ("NA") and Amino Acid ("AA") Sequences

iPS#	Ab	Type	LC	HC
iPS:336067	5G12.006	NA	GACATCCAGCTGACCCAGTCTCC ATCTCCCTGTCTGCATCTGTAG GAGACAGAGTCACTATCACTTGC CGGGCAAGTCAGACCATTAGCA GGTTTTAAATTGGTATCAGCAG AAACCTGGGAAAGCCCCTGAGCT CCTGATCTATGTTGCATCCAGTTT GCAAAGTGGGGTCCCATCAAGAT TCAGTGGCAGTGGTTCTGGGACA GATTTCACTCTCACCATCAGCAG TCTGCAACCTGAAGATTTTGCAA CTTACTACTGTCAACAGAGTTAC AGTACCCTGATCAGTTTTGGCCA GGGGACCAAGCTGGAGATCACA CGAACGGTGGCTGCACCATCTGT CTTTCATCTTCCCGCCATCTGATG AGCAGTTGAAATCTGGAAGTGCC TCTGTTGTGTGCCTGCTGAATAA CTTCTATCCCAGAGAGGCCAAAG TACAGTGGAAAGGTGGATAACGC CCTCCAATCGGGTAACTCCCAGG AGAGTGTCACAGAGCAGGACAG CAAGGACAGCACCTACAGCCTCA GCAGCACCTGACGCTGAGCAA AGCAGACTACGAGAAACACAAA GTCTACGCCTGCCAAGTACCCCA TCAGGGCCTGAGCTCGCCCGTCA CAAAGAGCTTCAACAGGGGAGA GTGT	CAGGTGCAGTTGGTGGAGTCTGG GGGAGGCGTGGTCCAGCCTGGG AGGTCCCTGAGACTCTCCTGTAC AGCGTCTGGATTACCTTCAGTA GCTATGGCATACTGGGTCCGC CAGGCTCCAGGCAAGGGGCTGG AGTGGGTGGCAGTTATATGGTAT GATGGAAGTAATAAGTTCCATGC AGACTCCGTGAAGGGCCGATTC ACCATCTCCAGAGACAATTCCAA GAACACGCTGTATCTGCAAATGA ACAGCCTGAGAGCCGAGGACTC GGCTATGTACTTCTGTGCGAGAG GAAAAGTGGCTGGTATGCCTGA AGCTTTTGAAATCTGGGGCCAAG GGACAAAGGTCACCGTCTCTTCA GCCTCCACCAAGGGCCCATCGGT CTTCCCCCTGGCACCCCTCCTCCA AGAGCACCTCTGGGGGCACAGC GGCCCTGGGCTGCCTGGTCAAGG ACTACTTCCCCGAACCGGTGACG GTGTCTGTGGAAGTCAAGGCGCCT GACCAGCGGCGTGCACACCTTCC CGGCTGTCCTACAGTCCTCAGGA CTCTACTCCCTCAGCAGCGTGGT GACCGTGCCCTCCAGCAGCTTGG GCACCCAGACCTACATCTGCAAC GTGAATCACAAGCCCAGCAACA CCAAGGTGGACAAGAAAGTTGA GCCCAAATCTTGTGACAAAATC ACACATGCCCACCGTGCCAGCA CCTGAACTCCTGGGGGGACCGTC

				AGTCTTCCTCTTCCCCCAAAC
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			CCAAGGACACCCTCATGATCTCC CGGACCCCTGAGGTCACATGCGT GGTGGTGGACGTGAGCCACGAA GACCCTGAGGTCAAGTTCAACTG GTACGTGGACGGCGTGGAGGTG CATAATGCCAAGACAAAGCCGT GTGAGGAGCAGTACGGCAGCAC GTACCGTTGTGTCAGCGTCCTCA CCGTCCTGCACCAGGACTGGCTG AATGGCAAGGAGTACAAGTGCA AGGTCTCCAACAAAGCCCTCCCA GCCCCATCGAGAAAACCATCTC CAAAGCCAAAGGGCAGCCCCGA GAACCACAGGTGTACACCCTGCC CCCATCCCGGGAGGAGATGACC AAGAACCAGGTCAGCCTGACCT GCCTGGTCAAAGGCTTCTATCCC AGCGACATCGCCGTGGAGTGGG AGAGCAATGGGCAGCCGGAGAA CAACTACAAGACCACGCCTCCCG TGCTGGACTCCGACGGCTCCTTC TTCCTCTATAGCAAGCTCACCGT GGACAAGAGCAGGTGGCAGCAG GGGAACGTCTTCTCATGCTCCGT GATGCATGAGGCTCTGCACAACC ACTACACGCAGAAGAGCCTCTCC CTGTCTCCGGGTAAA
			SEQ ID NO: 5
			SEQ ID NO: 6
	AA	DIQLTQSPSSLASVGDRVTTTCRA SQTISRFLNWWYQKPGKAPELLIY VASSLQSGVPSRFSGSGSGTDFLT ISSLQPEDFATYYCQQSYSTLISFG QGTKLEITRTVAAPSVFIFPPSDEQ LKSGTASVVCLLNNFYPREAKVQ WKVDNALQSGNSQESVTEQDSKD	QVQLVESGGGVVQPGRSLRLSCT ASGFTFSSYGIHWVRQAPGKGLE WVAWIWDGSKNFHADSVMKGRF TISRDNSKNTLYLQMNSLRAEDSA MYFCARGKVAGMPEAFIEWGQG TKVTVSSASTKGPSVFPLAPSSKST SGGTAALGCLVKDYFPEPVTWSW

		STYLSSTLTLSKADYEKHKVYAC EVTHQGLSSPVTKSFNRGEC	NSGALTSGVHTFPAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKP SNTKVDKKVEPKSCDKTHTCPPCP APELLGGPSVFLFPPKPKDTLMISR TPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPCEEQYGSTYR CVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQV
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			YTLPPSREEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTPP VLDSGDGSFFLYSKLTVDKSRWQQ GNVFSCSVMHEALHNHYTQKSLS LSPGK
		SEQ ID NO: 7	SEQ ID NO: 8

[0159] Complementarity determining regions (CDRs) and framework regions (FR) of a given antibody may be identified using the system described by Kabat et al. in Sequences of Proteins of Immunological Interest, 5th Ed., US Dept. of Health and Human Services, PHS, NIH, NIH Publication no. 91-3242, 1991. Certain antibodies that are disclosed herein comprise one or more amino acid sequences that are identical or have substantial sequence identity to the amino acid sequences of one or more of the CDRs presented in TABLES 4A and 4B. These CDRs use the system described by Kabat et al. as noted above.

[0160] The structure and properties of CDRs within a naturally occurring antibody has been described, *supra*. Briefly, in a traditional antibody, the CDRs are embedded within a framework in the heavy and light chain variable region where they constitute the regions responsible for antigen binding and recognition. A variable region comprises at least three heavy or light chain CDRs, *see, supra* (Kabat et al., 1991, Sequences of Proteins of Immunological Interest, Public Health Service N.I.H., Bethesda, MD; *see also* Chothia and Lesk, 1987, J. Mol. Biol. 196:901-917; Chothia et al., 1989, Nature 342: 877-883), within a framework region (designated framework regions 1-4, FR1, FR2, FR3, and FR4, by Kabat *et al.*, 1991, *supra*; *see also* Chothia and Lesk, 1987, *supra*). The CDRs provided herein, however, may not only be used to define the antigen binding domain of a traditional antibody structure, but may be embedded in a variety of other polypeptide structures, as described herein.

[0161] The antigen binding proteins that are provided include monoclonal antibodies. Monoclonal antibodies may be produced using any technique known in the art, e.g., by immortalizing spleen cells harvested from the transgenic animal after completion of the immunization schedule. The spleen cells can be immortalized using any technique known in the art, e.g., by fusing them with myeloma cells to produce hybridomas. Myeloma cells for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render them incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas). Examples of suitable cell lines for use in mouse fusions include Sp-20, P3-X63/Ag8, P3-X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XXO Bul; examples of cell lines used in rat fusions include R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210. Other cell lines useful for cell fusions are U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6.

[0162] In some instances, a hybridoma cell line is produced by immunizing an animal (e.g., a transgenic animal having human immunoglobulin sequences) with an immunogen; harvesting

spleen cells from the immunized animal; fusing the harvested spleen cells to a myeloma cell line, thereby generating hybridoma cells; establishing hybridoma cell lines from the hybridoma cells, and identifying a hybridoma cell line that produces an antibody that binds a target polypeptide. Such hybridoma cell lines, and monoclonal antibodies produced by them, are aspects of the present application.

[0163] Monoclonal antibodies secreted by a hybridoma cell line can be purified using any technique known in the art. Hybridomas or mAbs may be further screened to identify mAbs with particular properties.

[0164] Chimeric and humanized antibodies based upon the foregoing sequences are also provided. Monoclonal antibodies for use as therapeutic agents may be modified in various ways prior to use. One example is a chimeric antibody, which is an antibody composed of protein segments from different antibodies that are covalently joined to produce functional immunoglobulin light or heavy chains or immunologically functional portions thereof. Generally, a portion of the heavy chain and/or light chain is identical with or homologous to a corresponding sequence in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is/are identical with or homologous to a corresponding sequence in antibodies derived from another species or belonging to another antibody class or subclass. For methods relating to chimeric antibodies, see, for example, United States Patent No. 4,816,567; and Morrison et al., 1985, Proc. Natl. Acad. Sci. USA 81:6851-6855. CDR grafting is described, for example, in United States Patent No. 6,180,370, No. 5,693,762, No. 5,693,761, No. 5,585,089, and No. 5,530,101.

[0165] Generally, the goal of making a chimeric antibody is to create a chimera in which the number of amino acids from the intended patient species is maximized. One example is the "CDR-grafted" antibody, in which the antibody comprises one or more complementarity determining regions (CDRs) from a particular species or belonging to a particular antibody class or subclass, while the remainder of the antibody chain(s) is/are identical with or homologous to a corresponding sequence in antibodies derived from another species or belonging to another antibody class or subclass. For use in humans, the variable region or selected CDRs from a rodent antibody often are grafted into a human antibody, replacing the naturally-occurring variable regions or CDRs of the human antibody.

[0166] One useful type of chimeric antibody is a "humanized" antibody. Generally, a humanized antibody is produced from a monoclonal antibody raised initially in a non-human animal. Certain amino acid residues in this monoclonal antibody, typically from non-antigen recognizing portions of the antibody, are modified to be homologous to corresponding residues in a human antibody of corresponding isotype. Humanization can be performed, for example, using various methods by substituting at least a portion of a rodent variable region for the corresponding regions of a human antibody (see, e.g., United States Patent No. 5,585,089, and No. 5,693,762; Jones et al., 1986, Nature 321:522-525; Riechmann et al., 1988, Nature 332:323-27; Verhoeyen et al., 1988, Science 239:1534-1536).

[0167] In one aspect, the CDRs of the light and heavy chain variable regions of the antibodies provided herein are grafted to framework regions (FRs) from antibodies from the same, or a different, phylogenetic species. For example, the CDRs of the heavy and light chain variable regions V_H1 , V_H2 , V_H3 , V_H4 , V_H5 , V_H6 , V_H7 , V_H8 , V_H9 , V_H10 , V_H11 , V_H12 and/or V_L1 , and V_L2 can be grafted to consensus human FRs. To create consensus human FRs, FRs from several human heavy chain or light chain amino acid sequences may be aligned to identify a consensus amino acid sequence. In other embodiments, the FRs of a heavy chain or light chain disclosed herein are replaced with the FRs from a different heavy chain or light chain. In one aspect, rare amino acids in the FRs of the heavy and light chains of antibodies are not replaced, while the rest of the FR amino acids are replaced. A "rare amino acid" is a specific amino acid that is in a position in which this particular amino acid is not usually found in an FR. Alternatively, the grafted variable regions from the one heavy or light chain may be used with a constant region that is different from the constant region of that particular heavy or light chain as disclosed herein. In other embodiments, the grafted variable regions are part of a single chain Fv antibody.

[0168] In certain embodiments, constant regions from species other than human can be used along with the human variable region(s) to produce hybrid antibodies.

[0169] Fully human antibodies are also provided. Methods are available for making fully human antibodies specific for a given antigen without exposing human beings to the antigen ("fully human antibodies"). One specific means provided for implementing the production of fully human antibodies is the "humanization" of the mouse humoral immune system. Introduction of human immunoglobulin (Ig) loci into mice in which the endogenous Ig genes have been inactivated is one means of producing fully human monoclonal antibodies (mAbs) in mouse, an animal that can be immunized with any desirable antigen. Using fully human antibodies can minimize the immunogenic and allergic responses that can sometimes be caused by administering mouse or mouse-derived mAbs to humans as therapeutic agents.

[0170] Fully human antibodies can be produced by immunizing transgenic animals (usually mice) that are capable of producing a repertoire of human antibodies in the absence of endogenous immunoglobulin production. Antigens for this purpose typically have six or more contiguous amino acids, and optionally are conjugated to a carrier, such as a hapten. See, e.g., Jakobovits et al., 1993, Proc. Natl. Acad. Sci. USA 90:2551-2555; Jakobovits et al., 1993, Nature 362:255-258; and Bruggermann et al., 1993, Year in Immunol. 7:33. In one example of such a method, transgenic animals are produced by incapacitating the endogenous mouse immunoglobulin loci encoding the mouse heavy and light immunoglobulin chains therein, and inserting into the mouse genome large fragments of human genome DNA containing loci that encode human heavy and light chain proteins. Partially modified animals, which have less than the full complement of human immunoglobulin loci, are then cross-bred to obtain an animal having all of the desired immune system modifications. When administered an immunogen, these transgenic animals produce antibodies that are immunospecific for the immunogen but have human rather than murine amino acid sequences, including the variable regions. For further details of such methods, see, for example, WO96/33735 and WO94/02602. Additional

methods relating to transgenic mice for making human antibodies are described in United States Patent No. 5,545,807; No. 6,713,610; No. 6,673,986; No. 6,162,963; No. 5,545,807; No. 6,300,129; No. 6,255,458; No. 5,877,397; No. 5,874,299 and No. 5,545,806; in PCT publications WO91/10741, WO90/04036, and in EP 546073B1 and EP 546073A1.

[0171] The transgenic mice described above, referred to herein as "HuMab" mice, contain a human immunoglobulin gene minilocus that encodes unrearranged human heavy ([mu] and [gamma]) and [kappa] light chain immunoglobulin sequences, together with targeted mutations that inactivate the endogenous [mu] and [kappa] chain loci (Lonberg et al., 1994, Nature 368:856-859). Accordingly, the mice exhibit reduced expression of mouse IgM or [kappa] and in response to immunization, and the introduced human heavy and light chain transgenes undergo class switching and somatic mutation to generate high affinity human IgG [kappa] monoclonal antibodies (Lonberg *et al.*, *supra.*; Lonberg and Huszar, 1995, Intern. Rev. Immunol. 13: 65-93; Harding and Lonberg, 1995, Ann. N.Y Acad. Sci. 764:536-546). The preparation of HuMab mice is described in detail in Taylor et al., 1992, Nucleic Acids Research 20:6287-6295; Chen et al., 1993, International Immunology 5:647-656; Tuaillon et al., 1994, J. Immunol. 152:2912-2920; Lonberg et al., 1994, Nature 368:856-859; Lonberg, 1994, Handbook of Exp. Pharmacology 113:49-101; Taylor et al., 1994, International Immunology 6:579-591; Lonberg and Huszar, 1995, Intern. Rev. Immunol. 13:65-93; Harding and Lonberg, 1995, Ann. N.Y Acad. Sci. 764:536-546; Fishwild et al., 1996, Nature Biotechnology 14:845-851. See, further United States Patent No. 5,545,806; No. 5,569,825; No. 5,625,126; No. 5,633,425; No. 5,789,650; No. 5,877,397; No. 5,661,016; No. 5,814,318; No. 5,874,299; and No. 5,770,429; as well as United States Patent No. 5,545,807; International Publication Nos. WO 93/1227; WO 92/22646; and WO 92/03918. Technologies utilized for producing human antibodies in these transgenic mice are disclosed also in WO 98/24893, and Mendez et al., 1997, Nature Genetics 15: 146-156. For example, the HCo7 and HCo12 transgenic mice strains can be used to generate human monoclonal antibodies against a target antigen. Further details regarding the production of human antibodies using transgenic mice are provided below.

[0172] Using hybridoma technology, antigen-specific human mAbs with the desired specificity can be produced and selected from the transgenic mice such as those described above. Such antibodies may be cloned and expressed using a suitable vector and host cell, or the antibodies can be harvested from cultured hybridoma cells.

[0173] Fully human antibodies can also be derived from phage-display libraries (as disclosed in Hoogenboom et al., 1991, J. Mol. Biol. 227:381; and Marks et al., 1991, J. Mol. Biol. 222:581). Phage display techniques mimic immune selection through the display of antibody repertoires on the surface of filamentous bacteriophage, and subsequent selection of phage by their binding to an antigen of choice. One such technique is described in PCT Publication No. WO 99/10494.

[0174] Derivatives of the antigen binding proteins that are described herein are also provided. The derivatized antigen binding proteins can comprise any molecule or substance that imparts

a desired property to the antibody or fragment, such as increased half-life in a particular use. The derivatized antigen binding protein can comprise, for example, a detectable (or labeling) moiety (e.g., a radioactive, colorimetric, antigenic or enzymatic molecule, a detectable bead (such as a magnetic or electrodense (e.g., gold) bead), or a molecule that binds to another molecule (e.g., biotin or streptavidin)), a therapeutic or diagnostic moiety (e.g., a radioactive, cytotoxic, or pharmaceutically active moiety), or a molecule that increases the suitability of the antigen binding protein for a particular use (e.g., administration to a subject, such as a human subject, or other *in vivo* or *in vitro* uses). Examples of molecules that can be used to derivatize an antigen binding protein include albumin (e.g., human serum albumin) and polyethylene glycol (PEG). Albumin-linked and PEGylated derivatives of antigen binding proteins can be prepared using techniques well known in the art. Certain antigen binding proteins include a pegylated single chain polypeptide as described herein. In one embodiment, the antigen binding protein is conjugated or otherwise linked to transthyretin (TTR) or a TTR variant. The TTR or TTR variant can be chemically modified with, for example, a chemical selected from the group consisting of dextran, poly(n-vinyl pyrrolidone), polyethylene glycols, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols and polyvinyl alcohols.

[0175] Other derivatives include covalent or aggregative conjugates of antigen binding proteins with other proteins or polypeptides, such as by expression of recombinant fusion proteins comprising heterologous polypeptides fused to the N-terminus or C-terminus of an antigen binding protein. For example, the conjugated peptide may be a heterologous signal (or leader) polypeptide, e.g., the yeast alpha-factor leader, or a peptide such as an epitope tag. Antigen binding protein-containing fusion proteins can comprise peptides added to facilitate purification or identification of the antigen binding protein (e.g., poly-His). An antigen binding protein also can be linked to the FLAG peptide as described in Hopp et al., 1988, *Bio/Technology* 6: 1204; and United States Patent No. 5,011,912. The FLAG peptide is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody (mAb), enabling rapid assay and facile purification of expressed recombinant protein. Reagents useful for preparing fusion proteins in which the FLAG peptide is fused to a given polypeptide are commercially available (Sigma, St. Louis, MO).

[0176] In some embodiments, the antigen binding protein comprises one or more labels. The term "labeling group" or "label" means any detectable label. Examples of suitable labeling groups include, but are not limited to, the following: radioisotopes or radionuclides (e.g., ^3H , ^{14}C , ^{15}N , ^{35}S , ^{90}Y , ^{99}Tc , ^{111}In , ^{125}I , ^{131}I), fluorescent groups (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic groups (e.g., horseradish peroxidase, β -galactosidase, luciferase, alkaline phosphatase), chemiluminescent groups, biotinyl groups, or predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, the labeling group is coupled to the antigen binding protein *via* spacer arms of various lengths to reduce potential steric hindrance. Various methods for labeling proteins are known in the art and may be used as is seen fit.

[0177] The term "effector group" means any group coupled to an antigen binding protein that acts as a cytotoxic agent. Examples for suitable effector groups are radioisotopes or radionuclides (e.g., ^3H , ^{14}C , ^{15}N , ^{35}S , ^{90}Y , ^{99}Tc , ^{111}In , ^{125}I , ^{131}I). Other suitable groups include toxins, therapeutic groups, or chemotherapeutic groups. Examples of suitable groups include calicheamicin, auristatins, geldanamycin and maytansine. In some embodiments, the effector group is coupled to the antigen binding protein *via* spacer arms of various lengths to reduce potential steric hindrance.

[0178] In general, labels fall into a variety of classes, depending on the assay in which they are to be detected: a) isotopic labels, which may be radioactive or heavy isotopes; b) magnetic labels (e.g., magnetic particles); c) redox active moieties; d) optical dyes; enzymatic groups (e.g. horseradish peroxidase, β -galactosidase, luciferase, alkaline phosphatase); e) biotinylated groups; and f) predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags, etc.). In some embodiments, the labeling group is coupled to the antigen binding protein *via* spacer arms of various lengths to reduce potential steric hindrance. Various methods for labeling proteins are known in the art.

[0179] Specific labels include optical dyes, including, but not limited to, chromophores, phosphors and fluorophores, with the latter being specific in many instances. Fluorophores can be either "small molecule" fluors, or proteinaceous fluors.

[0180] By "fluorescent label" is meant any molecule that may be detected *via* its inherent fluorescent properties. Suitable fluorescent labels include, but are not limited to, fluorescein, rhodamine, tetramethylrhodamine, eosin, erythrosin, coumarin, methyl-coumarins, pyrene, Malachite green, stilbene, Lucifer Yellow, Cascade BlueJ, Texas Red, IAEDANS, EDANS, BODIPY FL, LC Red 640, Cy 5, Cy 5.5, LC Red 705, Oregon green, the Alexa-Fluor dyes (Alexa Fluor 350, Alexa Fluor 430, Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 568, Alexa Fluor 594, Alexa Fluor 633, Alexa Fluor 660, Alexa Fluor 680), Cascade Blue, Cascade Yellow and R-phycoerythrin (PE) (Molecular Probes, Eugene, OR), FITC, Rhodamine, and Texas Red (Pierce, Rockford, IL), Cy5, Cy5.5, Cy7 (Amersham Life Science, Pittsburgh, PA). Suitable optical dyes, including fluorophores, are described in Molecular Probes Handbook by Richard P. Haugland.

[0181] Suitable proteinaceous fluorescent labels also include, but are not limited to, green fluorescent protein, including a Renilla, Ptilosarcus, or Aequorea species of GFP (Chalfie et al., 1994, Science 263:802-805), EGFP (Clontech Labs., Inc., Genbank Accession Number U55762), blue fluorescent protein (BFP, Quantum Biotechnologies, Inc., Quebec, Canada; Stauber, 1998, Biotechniques 24:462-471; Heim et al., 1996, Curr. Biol. 6: 178-182), enhanced yellow fluorescent protein (EYFP, Clontech Labs., Inc.), luciferase (Ichiki et al., 1993, J. Immunol. 150:5408-5417), β galactosidase (Nolan et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:2603-2607) and Renilla (WO92/15673, WO95/07463, WO98/14605, WO98/26277, WO99/49019, United States Patents No. 5292658, No. 5418155, No. 5683888, No. 5741668, No. 5777079, No. 5804387, No. 5874304, No. 5876995, No. 5925558).

[0182] Nucleic acids that encode for the antigen binding proteins described herein, or portions thereof, are also provided, including nucleic acids encoding one or both chains of an antibody, or a fragment, derivative, mutein, or variant thereof, polynucleotides encoding heavy chain variable regions or only CDRs, polynucleotides sufficient for use as hybridization probes, PCR primers or sequencing primers for identifying, analyzing, mutating or amplifying a polynucleotide encoding a polypeptide, anti-sense nucleic acids for inhibiting expression of a polynucleotide, and complementary sequences of the foregoing. The nucleic acids can be any length. They can be, for example, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450, 500, 750, 1,000, 1,500, 3,000, 5,000 or more nucleotides in length, and/or can comprise one or more additional sequences, for example, regulatory sequences, and/or be part of a larger nucleic acid, for example, a vector. The nucleic acids can be single-stranded or double-stranded and can comprise RNA and/or DNA nucleotides, and artificial variants thereof (e.g., peptide nucleic acids). Any variable region provided herein may be attached to these constant regions to form complete heavy and light chain sequences. However, it should be understood that these constant regions sequences are provided as specific examples only. In some embodiments, the variable region sequences are joined to other constant region sequences that are known in the art.

[0183] Nucleic acids encoding certain antigen binding proteins, or portions thereof (e.g., full length antibody, heavy or light chain, variable domain, or CDRH1, CDRH2, CDRH3, CDRL1, CDRL2, or CDRL3) may be isolated from B-cells of mice that have been immunized with antigen. The nucleic acid may be isolated by conventional procedures such as polymerase chain reaction (PCR). Phage display is another example of a known technique whereby derivatives of antibodies and other antigen binding proteins may be prepared. In one approach, polypeptides that are components of an antigen binding protein of interest are expressed in any suitable recombinant expression system, and the expressed polypeptides are allowed to assemble to form antigen binding proteins.

[0184] An aspect further provides nucleic acids that hybridize to other nucleic acids under particular hybridization conditions. Methods for hybridizing nucleic acids are well-known in the art. See, e.g., Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. As defined herein, a moderately stringent hybridization condition uses a prewashing solution containing 5x sodium chloride/sodium citrate (SSC), 0.5% SDS, 1.0 mM EDTA (pH 8.0), hybridization buffer of about 50% formamide, 6x SSC, and a hybridization temperature of 55°C (or other similar hybridization solutions, such as one containing about 50% formamide, with a hybridization temperature of 42°C), and washing conditions of 60°C, in 0.5x SSC, 0.1% SDS. A stringent hybridization condition hybridizes in 6x SSC at 45°C, followed by one or more washes in 0.1x SSC, 0.2% SDS at 68°C. Furthermore, one of skill in the art can manipulate the hybridization and/or washing conditions to increase or decrease the stringency of hybridization such that nucleic acids comprising nucleotide sequences that are at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99% identical to each other typically remain hybridized to each other.

[0185] The basic parameters affecting the choice of hybridization conditions and guidance for devising suitable conditions are set forth by, for example, Sambrook, Fritsch, and Maniatis (2001, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., *supra*; and *Current Protocols in Molecular Biology*, 1995, Ausubel et al., eds., John Wiley & Sons, Inc., sections 2.10 and 6.3-6.4), and can be readily determined by those having ordinary skill in the art based on, e.g., the length and/or base composition of the nucleic acid.

[0186] Changes can be introduced by mutation into a nucleic acid, thereby leading to changes in the amino acid sequence of a polypeptide (e.g., an antibody or antibody derivative) that it encodes. Mutations can be introduced using any technique known in the art. In one embodiment, one or more particular amino acid residues are changed using, for example, a site-directed mutagenesis protocol. In another embodiment, one or more randomly selected residues is changed using, for example, a random mutagenesis protocol. However it is made, a mutant polypeptide can be expressed and screened for a desired property.

[0187] Mutations can be introduced into a nucleic acid without significantly altering the biological activity of a polypeptide that it encodes. For example, one can make nucleotide substitutions leading to amino acid substitutions at non-essential amino acid residues. Alternatively, one or more mutations can be introduced into a nucleic acid that selectively changes the biological activity of a polypeptide that it encodes. For example, the mutation can quantitatively or qualitatively change the biological activity. Examples of quantitative changes include increasing, reducing or eliminating the activity. Examples of qualitative changes include changing the antigen specificity of an antibody. In one embodiment, a nucleic acid encoding any antigen binding protein described herein can be mutated to alter the amino acid sequence using molecular biology techniques that are well-established in the art.

[0188] Another aspect provides nucleic acid molecules that are suitable for use as primers or hybridization probes for the detection of nucleic acid sequences. A nucleic acid molecule can comprise only a portion of a nucleic acid sequence encoding a full-length polypeptide, for example, a fragment that can be used as a probe or primer or a fragment encoding an active portion of a polypeptide.

[0189] Probes based on the sequence of a nucleic acid can be used to detect the nucleic acid or similar nucleic acids, for example, transcripts encoding a polypeptide. The probe can comprise a label group, e.g., a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used to identify a cell that expresses the polypeptide.

[0190] Another aspect provides vectors comprising a nucleic acid encoding a polypeptide or a portion thereof (e.g., a fragment containing one or more CDRs or one or more variable region domains). Examples of vectors include, but are not limited to, plasmids, viral vectors, non-episomal mammalian vectors and expression vectors, for example, recombinant expression vectors. The recombinant expression vectors can comprise a nucleic acid in a form suitable for expression of the nucleic acid in a host cell. The recombinant expression vectors include one

or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operably linked to the nucleic acid sequence to be expressed. Regulatory sequences include those that direct constitutive expression of a nucleotide sequence in many types of host cells (e.g., SV40 early gene enhancer, Rous sarcoma virus promoter and cytomegalovirus promoter), those that direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences, see, Voss et al., 1986, Trends Biochem. Sci. 11:287, Maniatis et al., 1987, Science 236: 1237), and those that direct inducible expression of a nucleotide sequence in response to particular treatment or condition (e.g., the metallothionin promoter in mammalian cells and the tet-responsive and/or streptomycin responsive promoter in both prokaryotic and eukaryotic systems (see, *id.*). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein.

[0191] Another aspect provides host cells into which a recombinant expression vector has been introduced. A host cell can be any prokaryotic cell (for example, *E. coli*) or eukaryotic cell (for example, yeast, insect, or mammalian cells (e.g., CHO cells)). Vector DNA can be introduced into prokaryotic or eukaryotic cells *via* conventional transformation or transfection techniques. For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., for resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die), among other methods.

[0192] Expression systems and constructs in the form of plasmids, expression vectors, transcription or expression cassettes that comprise at least one polynucleotide as described above are also provided herein, as well host cells comprising such expression systems or constructs.

[0193] The antigen binding proteins provided herein may be prepared by any of a number of conventional techniques. For example, antigen binding proteins may be produced by recombinant expression systems, using any technique known in the art. See, e.g., Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Kennet et al. (eds.) Plenum Press, New York (1980); and Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1988).

[0194] Antigen binding proteins can be expressed in hybridoma cell lines (e.g., in particular antibodies may be expressed in hybridomas) or in cell lines other than hybridomas. Expression

constructs encoding the antibodies can be used to transform a mammalian, insect or microbial host cell. Transformation can be performed using any known method for introducing polynucleotides into a host cell, including, for example packaging the polynucleotide in a virus or bacteriophage and transducing a host cell with the construct by transfection procedures known in the art, as exemplified by United States Patent No. 4,399,216; No. 4,912,040; No. 4,740,461; No. 4,959,455. The optimal transformation procedure used will depend upon which type of host cell is being transformed. Methods for introduction of heterologous polynucleotides into mammalian cells are well known in the art and include, but are not limited to, dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, mixing nucleic acid with positively-charged lipids, and direct microinjection of the DNA into nuclei.

[0195] Recombinant expression constructs typically comprise a nucleic acid molecule encoding a polypeptide comprising one or more of the following: one or more CDRs provided herein; a light chain constant region; a light chain variable region; a heavy chain constant region (*e.g.*, C_H1, C_H2 and/or C_H3); and/or another scaffold portion of an antigen binding protein. These nucleic acid sequences are inserted into an appropriate expression vector using standard ligation techniques. In one embodiment, the heavy or light chain constant region is appended to the C-terminus of the heavy or light chain variable region and is ligated into an expression vector. The vector is typically selected to be functional in the particular host cell employed (*i.e.*, the vector is compatible with the host cell machinery, permitting amplification and/or expression of the gene can occur). In some embodiments, vectors are used that employ protein-fragment complementation assays using protein reporters, such as dihydrofolate reductase (see, for example, U.S. Pat. No. 6,270,964). Suitable expression vectors can be purchased, for example, from Invitrogen Life Technologies or BD Biosciences (formerly "Clontech"). Other useful vectors for cloning and expressing the antibodies and fragments include those described in Bianchi and McGrew, 2003, *Biotech. Biotechnol. Bioeng.* 84:439-44. Additional suitable expression vectors are discussed, for example, in *Methods Enzymol.*, vol. 185 (D. V. Goeddel, ed.), 1990, New York: Academic Press.

[0196] Typically, expression vectors used in any of the host cells will contain sequences for plasmid maintenance and for cloning and expression of exogenous nucleotide sequences. Such sequences, collectively referred to as "flanking sequences" in certain embodiments will typically include one or more of the following nucleotide sequences: a promoter, one or more enhancer sequences, an origin of replication, a transcriptional termination sequence, a complete intron sequence containing a donor and acceptor splice site, a sequence encoding a leader sequence for polypeptide secretion, a ribosome binding site, a polyadenylation sequence, a polylinker region for inserting the nucleic acid encoding the polypeptide to be expressed, and a selectable marker element. Each of these sequences is discussed below.

[0197] Optionally, the vector may contain a "tag"-encoding sequence, *i.e.*, an oligonucleotide molecule located at the 5' or 3' end of the antigen binding protein coding sequence; the oligonucleotide sequence encodes polyHis (such as hexaHis), or another "tag" such as FLAG[®], HA (hemagglutinin influenza virus), or myc, for which commercially available antibodies

exist. This tag is typically fused to the polypeptide upon expression of the polypeptide, and can serve as a means for affinity purification or detection of the antigen binding protein from the host cell. Affinity purification can be accomplished, for example, by column chromatography using antibodies against the tag as an affinity matrix. Optionally, the tag can subsequently be removed from the purified antigen binding protein by various means such as using certain peptidases for cleavage.

[0198] Flanking sequences may be homologous (*i.e.*, from the same species and/or strain as the host cell), heterologous (*i.e.*, from a species other than the host cell species or strain), hybrid (*i.e.*, a combination of flanking sequences from more than one source), synthetic or native. As such, the source of a flanking sequence may be any prokaryotic or eukaryotic organism, any vertebrate or invertebrate organism, or any plant, provided that the flanking sequence is functional in, and can be activated by, the host cell machinery.

[0199] Flanking sequences useful in the vectors may be obtained by any of several methods well known in the art. Typically, flanking sequences useful herein will have been previously identified by mapping and/or by restriction endonuclease digestion and can thus be isolated from the proper tissue source using the appropriate restriction endonucleases. In some cases, the full nucleotide sequence of a flanking sequence may be known. Here, the flanking sequence may be synthesized using the methods described herein for nucleic acid synthesis or cloning.

[0200] Whether all or only a portion of the flanking sequence is known, it may be obtained using polymerase chain reaction (PCR) and/or by screening a genomic library with a suitable probe such as an oligonucleotide and/or flanking sequence fragment from the same or another species. Where the flanking sequence is not known, a fragment of DNA containing a flanking sequence may be isolated from a larger piece of DNA that may contain, for example, a coding sequence or even another gene or genes. Isolation may be accomplished by restriction endonuclease digestion to produce the proper DNA fragment followed by isolation using agarose gel purification, Qiagen[®] column chromatography (Chatsworth, CA), or other methods known to the skilled artisan. The selection of suitable enzymes to accomplish this purpose will be readily apparent to one of ordinary skill in the art.

[0201] An origin of replication is typically a part of those prokaryotic expression vectors purchased commercially, and the origin aids in the amplification of the vector in a host cell. If the vector of choice does not contain an origin of replication site, one may be chemically synthesized based on a known sequence, and ligated into the vector. For example, the origin of replication from the plasmid pBR322 (New England Biolabs, Beverly, MA) is suitable for most gram-negative bacteria, and various viral origins (*e.g.*, SV40, polyoma, adenovirus, vesicular stomatitis virus (VSV), or papillomaviruses such as HPV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (for example, the SV40 origin is often used only because it also contains the virus early promoter).

[0202] A transcription termination sequence is typically located 3' to the end of a polypeptide coding region and serves to terminate transcription. Usually, a transcription termination sequence in prokaryotic cells is a G-C rich fragment followed by a poly-T sequence. While the sequence is easily cloned from a library or even purchased commercially as part of a vector, it can also be readily synthesized using methods for nucleic acid synthesis such as those described herein.

[0203] A selectable marker gene encodes a protein necessary for the survival and growth of a host cell grown in a selective culture medium. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, tetracycline, or kanamycin for prokaryotic host cells; (b) complement auxotrophic deficiencies of the cell; or (c) supply critical nutrients not available from complex or defined media. Specific selectable markers are the kanamycin resistance gene, the ampicillin resistance gene, and the tetracycline resistance gene. Advantageously, a neomycin resistance gene may also be used for selection in both prokaryotic and eukaryotic host cells.

[0204] Other selectable genes may be used to amplify the gene that will be expressed. Amplification is the process wherein genes that are required for production of a protein critical for growth or cell survival are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Examples of suitable selectable markers for mammalian cells include dihydrofolate reductase (DHFR) and promoterless thymidine kinase genes. Mammalian cell transformants are placed under selection pressure wherein only the transformants are uniquely adapted to survive by virtue of the selectable gene present in the vector. Selection pressure is imposed by culturing the transformed cells under conditions in which the concentration of selection agent in the medium is successively increased, thereby leading to the amplification of both the selectable gene and the DNA that encodes another gene, such as an antigen binding protein that binds target polypeptide. As a result, increased quantities of a polypeptide such as an antigen binding protein are synthesized from the amplified DNA.

[0205] A ribosome-binding site is usually necessary for translation initiation of mRNA and is characterized by a Shine-Dalgarno sequence (prokaryotes) or a Kozak sequence (eukaryotes). The element is typically located 3' to the promoter and 5' to the coding sequence of the polypeptide to be expressed.

[0206] In some cases, such as where glycosylation is desired in a eukaryotic host cell expression system, one may manipulate the various pre- or pro-sequences to improve glycosylation or yield. For example, one may alter the peptidase cleavage site of a particular signal peptide, or add prosequences, which also may affect glycosylation. The final protein product may have, in the -1 position (relative to the first amino acid of the mature protein), one or more additional amino acids incident to expression, which may not have been totally removed. For example, the final protein product may have one or two amino acid residues found in the peptidase cleavage site, attached to the amino-terminus. Alternatively, use of some enzyme cleavage sites may result in a slightly truncated form of the desired polypeptide, if the enzyme cuts at such area within the mature polypeptide.

[0207] Expression and cloning will typically contain a promoter that is recognized by the host organism and operably linked to the molecule encoding the antigen binding protein. Promoters are untranscribed sequences located upstream (*i.e.*, 5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control transcription of the structural gene. Promoters are conventionally grouped into one of two classes: inducible promoters and constitutive promoters. Inducible promoters initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, such as the presence or absence of a nutrient or a change in temperature. Constitutive promoters, on the other hand, uniformly transcribe a gene to which they are operably linked, that is, with little or no control over gene expression. A large number of promoters, recognized by a variety of potential host cells, are well known. A suitable promoter is operably linked to the DNA encoding heavy chain or light chain comprising an antigen binding protein by removing the promoter from the source DNA by restriction enzyme digestion and inserting the desired promoter sequence into the vector.

[0208] Suitable promoters for use with yeast hosts are also well known in the art. Yeast enhancers are advantageously used with yeast promoters. Suitable promoters for use with mammalian host cells are well known and include, but are not limited to, those obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, retroviruses, hepatitis-B virus, and Simian Virus 40 (SV40). Other suitable mammalian promoters include heterologous mammalian promoters, for example, heat-shock promoters and the actin promoter.

[0209] An enhancer sequence may be inserted into the vector to increase transcription of DNA encoding light chain or heavy chain comprising an antigen binding protein by higher eukaryotes. Enhancers are cis-acting elements of DNA, usually about 10-300 bp in length, that act on the promoter to increase transcription. Enhancers are relatively orientation and position independent, having been found at positions both 5' and 3' to the transcription unit. Several enhancer sequences available from mammalian genes are known (*e.g.*, globin, elastase, albumin, alpha-feto-protein and insulin). Typically, however, an enhancer from a virus is used. The SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer, and adenovirus enhancers known in the art are exemplary enhancing elements for the activation of eukaryotic promoters. While an enhancer may be positioned in the vector either 5' or 3' to a coding sequence, it is typically located at a site 5' from the promoter. A sequence encoding an appropriate native or heterologous signal sequence (leader sequence or signal peptide) can be incorporated into an expression vector, to promote extracellular secretion of the antibody. The choice of signal peptide or leader depends on the type of host cells in which the antibody is to be produced, and a heterologous signal sequence can replace the native signal sequence. Examples of signal peptides that are functional in mammalian host cells include the following: the signal sequence for interleukin-7 (IL-7) described in US Patent No. 4,965,195; the signal sequence for interleukin-2 receptor described in Cosman et al., 1984, Nature 312:768; the interleukin-4 receptor signal peptide described in EP Patent No. 0367 566; the type I interleukin-1 receptor signal peptide described in U.S. Patent No. 4,968,607; the type

II interleukin-1 receptor signal peptide described in EP Patent No. 0 460 846.

[0210] In one embodiment the leader sequence comprises SEQ ID NO: 21 (MDMRVPAQLL GLLLLWLRGA RC) which is encoded by SEQ ID NO: 22 (atggacatga gaggctgc acagctgctg ggcctgctgc tgctgtggct gagaggcgcc agatgc). In another embodiment the leader sequence comprises SEQ ID NO: 23 (MAWALLLT LTQGTGSWA) which is encoded by SEQ ID NO: 24 (atggcctggg ctctgctgct cctaccctc ctactcagg gcacagggtc ctgggcc).

[0211] The expression vectors that are provided may be constructed from a starting vector such as a commercially available vector. Such vectors may or may not contain all of the desired flanking sequences. Where one or more of the flanking sequences described herein are not already present in the vector, they may be individually obtained and ligated into the vector. Methods used for obtaining each of the flanking sequences are well known to one skilled in the art.

[0212] After the vector has been constructed and a nucleic acid molecule encoding light chain, a heavy chain, or a light chain and a heavy chain comprising an antigen binding sequence has been inserted into the proper site of the vector, the completed vector may be inserted into a suitable host cell for amplification and/or polypeptide expression. The transformation of an expression vector for an antigen-binding protein into a selected host cell may be accomplished by well-known methods including transfection, infection, calcium phosphate co-precipitation, electroporation, microinjection, lipofection, DEAE-dextran mediated transfection, or other known techniques. The method selected will in part be a function of the type of host cell to be used. These methods and other suitable methods are well known to the skilled artisan, and are set forth, for example, in Sambrook *et al.*, 2001, *supra*.

[0213] A host cell, when cultured under appropriate conditions, synthesizes an antigen binding protein that can subsequently be collected from the culture medium (if the host cell secretes it into the medium) or directly from the host cell producing it (if it is not secreted). The selection of an appropriate host cell will depend upon various factors, such as desired expression levels, polypeptide modifications that are desirable or necessary for activity (such as glycosylation or phosphorylation) and ease of folding into a biologically active molecule.

[0214] Mammalian cell lines available as hosts for expression are well known in the art and include, but are not limited to, immortalized cell lines available from the American Type Culture Collection (ATCC), including but not limited to Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), and a number of other cell lines. In another embodiment, a cell line from the B cell lineage that does not make its own antibody but has a capacity to make and secrete a heterologous antibody can be selected.

[0215] A "linker moiety" as used herein refers to a biologically acceptable peptidyl or non-peptidyl organic group that is covalently bound to an amino acid residue of a toxin peptide analog or other polypeptide chain (e.g., an immunoglobulin HC or LC or immunoglobulin Fc

domain) contained in the inventive composition, which linker moiety covalently joins or conjugates the toxin peptide analog or other polypeptide chain to another peptide or polypeptide chain in the composition, or to a half-life extending moiety. In some embodiments of the composition, a half-life extending moiety, as described herein, is conjugated, i.e., covalently bound directly to an amino acid residue of the toxin peptide analog itself, or optionally, to a peptidyl or non-peptidyl linker moiety (including but not limited to aromatic or aryl linkers) that is covalently bound to an amino acid residue of the toxin peptide analog. The presence of any linker moiety is optional. When present, its chemical structure is not critical, since it serves primarily as a spacer to position, join, connect, or optimize presentation or position of one functional moiety in relation to one or more other functional moieties of a molecule of the inventive composition. The presence of a linker moiety can be useful in optimizing pharmacological activity of some embodiments of the inventive composition. The linker, if present, can be made up of amino acids linked together by peptide bonds. The linker moiety, if present, can be independently the same or different from any other linker, or linkers, that may be present in the inventive composition. In some embodiments the linker can be a multivalent linker that facilitates multivalent display of toxin peptide analogs of the present invention; multivalent display of such biologically active compounds can increase binding affinity and/or potency through avidity. The *in vivo* properties of a therapeutic can be altered (i.e., specific targeting, half-life extension, distribution profile, etc.) through conjugation to a polymer or protein.

[0216] Peptidyl linkers. As stated above, the linker moiety, if present (whether within the primary amino acid sequence of the toxin peptide analog, or as a linker for attaching a half-life extending moiety to the toxin peptide analog), can be "peptidyl" in nature (i.e., made up of amino acids linked together by peptide bonds) and made up in length, preferably, of from 1 up to about 40 amino acid residues, more preferably, of from 1 up to about 20 amino acid residues, and most preferably of from 1 to about 10 amino acid residues. Preferably, but not necessarily, the amino acid residues in the linker are from among the twenty canonical amino acids, more preferably, cysteine, glycine, alanine, proline, asparagine, glutamine, and /or serine. Even more preferably, a peptidyl linker is made up of a majority of amino acids that are sterically unhindered, such as glycine, serine, and alanine linked by a peptide bond. It is also desirable that, if present, a peptidyl linker be selected that avoids rapid proteolytic turnover in circulation *in vivo*. Some of these amino acids may be glycosylated, as is well understood by those in the art. For example, a useful linker sequence constituting a sialylation site is $X_1X_2NX_4X_5G$ (SEQ ID NO: 25), wherein X_1 , X_2 , X_4 and X_5 are each independently any amino acid residue.

[0217] In other embodiments, the 1 to 40 amino acids of the peptidyl linker moiety are selected from glycine, alanine, proline, asparagine, glutamine, and lysine. Preferably, a linker is made up of a majority of amino acids that are sterically unhindered, such as glycine and alanine. Thus, preferred linkers include polyglycines, polyserines, and polyalanines, or combinations of any of these. Some exemplary peptidyl linkers are poly(Gly)₁₋₈, particularly (Gly)₃, (Gly)₄ (SEQ ID NO:26), (Gly)₅ (SEQ ID NO:27) and (Gly)₇ (SEQ ID NO:28), as well as, GlySer and poly(Gly)₄Ser, such as "L15" (GGGGSGGGSGGGGS; SEQ ID NO:29), poly(Gly-Ala)₂₋₄ and

poly(Ala)₁₋₈. Other specific examples of peptidyl linkers include (Gly)_sLys (SEQ ID NO:30), and (Gly)_sLysArg (SEQ ID NO:31). Other examples of useful peptidyl linkers are: Other examples of useful peptidyl linkers are:

(Gly)₃Lys(Gly)₄ (SEQ ID NO:32);

(Gly)₃AsnGlySer(Gly)₂ (SEQ ID NO:33);

(Gly)₃Cys(Gly)₄ (SEQ ID NO:34); and

GlyProAsnGlyGly (SEQ ID NO:35).

[0218] To explain the above nomenclature, for example, (Gly)₃Lys(Gly)₄ means Gly-Gly-Gly-Lys-Gly-Gly-Gly-Gly (SEQ ID NO:36). Other combinations of Gly and Ala are also useful.

[0219] Other preferred linkers are those identified herein as "L5" (GGGGS; or "G₄S"; SEQ ID NO:37), "L10" (GGGGSGGGGS; SEQ ID NO:38); "L20" (GGGGSGGGGSGGGGSGGGGS; SEQ ID NO:39); "L25" (GGGGSGGGGSGGGGSGGGGS; SEQ ID NO:40) and any linkers used in the working examples hereinafter.

[0220] In some embodiments of the compositions of this invention, which comprise a peptide linker moiety, acidic residues, for example, glutamate or aspartate residues, are placed in the amino acid sequence of the linker moiety. Examples include the following peptide linker sequences:

GGEGGG (SEQ ID NO:41);

GGEEEGGG (SEQ ID NO:42);

GEEEG (SEQ ID NO:43);

GEEE (SEQ ID NO:44);

GGDGGG (SEQ ID NO:45);

GGDDDGG (SEQ ID NO:46);

GDDDG (SEQ ID NO:47);

GDDD (SEQ ID NO:48);

GGGGSDDSDGSDGEDGGGGS (SEQ ID NO:49);

WEWEW (SEQ ID NO:50);

FEFEF (SEQ ID NO:51);

EEEWWW (SEQ ID NO:52);

EEEEFF (SEQ ID NO:53);

WWEEEEWW (SEQ ID NO:54); or

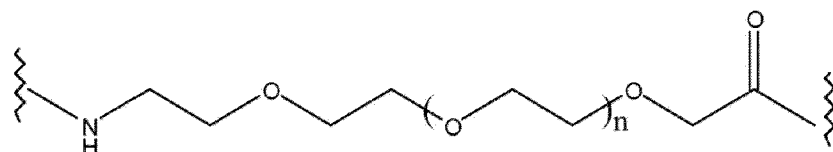
FFEEEEFF (SEQ ID NO:55).

[0221] In other embodiments, the linker constitutes a phosphorylation site, e.g., $X_1X_2YX_4X_5G$ (SEQ ID NO:56), wherein X_1 , X_2 , X_4 , and X_5 are each independently any amino acid residue; $X_1X_2SX_4X_5G$ (SEQ ID NO:57), wherein X_1 , X_2 , X_4 and X_5 are each independently any amino acid residue; or $X_1X_2TX_4X_5G$ (SEQ ID NO:58), wherein X_1 , X_2 , X_4 and X_5 are each independently any amino acid residue.

[0222] The linkers shown here are exemplary; peptidyl linkers within the scope of this invention may be much longer and may include other residues. A peptidyl linker can contain, e.g., a cysteine, another thiol, or nucleophile for conjugation with a half-life extending moiety. In another embodiment, the linker contains a cysteine or homocysteine residue, or other 2-amino-ethanethiol or 3-amino-propanethiol moiety for conjugation to maleimide, iodoacetaamide or thioester, functionalized half-life extending moiety.

[0223] Another useful peptidyl linker is a large, flexible linker comprising a random Gly/Ser/Thr sequence, for example: GSGSATGGSGSTASSGSGSATH (SEQ ID NO:59) or HGSGSATGGSGSTASSGSGSAT (SEQ ID NO:60), that is estimated to be about the size of a 1 kDa PEG molecule. Alternatively, a useful peptidyl linker may be comprised of amino acid sequences known in the art to form rigid helical structures (e.g., Rigid linker: -AEAAAKEAAAKEAAAKAGG-// SEQ ID NO:61). Additionally, a peptidyl linker can also comprise a non-peptidyl segment such as a 6 carbon aliphatic molecule of the formula $-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-$. The peptidyl linkers can be altered to form derivatives as described herein.

[0224] Non-peptidyl linkers. Optionally, a non-peptidyl linker moiety is also useful for conjugating the half-life extending moiety to the peptide portion of the half-life extending moiety-conjugated toxin peptide analog. For example, alkyl linkers such as $-NH-(CH_2)_s-C(O)-$, wherein $s = 2-20$ can be used. These alkyl linkers may further be substituted by any non-sterically hindering group such as lower alkyl (e.g., C_1-C_6) lower acyl, halogen (e.g., Cl, Br), CN, NH_2 , phenyl, etc. Exemplary non-peptidyl linkers are PEG linkers (e.g., shown below):



wherein n is such that the linker has a molecular weight of about 100 to about 5000 Daltons (Da), preferably about 100 to about 500 Da.

[0225] In one embodiment, the non-peptidyl linker is aryl. The linkers may be altered to form derivatives in the same manner as described herein. "Aryl" is phenyl or phenyl vicinally-fused with a saturated, partially-saturated, or unsaturated 3-, 4-, or 5 membered carbon bridge, the phenyl or bridge being substituted by 0, 1, 2 or 3 substituents selected from C₁₋₈ alkyl, C₁₋₄ haloalkyl or halo. "Heteroaryl" is an unsaturated 5, 6 or 7 membered monocyclic or partially-saturated or unsaturated 6-, 7-, 8-, 9-, 10- or 11 membered bicyclic ring, wherein at least one ring is unsaturated, the monocyclic and the bicyclic rings containing 1, 2, 3 or 4 atoms selected from N, O and S, wherein the ring is substituted by 0, 1, 2 or 3 substituents selected from C₁₋₈ alkyl, C₁₋₄ haloalkyl and halo.

[0226] Non-peptide portions of the inventive composition of matter, such as non-peptidyl linkers or non-peptide half-life extending moieties can be synthesized by conventional organic chemistry reactions.

[0227] Other embodiments of the multivalent linker comprise a rigid polyheterocyclic core of controlled length. The linkers are chemically differentiated on either end to accommodate orthogonal coupling chemistries (i.e. azide "Click", amide coupling, thioether formation by alkylation with maleimide or haloacetamide, oxime formation, reductive amination, etc.).

[0228] The above is merely illustrative and not an exhaustive treatment of the kinds of linkers that can optionally be employed in accordance with the present invention.

Example 1

Generation of anti-GIPR/GLP-1 peptide conjugates

[0229] Anti-GIPR antibody 2G10_LC1.003 was engineered to have a E70C mutation in SEQ ID NO: 151 (light chain) or to have an E275C mutation in SEQ ID NO: 152 (heavy chain). Bis-cysteamine-capped anti-GIPR Cys mAb (3-12 mg/mL IgG1 in 20 mM sodium acetate, pH 5.0) was partially reduced using 2-4 equivalents of triphenylphosphine-3,3',3''-trisulfonate at RT. Cation Exchange Chromatography (CEX) was used to monitor the reaction progression (typically complete in 1-2 hours). The liberated cysteamine was purged from the partially over-reduced IgG1 by buffer exchange into 20 mM sodium acetate, pH 5.0. The resulting partially over-reduced and cysteamine-free Cys mAb (3-12 mg/mL) was reoxidized by addition of 4-7 equiv. of 4 mM dehydroascorbic acid, 0.5 M aq. Na₂HPO₄ to pH 7.0-7.5 followed by incubation at 2-8 °C. The reoxidation progress was monitored by Reversed Phase HPLC. As soon as IgG1 was fully reformed (typically 1-3 hours) 2-3 equivalents of bromoacetyl-GLP-1 peptide (SEQ ID NO: 129) with C-terminal linker (SEQ ID NO: 29) was added and the reaction mixture was further incubated at 2-8 °C. The alkylation reaction progress was monitored by LC/MS and/or CEX until the target Peptide-to-Antibody Ratio (PAR) profile is obtained (e.g. ≥95%

PART, <5% PAR0+PAR1). The reaction mixture was quenched by adjusting the pH down to 5.0 using acetic acid. The desired PART anti-GIPR/GLP-1 conjugate was purified using Hydrophobic Interaction Chromatography (HIC) followed by UF/DF formulation into 10% sodium acetate, 9% sucrose, pH 5.2.

Table 6. Amino acid SEQ ID NOs.

Identifier	Construct	VL	VH	LC	HC	CDR L1	CDR L2	CDR L3	CDR H1	CDR H2	CDR H3
iPS:361172	2G10_LC1.003	147	148	151	152	156	157	158	162	163	164

Table 7. Nucleic acid SEQ ID NOs.

Identifier	Construct	VL	VH	LC	HC	CDR L1	CDR L2	CDR L3	CDR H1	CDR H2	CDR H3
iPS:361172	2G10_LC1.003	145	146	149	150	153	154	155	159	160	161

Table 8. Variable Light and Variable Heavy Regions: Nucleic Acid ("NA") and Amino Acid ("AA") Sequences

iPS #	Ab	Type	VL	VH
iPS:361172	2G10_LC1.003	NA	GAAATAGTGATGACGCAGTCTCC AGCCACCCTGTCTGTGTCTCCAG GGGAAAGAGCCACCCTCTCCTGC AGGGCCAGTCAGAGTGTTAGCAG CAACTTAGCCTGGTACCAGCAGA AACCTGGCCAGGCTCCCAGGCTC CTCATCTATGGTGCAGCCACCAG GGCCACTGGTATCCCAGCCAGGT TCAGTGGCAGTGGGTCTGGGACA GAGTTCACCTCTCACCATCAGCAG CCTGCAGTCTGAAGATTTTGCAG TTTATTACTGTCAGCAGTATAATA ACTGGCCTCTCACTTTTCGGCGGA GGGACCAAGGTGGAGATCAAAC GA	CAGGTGCAGCTGGTGGAGTCTGG GGGAGGCGTGGTCCAGCCTGGGA GGTCCCTGAGACTCTCCTGTGCA GCATCTGGATTACCTTCAGTAAC TATGGCATGCACTGGGTCCGCCA GGCTCCAGGCGAGGGGCTGGAGT GGGTGGCAGCTATATGGTTTGAT GCAAGTGATAAATACTATGCAGA CGCCGTGAAGGGCCGATTACCA TCTCCAGAGACAACTCCAAGAAC ACGCTGTATCTGCAAATGAACAG CCTGAGAGCCGAGGACACGGCTG TGTATTACTGTGCGAGAGATCAG GCGATTTTGGAGTGGTCCCCGA CTACTGGGGCCAGGGAACCCTGG

				TCACCGTCTCCTCA
			SEQ ID NO: 145	SEQ ID NO: 146
		AA	EIVMTQSPATLSVSPGERATLSCRA SQSVSSNLAWYQQKPGQAPRLLIY GAATRATGIPARFSGSGSGTEFTLT SSLQSEDFAVYYCQQYNNWPLTFG GGTKVEIKR	QVQLVESGGGVVQPGRSLRLSCAA SGFTFSNYGMHWVRQAPGEGLEW VAAIWFDASDKYYADAVKGRFTIS RDNSKNTLYLQMNSLRAEDTAVY YCARDQAIFGVVPDYWGQGTLVT VSS
			SEQ ID NO: 147	SEQ ID NO: 148

Table 9A. CDRL1, CDRL2, and CDRL3 Nucleic Acid ("NA") and Amino Acid ("AA") Sequences

iPS #	Ab	Type	CDRL1	CDRL2	CDRL3
iPS:361172	2G10_LC1.003	NA	AGGGCCAGTCAGAGT GTTAGCAGCAACTTA GCC	GGTGCAGCCACCA GGGCCACT	CAGCAGTATAATAAC TGGCCTCTCACT
			SEQ ID NO: 153	SEQ ID NO: 154	SEQ ID NO: 155
		AA	RASQSVSSNLA	GAATRAT	QQYNNWPLT
			SEQ ID NO: 156	SEQ ID NO: 157	SEQ ID NO: 158

Table 9B. CDRH1, CDRH2, and CDRH3 Nucleotide and Amino Acid Sequences

iPS#	Ab	Type	CDRH1	CDRH2	CDRH3
iPS:361172	2G10_LC1.003	NA	AACATATGGCATGCAC	GCTATATGGTTTG ATGCAAGTGATAA ATACTATGCAGAC GCCGTGAAGGGC	GATCAGGCGATTTTT GGAGTGGTCCCCGAC TAC
			SEQ ID NO: 159	SEQ ID NO: 160	SEQ ID NO: 161
		AA	NYGMH	AIWFDASDKYYAD AVKG	DQAIFGVVPDY
			SEQ ID NO: 162	SEQ ID NO: 163	SEQ ID NO: 164

Table 10. Light and Heavy Chain Nucleic Acid ("NA") and Amino Acid ("AA") Sequences

iPS #	Ab	Type	LC	HC
iPS:361172	2G10_LC1.003	NA	GAAATAGTGATGACGCAGTCTC CAGCCACCCTGTCTGTGTCTCC AGGGGAAAGAGCCACCCTCTCC TGCAGGGCCAGTCAGAGTGTTA GCAGCAACTTAGCCTGGTACCA GCAGAAACCTGGCCAGGCTCCC AGGCTCCTCATCTATGGTGCAG	CAGGTGCAGCTGGTGGAGTCTGGG GGAGGCGTGGTCCAGCCTGGGAG GTCCCTGAGACTCTCCTGTGCAGC ATCTGGATTACCTTCAGTAACTA TGGCATGCACTGGGTCCGCCAGGC TCCAGGCGAGGGGCTGGAGTGGG TGGCAGCTATATGGTTTGATGCAA

			CCACCAGGGCCACTGGTATCCC AGCCAGGTTCAGTGGCAGTGGG	GTGATAAATACTATGCAGACGCCG TGAAGGGCCGATTCACCATCTCCA
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			TCTGGGACAGAGTTCACTCTCA CCATCAGCAGCCTGCAGTCTGA AGATTTTGCAGTTTATTACTGTC AGCAGTATAATAACTGGCCTCT CACTTTCGGCGGAGGGACCAAG GTGGAGATCAAACGAACGGTG GCTGCACCATCTGTCTTCATCTT CCCGCCATCTGATGAGCAGTTG AAATCTGGAAGTGCCTCTGTTG TGTGCCTGCTGAATAACTTCTAT CCCAGAGAGGCCAAAGTACAGT GGAAGGTGGATAACGCCCTCCA ATCGGGTAACTCCCAGGAGAGT GTCACAGAGCAGGACAGCAAG GACAGCACCTACAGCCTCAGCA GCACCCTGACGCTGAGCAAAGC AGACTACGAGAAACACAAAGT CTACGCCTGCGAAGTCACCCAT CAGGGCCTGAGCTCGCCCGTCA CAAAGAGCTTCAACAGGGGAG AGTGT	GAGACAACTCCAAGAACACGCTG TATCTGCAAATGAACAGCCTGAGA GCCGAGGACACGGCTGTGTATTAC TGTGCGAGAGATCAGGCGATTTT GGAGTGGTCCCCGACTACTGGGGC CAGGGAACCCTGGTCACCGTCTCC TCAGCCTCCACCAAGGGCCCATCG GTCTTCCCCCTGGCACCTCCTCC AAGAGCACCTCTGGGGGCACAGC GGCCCTGGGCTGCCTGGTCAAGGA CTACTTCCCCGAACCGGTGACGGT GTCGTGGAAGTCAAGGCGCCCTGAC CAGCGGCGTGACACCTTCCCGGC TGTCTACAGTCCTCAGGACTCTA CTCCCTCAGCAGCGTGGTGACCGT GCCCTCCAGCAGCTTGGGCACCCA GACCTACATCTGCAACGTGAATCA CAAGCCCAGCAACACCAAGGTGG ACAAGAAAGTTGAGCCCAAATCTT GTGACAAAACACACATGCCCAC CGTGCCAGCACCTGAACTCCTGG GGGGACCGTCAGTCTTCTCTTCC CCCCAAAACCAAGGACACCCTCA TGATCTCCCGGACCCCTGAGGTCA CATGCGTGGTGGTGGACGTGAGCC ACGAAGACCCTGAGGTCAAGTTCA ACTGGTACGTGGACGGCGTGGAG GTGCATAATGCCAAGACAAAGCC GTGTGAGGAGCAGTACGGCAGCA CGTACCGTTGTGTCAGCGTCCTCA CCGTCCTGCACCAGGACTGGCTGA ATGGCAAGGAGTACAAGTGCAAG GTCTCCAACAAAGCCCTCCCAGCC CCCATCGAGAAAACCATCTCCAAA GCCAAAGGGCAGCCCCGAGAACC ACAGGTGTACACCCTGCCCCCATC
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			CCGGGAGGAGATGACCAAGAACC AGGTCAGCCTGACCTGCCTGGTCA AAGGCTTCTATCCCAGCGACATCG CCGTGGAGTGGGAGAGCAATGGG
--	--	--	--

		CAGCCGGAGAACAACACTACAAGAC CACGCCTCCCGTGCTGGACTCCGA CGGCTCCTTCTTCTCTATAGCAA GCTCACCGTGGACAAGAGCAGGT GGCAGCAGGGGAACGTCTTCTCAT GCTCCGTGATGCATGAGGCTCTGC ACAACCACTACACGCAGAAGAGC CTCTCCCTGTCTCCGGGTAAA
	SEQ ID NO: 149	SEQ ID NO: 150
AA	EIVMTQSPATLSVSPGERATLSCR ASQSVSSNLAWYQQKPGQAPRL LIYGAATRATGIPARFSGSGSGTE FTLTISLQSEDAVYYCQQYNN WPLTFGGGTKVEIKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFY PREAKVQWKVDNALQSGNSQES VTEQDSKDYSLSSLTSLTKAD YEKHKVYACEVTHQGLSSPVTKS FINRGEC	QVQLVESGGGVVQPGRSLRLSCAA SGFTFSNYGMHWVRQAPGEGLEW VAAIWFDASDKYYADAVKGRFTIS RDNSKNTLYLQMNSLRAEDTAVYY CARDQAIFGVVPDYWGQGLTVTS SASTKGPSVFPLAPSSKSTSGGTAAL GCLVKDYFPEPTVSWNSGALTSG VHTFPAVLQSSGLYSLSSVTVPSST LGTQTYICNVNHKPSNTKVDKKVEP KSCDKTHTCPPCPAPELLGGPSVFLF PPKPKDTLMISRTPEVTCVVVDVSH EDPEVKFNWYVDGVEVHNAKTKPC EEQYGSTYRCVSVLTVLHQDWLNG KEYKCKVSNKALPAPIEKTISKAKG QPREPQVYTLPPSREEMTKNQVSLT CLVKGFYPSDIAVEWESNGQPENNY KTTTPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQK SLSLSPGK
	SEQ ID NO: 151	SEQ ID NO: 152

[0230] A "GLP-1 receptor agonist" or "GLP-1 peptide" refers to compounds having GLP-1 receptor activity. Such exemplary compounds include exendins, exendin analogs, exendin agonists, GLP-1(7-37), GLP-1(7-37) analogs, GLP-1(7-37) agonists, and the like. The GLP-1 receptor agonist compounds may optionally be amidated. The terms "GLP-1 receptor agonist" and "GLP-1 receptor agonist compound" have the same meaning.

Table 1. Examples of GLP-1 receptor agonist Sequences

SEQ ID NO:	Sequence
62	HGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSSGAPPPS
63	HSDGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSSGAPPPS
64	HGEGTFTSDLSKQLEEEEAVRLFIEWLKNGGPSSGAPPPS

SEQ ID NO:	Sequence
65	HGEGTFTSDLSKQLEEEAVRLFIEFLKNGGPSSGAPPPS
66	HGEGTFTSDLSKQLEEEAARLFIEFLKNGGPSSGAPPPS
67	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGG
68	HGEGTFTSDLSKQLEEEAVRLFIEWLKNGG
69	HGEGTFTSDLSKQLEEEAVRLFIEFLKNGG
70	HGEGTFTSDLSKQLEEEAARLFIEFLKNGG
71	HGEGTFTSDLSKQMEEEAVRLFIEWLKN
72	HGEGTFTSDLSKQLEEEAVRLFIEWLKN
73	HGEGTFTSDLSKQLEEEAVRLFIEFLKN
74	HGEGTFTSDLSKQLEEEAARLFIEFLKN
75	HGEGTFTSDLSKQLEEKAAKEFIEFLKQGGPSSGAPPPS
76	HGEGTFTSDLSKQLEEKAAKEFIEWLKQGGPSSGAPPPS
77	HGEGTFTSDLSKQ(octylG)EEEAVRLFIEWLKQGGPSSGAPPPS
78	HGEGTFTSDLSKQLEEEAVRLFIEWLKQGGPSS(octylG)APPPS
79	HGEFTFTSDLSKQLEEEAVRLFIEWLKQGGPSKEIIS
80	HGEFTFTSDLSKQLEEKAAKEFIEWLKQGGPSSGAPPPS
81	HGEGTFTSDLVKILEAEAVRKFIIEFLKNGGPSSGAPPPS
82	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPSK
83	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG
84	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR
85	H(Aib)EGTFTSDVSSYLEGQAAREFIAFLVR(Aib)R
86	HXaa ₈ EGTFTSDVSSYLEXaa ₂₂ Xaa ₂₃ AAKEFI
	Xaa ₃₀ WLXaa ₃₃ Xaa ₃₄ GXaa ₃₆ Xaa ₃₇
	wherein Xaa ₈ is A, V, or G
	Xaa ₂₂ is G, K, or E
	Xaa ₂₃ is Q or K
	Xaa ₃₀ is A or E
	Xaa ₃₃ is V or K
	Xaa ₃₄ is K, N, or R
	Xaa ₃₆ is R or G
	and Xaa ₃₇ is G, H, P, or absent
87	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG
88	HAEGTFTSDVSSYLEGQAAKEFIEWLVKGRG

SEQ ID NO:	Sequence
89	HAEGTFTSDVSSYLEKQAAKEFIAWLVKGRG
90	HGEGTFTSDVSSYLEEQAAKEFIAWLVKGGG
91	HVEGTFTSDVSSYLEEQAAKEFIAWLVKGGG
92	HGEGTFTSDVSSYLEEQAAKEFIAWLKNGGG
93	HVEGTFTSDVSSYLEEQAAKEFIAWLKNGGG
94	HGEGTFTSDVSSYLEEQAAKEFIAWLVKGGP
95	HVEGTFTSDVSSYLEEQAAKEFIAWLVKGGP
96	HGEGTFTSDVSSYLEEQAAKEFIAWLKNGGP
97	HVEGTFTSDVSSYLEEQAAKEFIAWLKNGGP
98	HGEGTFTSDVSSYLEEQAAKEFIAWLVKGG
99	HVEGTFTSDVSSYLEEQAAKEFIAWLVKGG
100	HVEGTFTSDVSSYLEEQAAKEFIAWLVNGG
101	HGEGTFTSDVSSYLEEQAAKEFIAWLVNGG
102	HXaa ₈ EGTFTSDVS
	SYLEXaa ₂₂ QAAKEFIAWLXaa ₃₃ KGGPSSGAPPPC ₄₅ C ₄₆ -Z, wherein
	Xaa ₈ is: D-Ala, G, V, L, I, S or T
	Xaa ₂₂ is G, E, D or K
	Xaa ₃₃ is: V or I
	and Z is OH or NH ₂ and, optionally, wherein (i) one polyethylene glycol moiety is covalently attached to C ₄₅ , (ii) one polyethylene glycol moiety is covalently attached to C ₄₆ , or (iii) one polyethylene glycol moiety is attached to C ₄₅ and one polyethylene glycol moiety is attached to C ₄₆ .
103	HVEGTFTSDVSSYLEEQAAKEFIAWLKGGPSSGAPPPC ₄₅ C ₄₆ -NH ₂ and, optionally, wherein (i) one polyethylene glycol moiety is covalently attached to C ₄ , (ii) one polyethylene glycol moiety is covalently attached to C ₄₆ , or (iii) one polyethylene glycol moiety is attached to C ₄₅ and one polyethylene glycol moiety is attached to C ₄₆
104	HGEGTFTSDLSKQMEEEEAVKLFIEWLKNGGPSSGAPPPS
105	HGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPKSGAPPPS
106	GEGTFTSDLSKQMEEEEAVKLFIEWLKNGGPSSGAPPPS
107	HGEGTFTSDLSRQNorLeEEEEAVRLFIEWLRNGGPKSGAPPPS
108	HGEGTFTSDLSKQMEEEEAVKLFIEWLKNGGPSSGAPPPS
109	HGEGTFTSDLSKQMEEEEAVKLFIEWLKNGGPSSGAPPPS
110	HGEGTFTSDLSKQMEEEEAVKLFIEWLKNGGPSSGAPPPS

SEQ ID NO:	Sequence
111	HGEGTFTSDLSKQMEEEEAVKLFIEWLKNGGPSSGAPPPS
112	HGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSSGAPPPSK
113	HGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSSGAPPPSK
114	HGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSSGAPPPSK
115	HGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSSGAPPPSK
116	HGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSSGAPPPSK
117	HGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSSGAPPPSK
118	AEGTFTSDVSSYLEGQAAREFIAWLVKGRG
119	AEGTFTSDVSSYLEGQAAREFIAWLVKGRG
120	HAEGTFTSDVSSYLEGQAAREFIAWLVRGRGK
121	HAEGTFTSDVSSYLEGQAAREFIAWLVRGRGK
122	{H2}H[Aib]EGTFTSDVSSYLE[Aib]QAAKEFIAWLKNGG[Aeea][Aeea]K{CONH2}
123	{H2}H[Aib]EGTFTSDVSSYLE[Aib]QAAKEFIAWLVKGGG
124	{H2}H[Aib]EGTFTSDVSSYLEGEAAKKFIAWLVKGGG
125	{H2}H[Aib]EGTFTSDVSSYLEEQAAKEFIAWLVKGGK{CONH2}
126	{H2}H[Aib]EGTFTSDVSSYLE[Aib]EAVRLFIEWLKNGGPSSGAPPPS
127	{H2}H[Aib]EGTFTSDVSSYLEEQAAKEFIAWLVKGGG
128	{H2}H[Aib]EGTFTSDVSSYLEEQAAKEFIAWLVKGGG
129	{H2}H[Aib]EGTFTSDYSSYLEEQAAKEFIAWLVKGGG
130	{H2}H[Aib]EGTFTSDVSKYLEEEAVRLFIEWLKNGGG
131	{H2}H[Aib]EGTFTSDVSKYLEEEAAKLFIEWLKNGGG
132	{H2}H[Aib]EGTFTSDVSKYLEEEAAKLFIEWLKNGGG
133	{H2}H[Aib]IGTFTSDVSSYLE[Aib]QAAKEFIAWLVKGG
134	{H2}H[Aib]EGTFTSEVSSYLE[Aib]QAAKEFIAWLVKGG
135	{H2}H[Aib]AGTFTSDVSSYLE[Aib]QAAKEFIAWLVKGG
136	{H2}H[Aib]EGTFTSDVSSYLE[Aib]QAAKEFAAWLVKGG
137	{H2}H[Aib]EGTFTSDVSSYLE[Aib]QAAKEFIAALVKGG
138	{H2}H[Aib]TGTFTSDVSSYLE[Aib]QAAKEFIAWLVKGGG
139	{H2}H[Aib]EGTFTSEVSSYLE[Aib]QAAKEFIAALVKGGG
140	{H2}H[Aib]EGTFTSDVSSYLE[Aib]QAAKEFIAALVKGGG
141	{H2}H[Aib]EGTFTSDVSSYLEEEAVRLFIEWLKNGGPSSGAPPPS
142	{H2}H[Aib]EGTFTSDYSSYLE[Aib]QAAKEFIAWLVKGGG

SEQ ID NO:	Sequence
143	{H2}H[Aib]EGTFTSDVSSYLE[Aib]QAAKEFIAWLVKGGG{CONH2}
144	{Dmia}SQGTFTSDYSKYLDERRAKDFVQWLMNT
165	{H2}HGEGTFTSDLKQMEEEEAVRLFIEWLKNGGPSSGAPPPS
AEEA refers to [2-(2-amino)ethoxy]acetic acid EDA refers to ethylenediamine. MPA refers to maleimidopropionic acid.	

Results and Discussion

[0231] A successful site-specific conjugation with Cys mAb protein (IgG1) is critically reliant on the ability to selectively reduce ("un-cap") the cysteine residues that were engineered into the disulfidebridged IgG1 scaffold. This practically challenging process aims to reduce only the disulfide bonds of

the **two** engineered cysteines in the presence of **at least sixteen** native disulfide bonds holding together the IgG1 tetramer. A single step **Selective Reduction** would be highly desirable, but is currently not feasible (Fig. 1). A two-step **Net Selective Reduction** was developed instead (Fig. 2):

[0232] *Reduction Step:* Cys mAb allowed to react with a reducing agent (typically a phosphine) to effect complete reduction ("un-capping") of the engineered cysteine residues. Some native disulfide bonds are also reduced. The degree of this undesirable side-reaction is highly dependent on the reaction parameters including post-translational modifications (e.g. identity of "caps") and reaction conditions (e.g. temperature, type and amount a reducing agent, reaction buffer). The result is "un-capped" and over-reduced Cys mAb.

[0233] *Oxidation Step:* The thiol "caps" liberated in the Reduction Step must be cleared before proceeding to prevent the undesirable "re-capping" of the Cys mAb. The native disulfide bonds are then restored in the presence of an oxidant (typically dehydroascorbic acid).

[0234] This two-step protocol generates "Un-capped" Cys mAb protein ready for the site-specific conjugation via S-alkylation reaction (Fig. 3).

[0235] In order to achieve virtually homogenous Cys mAb conjugate (PART; Peptide-to-Antibody Ratio of 2) it is imperative to keep the over-reduction to minimum since restoration of the native disulfide bonds via oxidation is not perfect. On the other hand under-reduction is equally undesirable as it leads to under-alkylated impurities (PAR1, PAR0). The optimal reduction profile consisting of complete un-capping with minimal over-reduction requires proper combination of several parameters:

Positively charged cysteine "cap" (e.g. cysteamine, CA) must be paired with negatively charged

reducing agent. Triphenylphosphine-3,3',3''-trisulfonate (TPPTS) was found to be particularly effective. Other anionic phosphines e.g. TCEP, TPPDS were functional, but their performance was inferior to TPPTS (Fig.). Mismatched pairs e.g. mercaptoethanesulfonate (MES, negatively charged "cap") and tris(2-carboxyethyl)phosphine (TCEP, negatively charged reducing agent) led to sluggish, non-selective and/or incomplete reactions (Fig. 4).

[0236] Reaction buffers must be of low ionic strength (e.g. 20 mM sodium acetate) to maximize the attractive Coulombic interactions between oppositely charged reaction partners (e.g. positively charged cysteamine-capped IgG1 and negatively TPPTS). Increasing ionic strength of the reaction medium make the reduction sluggish and non-selective (Fig.).

[0237] Reaction pH must be sufficiently low (e.g. pH 5) to keep the liberated thiols ("caps") from undergoing secondary side-reactions e.g. disulfide exchange with the native disulfides of IgG1.

[0238] Proper combination of the above variables allows for complete un-capping using only a small excess of the reducing agent (e.g. 1.5 equiv. excess, 3.5 equiv. per Cys mAb total) consequently leading to minimal over-reduction. Such minimal degree of over-reduction is readily corrected in the following Oxidation Step using dehydroascorbic acid (DHAA, e.g. 4-6 equiv.) as a mild oxidant. The resulting virtually homogenous "un-capped" Cys-mAb can be cleanly alkylated using only a small excess (e.g. 0.2 equiv. excess, 2.2 equiv. per Cys mAb total) of bromoacetamides (e.g. bromoacetamide derivatives of synthetic peptides) to afford $\geq 95\%$ of PAR2 conjugate (Fig.).

REFERENCES CITED IN THE DESCRIPTION

Cited references

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Patent documents cited in the description

- [WO2016067013A1 \[0004\]](#)
- [WO2009012256A1 \[0004\]](#)
- [WO2005084390A2 \[0004\]](#)

- [WO2018136440A1 \[0004\]](#)
- [WO2017137628A1 \[0004\]](#)
- [WO2015086853A1 \[0004\]](#)
- [WO2017055582A1 \[0004\]](#)
- [US6660843B \[0072\]](#)
- [US6926898B \[0072\]](#)
- [US20050054051A \[0072\]](#)
- [US6887470B \[0072\]](#)
- [US20030195154A1 \[0072\]](#)
- [US20030191056A1 \[0072\]](#)
- [US5714142A \[0073\]](#)
- [US20030069395A1 \[0073\]](#)
- [US6342225B \[0073\]](#)
- [WO8801649A \[0108\]](#)
- [US4946778A \[0108\]](#)
- [US5260203A \[0108\]](#)
- [US4816567A \[0164\]](#)
- [US6180370B \[0164\]](#)
- [US5693762A \[0164\] \[0166\]](#)
- [US5693761A \[0164\]](#)
- [US5585089A \[0164\] \[0166\]](#)
- [US5530101A \[0164\]](#)
- [WO9633735A \[0170\]](#)
- [WO9402602A \[0170\]](#)
- [US5545807A \[0170\] \[0170\] \[0171\]](#)
- [US6713610B \[0170\]](#)
- [US6673986B \[0170\]](#)
- [US6162963A \[0170\]](#)
- [US6300129B \[0170\]](#)
- [US6255458B \[0170\]](#)
- [US5877397A \[0170\] \[0171\]](#)
- [US5874299A \[0170\] \[0171\]](#)
- [US5545806A \[0170\] \[0171\]](#)
- [WO9110741A \[0170\]](#)
- [WO9004036A \[0170\]](#)
- [EP546073B1 \[0170\]](#)
- [EP546073A1 \[0170\]](#)
- [US5569825A \[0171\]](#)
- [US5625126A \[0171\]](#)
- [US5633425A \[0171\]](#)
- [US5789650A \[0171\]](#)
- [US5661016A \[0171\]](#)
- [US5814318A \[0171\]](#)
- [US5770429A \[0171\]](#)

- [WO931227A \[0171\]](#)
- [WO9222646A \[0171\]](#)
- [WO9203918A \[0171\]](#)
- [WO9824893A \[0171\]](#)
- [WO9910494A \[0173\]](#)
- [US5011912A \[0175\]](#)
- [WO9215673A \[0181\]](#)
- [WO9507463A \[0181\]](#)
- [WO9814605A \[0181\]](#)
- [WO9826277A \[0181\]](#)
- [WO9949019A \[0181\]](#)
- [US5292658A \[0181\]](#)
- [US5418155A \[0181\]](#)
- [US5683888A \[0181\]](#)
- [US5741668A \[0181\]](#)
- [US5777079A \[0181\]](#)
- [US5804387A \[0181\]](#)
- [US5874304A \[0181\]](#)
- [US5876995A \[0181\]](#)
- [US5925558A \[0181\]](#)
- [US4399216A \[0194\]](#)
- [US4912040A \[0194\]](#)
- [US4740461A \[0194\]](#)
- [US4959455A \[0194\]](#)
- [US6270964B \[0195\]](#)
- [US4965195A \[0209\]](#)
- [EP0367566A \[0209\]](#)
- [US4968607A \[0209\]](#)
- [EP0460846A \[0209\]](#)

Non-patent literature cited in the description

- **JAGATH R JUNUTULA et al.** Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index *Nature Biotechnology*, 2008, vol. 26, 8 [\[0004\]](#)
- **CHRISTOPHER R BEHRENS et al.** Methods for site-specific drug conjugation to antibodies *MABS*, 2014, vol. 6, 1 [\[0004\]](#)
- **SAMBROOK et al.** *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratory Press 1989 0000 [\[0074\]](#)
- *Current Protocols in Molecular Biology* Green Publishers Inc. and Wiley and Sons 1994 0000 [\[0074\]](#)

- **SAMBROOK et al.** Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press 20010000 [0077]
- **AUSUBEL et al.** Current Protocols in Molecular Biology Greene Publishing Associates 19920000 [0077]
- **HARLOW LANE** Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press 19900000 [0077]
- **Computational Molecular Biology** University Press 19880000 [0088]
- **Biocomputing Informatics and Genome Projects** Academic Press 19930000 [0088]
- **Computer Analysis of Sequence Data** Humana Press 19940000 [0088]
- **ON HEINJE, G.** Sequence Analysis in Molecular Biology Academic Press 19870000 [0088]
- **Sequence Analysis Primer** M. Stockton Press 19910000 [0088]
- **CARILLO et al.** SIAM J. Applied Math., 1988, vol. 48, 1073- [0088]
- **Nucl. Acid Res. DEVEREUX et al.** Genetics Computer Group University of Wisconsin 19840000 vol. 12, 387- [0089]
- **DAYHOFF et al.** Atlas of Protein Sequence and Structure, 1978, vol. 5, 345-352 [0089]
- **HENIKOFF et al.** Proc. Natl. Acad. Sci. U.S.A., 1992, vol. 89, 10915-10919 [0089]
- **NEEDLEMAN et al.** J. Mol. Biol., 1970, vol. 48, 443-453 [0090]
- **SONGSIVILAILACHMANN** Clin. Exp. Immunol., 1990, vol. 79, 315-321 [0112]
- **KOSTELNY et al.** J. Immunol., 1992, vol. 148, 1547-1553 [0112]
- **STAHLI et al.** Methods in Enzymology, 1983, vol. 9, 242-253 [0113]
- **KIRKLAND et al.** J. Immunol., 1986, vol. 137, 3614-3619 [0113]
- **HARLOW LANE** Antibodies, A Laboratory Manual Cold Spring Harbor Press 19880000 [0113]
- **MOREL et al.** Molec. Immunol., 1988, vol. 25, 7-15 [0113]
- **CHEUNG et al.** Virology, 1990, vol. 176, 546-552 [0113]
- **MOLDENHAUER et al.** Scand. J. Immunol., 1990, vol. 32, 77-82 [0113]
- **CREIGHTON** PROTEINS: STRUCTURE AND MOLECULAR PROPERTIES W.H. Freeman and Company 19840000 [0134]
- **VAN HEEKESCHUSTER** J. Biol. Chem., 1989, vol. 264, 5503-5509 [0138]
- **BOOTCOV MR** Proc. Natl. Acad. Sci. USA, 1997, vol. 94, 11514-9 [0145]
- **FAIRLIE WD** Gene, 2000, vol. 254, 67-76 [0145]
- **Fundamental Immunology** Raven Press 19890000 [0151]
- **CHOTHIA LESKJ.** Mol. Biol., 1987, vol. 196, 901-917 [0152] [0160]
- **CHOTHIA et al.** Nature, 1989, vol. 342, 878-883 [0152]
- **BONG et al.** Chemoselective Pd(0)-catalyzed peptide coupling in water Organic Letters, 2001, vol. 3, 162509-11 [0154]
- **DIBOWSKI et al.** Bioconjugation of peptides by palladium-catalyzed C-C cross-coupling in water Angew. Chem. Int. Ed, 1998, vol. 37, 4476-78 [0154]
- **DEVASHER et al.** Aqueous-phase, palladium-catalyzed cross-coupling of aryl bromides under mild conditions, using water-soluble, sterically demanding alkylphosphines J. Org. Chem., 2004, vol. 69, 7919-27 [0154]
- **SHAUGNESSY et al.** J. Org. Chem, 2003, vol. 68, 6767-6774 [0154]
- **PRESCHER, JABERTOZZI CR** Chemistry in living system, Nature Chemical Biology, 2005, vol. 1, 113-21 [0154]

- **KABAT et al.** Sequences of Proteins of Immunological Interest US Dept. of Health and Human Services 19910000 [0159]
- **KABAT et al.** Sequences of Proteins of Immunological Interest Public Health Service 19910000 [0160]
- **CHOTHIA et al.** Nature, 1989, vol. 342, 877-883 [0160]
- **MORRISON et al.** Proc. Natl. Acad. Sci. USA, 1985, vol. 81, 6851-6855 [0164]
- **JONES et al.** Nature, 1986, vol. 321, 522-525 [0166]
- **RIECHMANN et al.** Nature, vol. 332, 323-27 [0166]
- **VERHOEYEN et al.** Science, 1988, vol. 239, 1534-1536 [0166]
- **JAKOBOVITS et al.** Proc. Natl. Acad. Sci. USA, vol. 90, 2551-2555 [0170]
- **JAKOBOVITS et al.** Nature, 1993, vol. 362, 255-258 [0170]
- **BRUGGERMANN et al.** Year in Immunol., 1993, vol. 7, 33- [0170]
- **LONBERG et al.** Nature, 1994, vol. 368, 856-859 [0171] [0171]
- **LONBERGHUSZAR** Intern. Rev. Immunol, 1995, vol. 13, 65-93 [0171]
- **HARDINGLONBERG** Ann. N.Y Acad. Sci., 1995, vol. 764, 536-546 [0171]
- **TAYLOR et al.** Nucleic Acids Research, 1992, vol. 20, 6287-6295 [0171]
- **CHEN et al.** International Immunology, 1993, vol. 5, 647-656 [0171]
- **TUAILLON et al.** J. Immunol., 1994, vol. 152, 2912-2920 [0171]
- **LONBERG** Handbook of Exp. Pharmacology, 1994, vol. 113, 49-101 [0171]
- **TAYLOR et al.** International Immunology, 1994, vol. 6, 576-591 [0171]
- **LONBERGHUSZAR** Intern. Rev. Immunol., 1995, vol. 13, 65-93 [0171]
- **HARDINGLONBERG** Ann. N.Y Acad. Sci., 1995, vol. 764, 536-546 [0171]
- **FISHWILD et al.** Nature Biotechnology, 1996, vol. 14, 845-851 [0171]
- **MENDEZ et al.** Nature Genetics, 1997, vol. 15, 146-156 [0171]
- **HOOGENBOOM et al.** J. Mol. Biol., 1991, vol. 227, 381- [0173]
- **MARKS et al.** J. Mol. Biol., 1991, vol. 222, 581- [0173]
- **HOPP et al.** Bio/Technology, 1988, vol. 6, 1204- [0175]
- **CHALFIE et al.** Science, 1994, vol. 263, 802-805 [0181]
- **STAUBER** Biotechniques, 1998, vol. 24, 462-471 [0181]
- **HEIM et al.** Curr. Biol., 1996, vol. 6, 178-182 [0181]
- **ICHIKI et al.** J. Immunol., 1993, vol. 150, 5408-5417 [0181]
- **NOLAN et al.** Proc. Natl. Acad. Sci. U.S.A., vol. 85, 2603-2607 [0181]
- Current Protocols in Molecular Biology John Wiley & Sons 198900006.3.1-6.3.6 [0184]
- **SAMBROOKFRITSCHMANIATIS** Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press 20010000 [0185]
- Current Protocols in Molecular Biology John Wiley & Sons, Inc. 19950000 [0185]
- **VOSS et al.** Trends Biochem. Sci., 1986, vol. 11, 287- [0190]
- **MANIATIS et al.** Science, 1987, vol. 236, 1237- [0190]
- Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses Plenum Press 19800000 [0193]
- Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press 19880000 [0193]
- **BIANCHIMCGREW** Biotech. Biotechnol. Bioeng, 2003, vol. 84, 439-44 [0195]
- Methods Enzymol. Academic Press 19900000 vol. 185, [0195]
- **COSMAN et al.** Nature, 1984, vol. 312, 768- [0209]

Patentkrav

1. Fremgangsmåde til fremstilling af et antistofkonjugat eller antistoffragmentkonjugat, hvilken fremgangsmåde omfatter

5 trinene:

a) opnåelse af en sammensætning, der omfatter et antistof eller et antistoffragment;

b) eksponering af antistoffet eller antistoffragmentet for et cysteinblokeringsmiddel, hvor cysteinblokeringsmidlet er

10 cysteamin, og cysteinblokeringsmidlet danner et stabilt blandet disulfid med mindst én cysteinrest i antistoffet eller antistoffragmentet;

c) tilsætning af et reduktionsmiddel til sammensætningen til frembringelse af en reduktionsblanding og muliggørelse af, at

15 en reduktionsreaktion kan finde sted, således at reduktionsblandingen omfatter et reduceret antistof eller reduceret antistoffragment, hvor reduktionsmidlet er triphenylphosphin-3,3',3"-trisulfonat ("TPPTS");

d) tilsætning af et oxidationsmiddel til reduktionsblandingen

20 til frembringelse af en oxideret blanding og muliggørelse af, at en oxideringsreaktion kan finde sted, således at oxideringsblandingen omfatter et oxideret antistof eller oxideret antistoffragment; og

e) tilsætning af en aktiveret kemisk del til den oxiderede

25 blanding til frembringelse af en konjugationsblanding og muliggørelse af, at en konjugationsreaktion kan finde sted, således at der frembringes et antistofkonjugat eller antistoffragmentkonjugat.

30 2. Fremgangsmåde ifølge krav 1, hvor det blandede disulfid er et antistof eller et antistoffragment med en cappet fri cystein.

3. Fremgangsmåde ifølge krav 1, hvor der efter trin b) og før trin c) udføres kationbytningskromatografi til fjernelse af

35 overskydende cysteinblokeringsmiddel.

4. Fremgangsmåde ifølge krav 1, hvor forholdet mellem reduktionsmiddel og antistof eller antistoffragment er 2 til 4:1

(mol/mol).

5. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor der efter trin c) og før trin d) udføres et
5 bufferudskiftningstrin til fjernelse af reduktionsmidlet.

6. Fremgangsmåde ifølge krav 5, hvor bufferudskiftningstrinet er ultrafiltrering/diafiltrering.

10 7. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor oxidationsmidlet er dehydroascorbinsyre ("DHAA").

8. Fremgangsmåde ifølge krav 7, hvor forholdet mellem oxidationsmiddel og antistof eller antistoffragment er 3 til 6:1
15 (mol/mol).

9. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor den aktiverede kemiske del er et peptid, der omfatter et halogen, hvor halogenet er valgt fra gruppen, der består af
20 Br, I og Cl.

10. Fremgangsmåde ifølge krav 9, hvor forholdet mellem aktiveret kemisk del og antistof eller antistoffragment er 2 til 3:1 (mol/mol).

25 11. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor der efter trin e) udføres et oprensningstrin til fjernelse af den aktiverede kemiske del.

30 12. Fremgangsmåde ifølge krav 11, hvor oprensningstrinet indbefatter hydrofob interaktionskromatografi ("HIC"), ultrafiltrering/diafiltrering eller hydrophob interaktionskromatografi ("HIC") efterfulgt af ultrafiltrering/diafiltrering.

35 13. Fremgangsmåde til fremstilling af et antistofkonjugat eller antistoffragmentkonjugat, hvilken fremgangsmåde omfatter trinene:

a) opnåelse af en sammensætning, der omfatter et blandet disulfid, der omfatter et antistof eller antistoffragment med en cappel fri cystein, der omfatter en cysteamincap;

b) tilsætning af et reduktionsmiddel til sammensætningen til frembringelse af en reduktionsblanding og muliggørelse af, at en reduktionsreaktion kan finde sted, således at reduktionsblandingen omfatter et reduceret antistof eller reduceret antistoffragment, hvor reduktionsmidlet er triphenylphosphin-3,3',3"-trisulfonat ("TPPTS");

c) tilsætning af et oxidationsmiddel til reduktionsblandingen til frembringelse af en oxideret blanding og muliggørelse af, at en oxideringsreaktion kan finde sted, således at oxideringsblandingen omfatter et oxideret antistof eller oxideret antistoffragment; og

d) tilsætning af en aktiveret kemisk del til den oxiderede blanding til frembringelse af en konjugationsblanding og muliggørelse af, at en konjugationsreaktion kan finde sted, således at der frembringes et antistofkonjugat eller antistoffragmentkonjugat.

14. Fremgangsmåde ifølge krav 13, hvor der efter trin a) og før trin b) udføres kationbytningskromatografi til fjernelse af overskydende cysteinblokeringsmiddel.

15. Fremgangsmåde ifølge krav 13, hvor forholdet mellem reduktionsmiddel og antistof eller antistoffragment er 2 til 4:1 (mol/mol).

16. Fremgangsmåde ifølge et hvilket som helst af kravene 13-15, hvor der efter trin b) og før trin c) udføres et bufferudskiftningstrin til fjernelse af reduktionsmidlet.

17. Fremgangsmåde ifølge krav 16, hvor bufferudskiftningstrinet er ultrafiltrering/diafiltrering.

18. Fremgangsmåde ifølge et hvilket som helst af kravene 13-17, hvor oxidationsmidlet er dehydroascorbinsyre ("DHAA").

19. Fremgangsmåde ifølge krav 18, hvor forholdet mellem oxidationsmiddel og antistof eller antistoffragment er 3 til 6:1 (mol/mol).

5 20. Fremgangsmåde ifølge et hvilket som helst af kravene 13-19, hvor den aktiverede kemiske del er et peptid, der omfatter et halogen, hvor halogenet er valgt fra gruppen, der består af Br, I og Cl.

10 21. Fremgangsmåde ifølge krav 20, hvor forholdet mellem aktiveret kemisk del og antistof eller antistoffragment er 2 til 3:1 (mol/mol).

15 22. Fremgangsmåde ifølge et hvilket som helst af kravene 13-21, hvor der efter trin d) udføres et oprensningstrin til fjernelse af den aktiverede kemiske del.

20 23. Fremgangsmåde ifølge krav 22, hvor oprensningstrinet indbefatter hydrofob interaktionskromatografi ("HIC"), ultrafiltrering/diafiltrering eller hydrofob interaktionskromatografi ("HIC") efterfulgt af ultrafiltrering/diafiltrering.

25 24. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor antistoffet eller antistoffragmentet omfatter en cysteinrest i en position valgt fra gruppen, der består af D70 i antistoffets lette kæde i forhold til referencesekvensen SEQ ID NO: 7, E276 i antistoffets tunge kæde i forhold til referencesekvensen SEQ ID NO: 8 og T363 i antistoffets tunge
30 kæde i forhold til referencesekvensen SEQ ID NO: 8.

FIG. 2

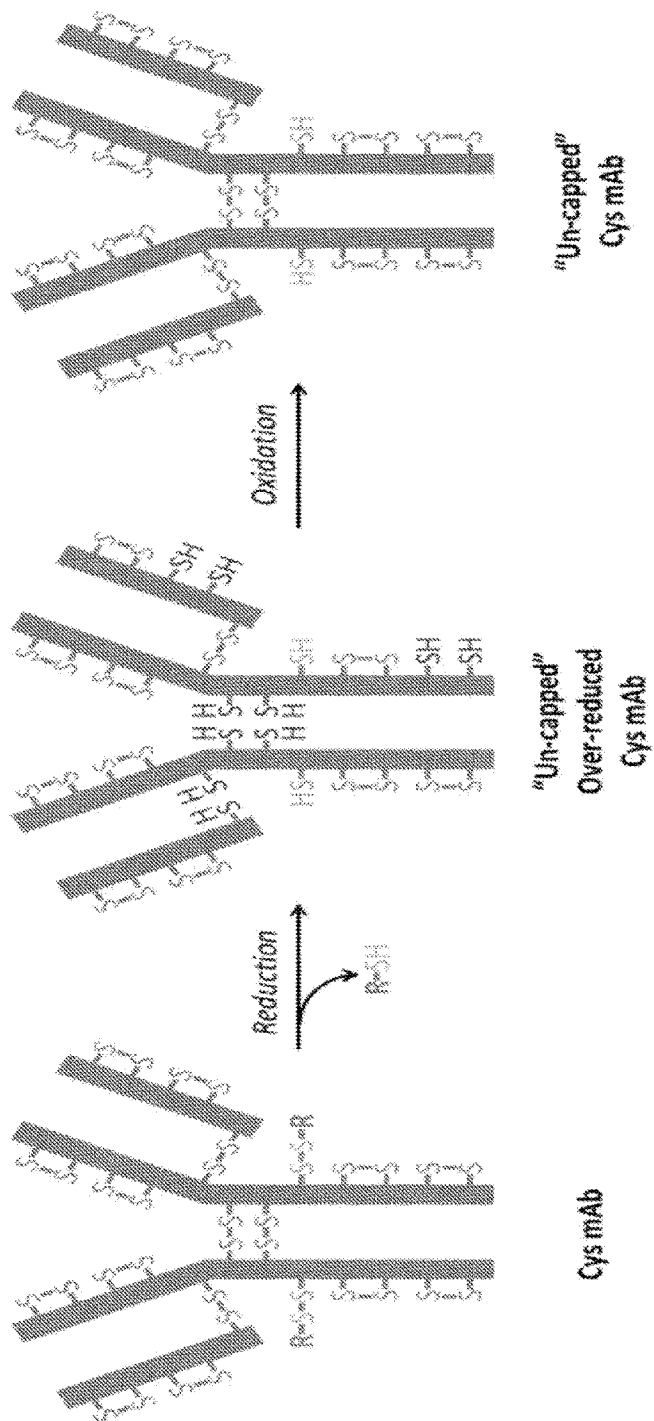


FIG. 3

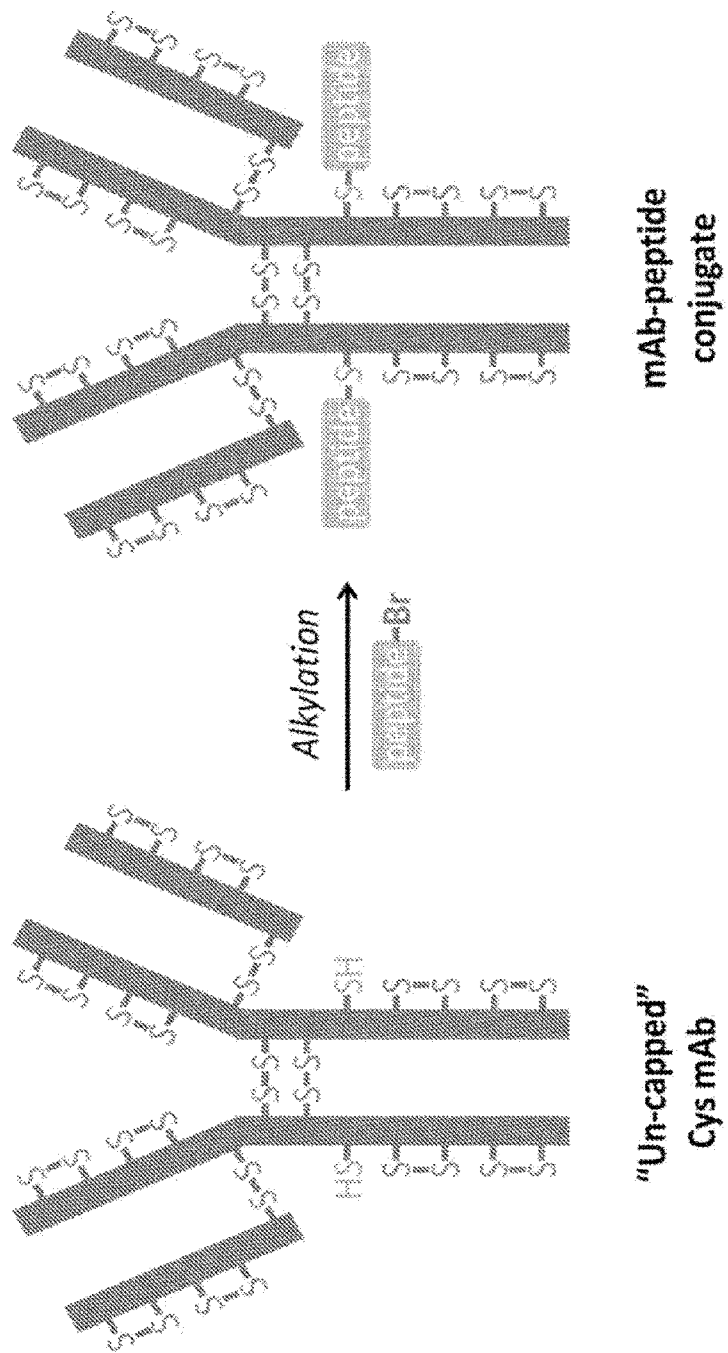


FIG. 4

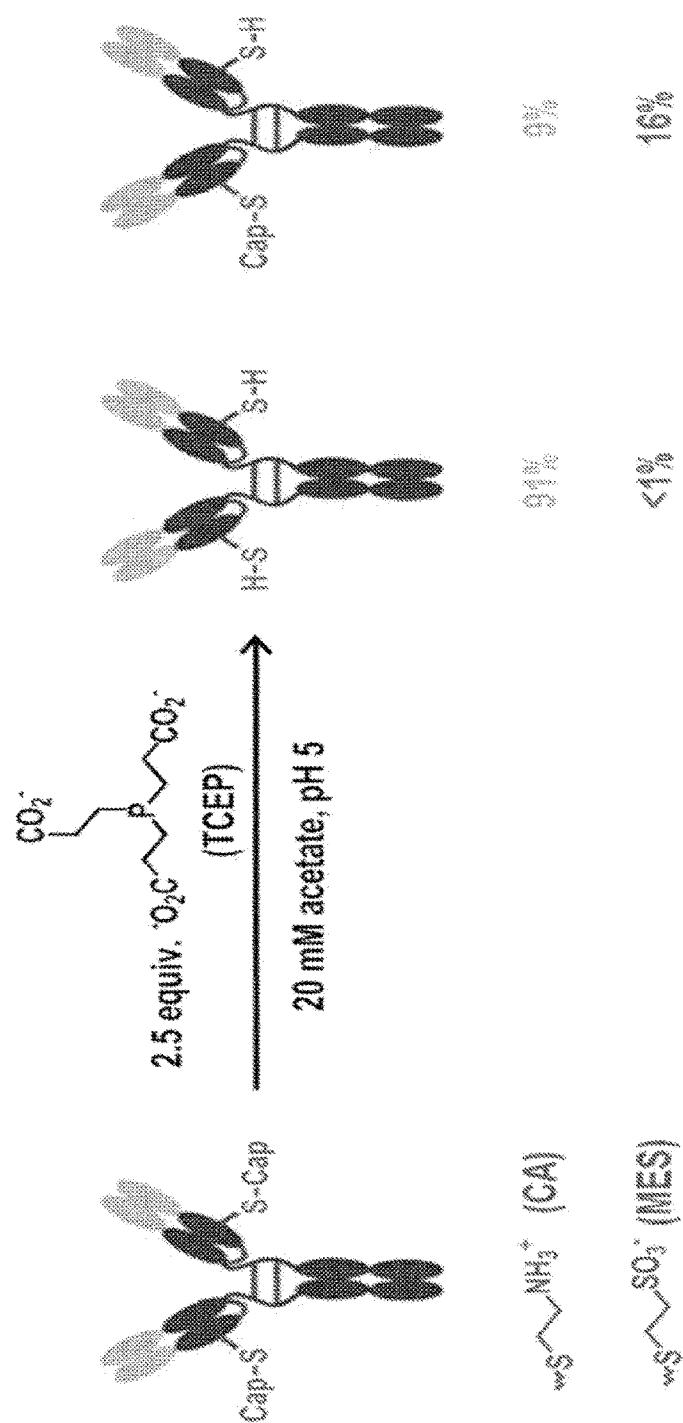


FIG. 5

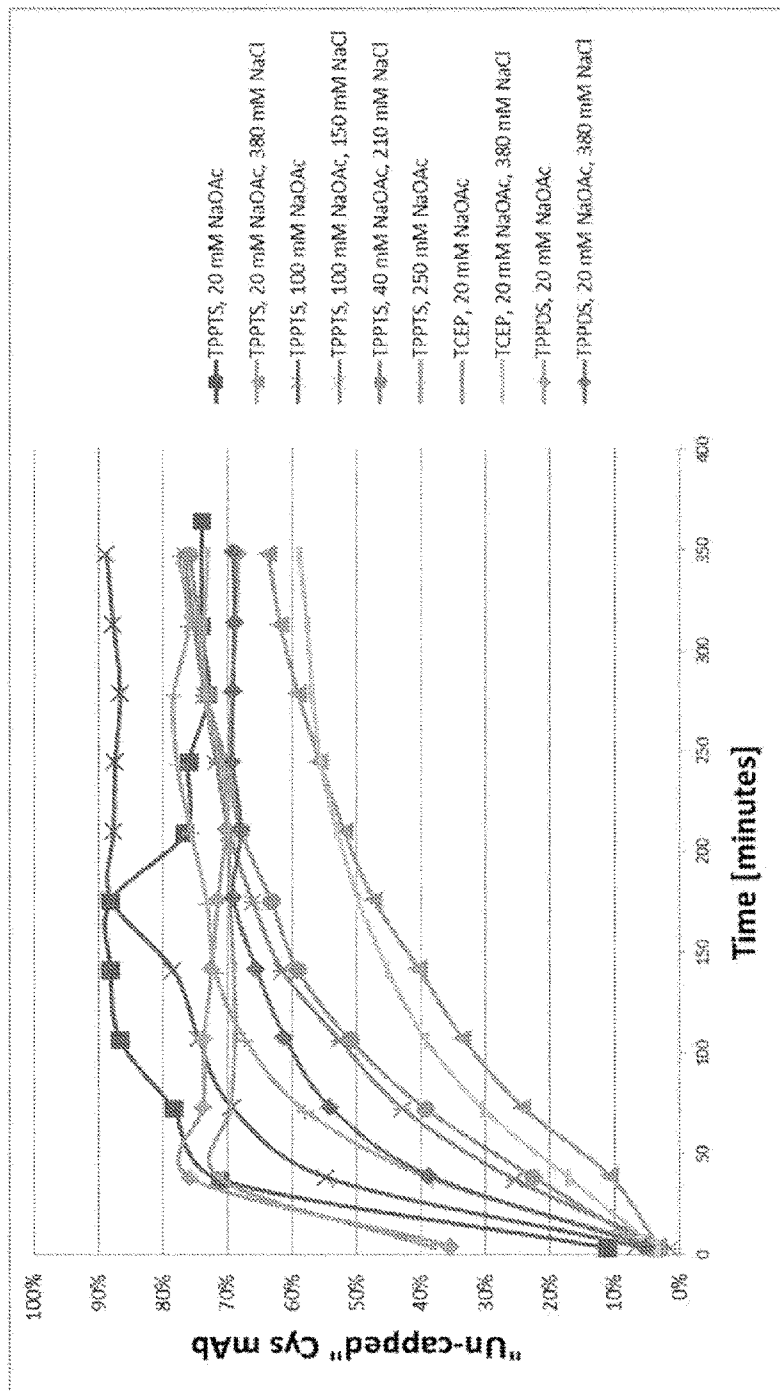
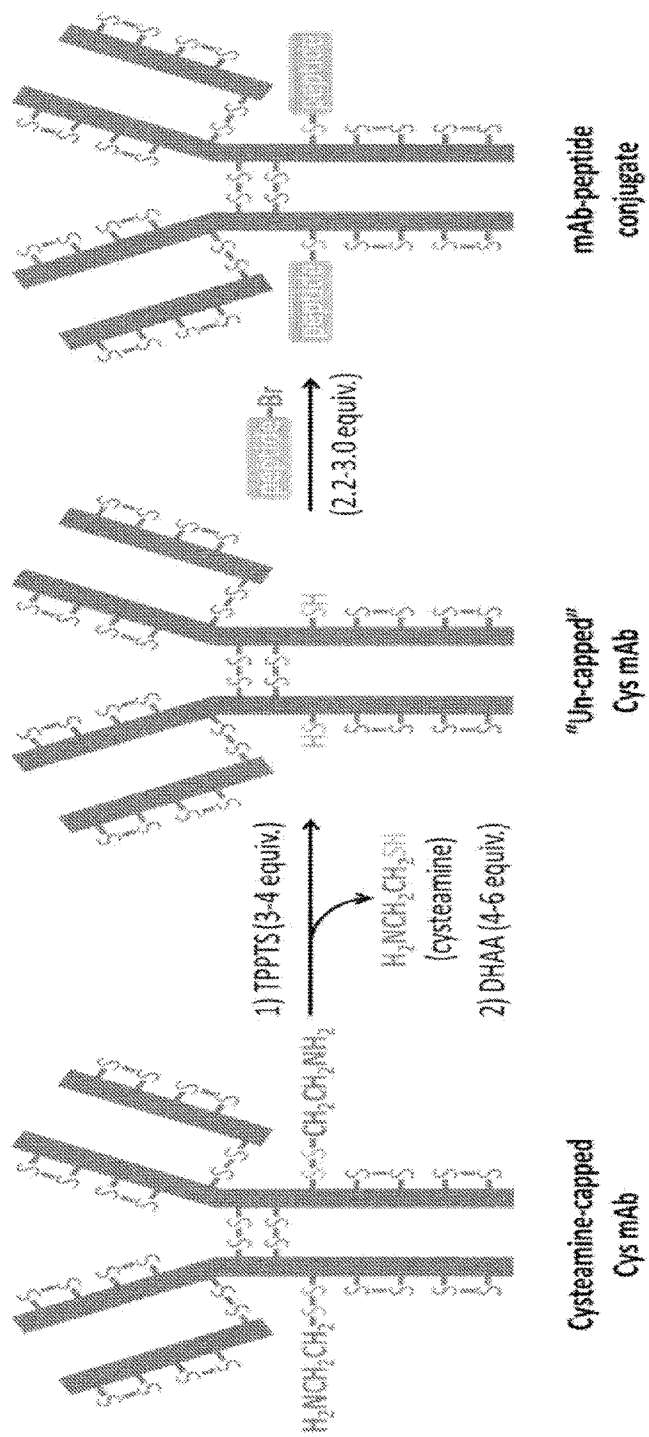


FIG. 6



SEKVENSLISTE

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