SITE-SPECIFIC GLYCOENGINEERING OF TARGETING MOIETIES

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on March 5, 2015, is named 566622SA9-148PC_SL.txt and is 115,860 bytes in size.

BACKGROUND

Use of specific antibodies to treat people and other animals is a powerful tool that has been very effective in treating many conditions and disorders. However, there is great demand for more effective targeted therapeutics, especially target specific therapies with higher efficacy and greater therapeutic windows. One of these target specific treatments employs antibody-effector moiety conjugates in which a targeting moiety directs a specific antibody to a desired treatment site. These molecules have shown improved therapeutic index – higher efficacy and/or lower toxicity profiles than the un-targeted antibody in a clinical setting. However, development of such therapeutics can be challenging as many factors, including physical and/or structural properties of the antibody itself as well as linkage stability, can have significant impact on the disease target (e.g. tumor) specificity, thereby reducing efficacy. With high non-specific binding and low stability in circulation, the antibody-effector moiety conjugate is typically cleared through normal tissues before reaching the target site. Moreover, antibody-effector moiety conjugates with significant subpopulations of high drug loading could generate aggregates which would be eliminated by macrophages, leading to shorter half-life. Thus, there are increasing needs for critical process control and improvement as well as preventing complications, such as antibody aggregation and nonspecific antibody-mediated toxicity.

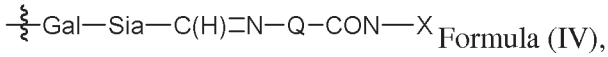
Although antibody-effector moiety conjugates generated according to current methods can be effective, development of such therapeutics are challenging, as heterogeneous mixtures are often a consequence of the conjugation chemistries used. For example, effector

moiety conjugation to antibody lysine residues is complicated by the fact that there are many lysine residues (~30) in an antibody available for conjugation. Since the optimal number of conjugated effector moiety to antibody ratio (DAR) is much lower to minimize loss of function of the antibody (e.g., around 4:1), lysine conjugation often generates a very heterogeneous profile. Furthermore, many lysines are located in critical antigen binding sites of the CDR region, and drug conjugation may lead to a reduction in antibody affinity. Thiol mediated conjugation mainly targets the eight cysteines involved in hinge disulfide bonds. However, it is still difficult to predict and identify which four of eight cysteines are consistently conjugated among the different preparations. More recently, genetic engineering of free cysteine residues has enabled site-specific conjugation with thiol-based chemistries, but such linkages often exhibit highly variable stability, with the linker undergoing exchange reactions with albumin and other thiol-containing serum molecules. Therefore, a site-specific conjugation strategy which generates an antibody conjugate with a defined conjugation site and stable linkage would be useful to enable effector moiety conjugation while minimizing adverse effects on antibody structure or function.

SUMMARY

The current disclosure provides binding polypeptides (e.g., antibodies), and targeting moiety conjugates thereof. In certain embodiments, the conjugates comprise a site-specifically engineered targeting-moiety glycan linkage within native or modified glycans of the binding polypeptide. The current disclosure also provides nucleic acid sequences encoding the antigen-binding polypeptides, recombinant expression vectors, and host cells for making such antigen-binding polypeptides. Methods of using the antigen-binding polypeptides disclosed herein to treat disease are also provided.

Accordingly, in one aspect, the invention provides a binding polypeptide comprising at least one modified glycan comprising at least one moiety of Formula (IV):



Formula (IV),

wherein:

A) Q is NH or O;

B) CON is a connector moiety;

C) X is a targeting moiety;

D) Gal is a component derived from galactose; and

E) Sia is a component derived from sialic acid;

wherein Sia is present or absent, and wherein the targeting moiety binds to a cell.

In one embodiment, the cell is a mammalian cell. In a further embodiment, the cell is selected from an immune cell, a liver cell, a tumor cell, a vascular cell, an epithelial cell, or a mesenchymal cell. In yet another embodiment, the cell is selected from a B cell, a T cell, a dendritic cell, a natural killer (NK) cell, a macrophage, a neutrophil, a hepatocyte, a liver sinusoidal endothelial cell, or a hepatoma cell.

In one embodiment, the binding polypeptide is internalized by the cell. In another embodiment, the amount of the binding polypeptide internalized by the cell is greater than the amount of a reference binding polypeptide lacking a targeting moiety internalized by the cell.

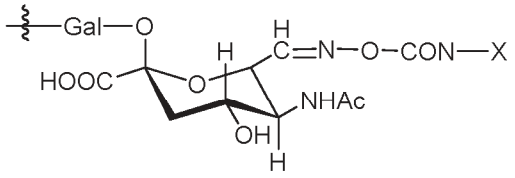
In one embodiment, the targeting moiety binds to a mannose 6 phosphate receptor on the cell. In another embodiment, the targeting moiety comprises a mannose 6 phosphate (Man 6-P) moiety.

In one embodiment, the targeting moiety binds to a Siglec on the cell. In a further embodiment, the Siglec is sialoadhesin (Siglec-1), CD22 (Siglec-2), CD33 (Siglec-3), MAG (Siglec-4), Siglec-5, Siglec-6, Siglec-7, Siglec-8, Siglec-9, Siglec-10, Siglec-11, Siglec-12, Siglec-14, or Siglec-15. In another embodiment, the targeting moiety comprises an α2,3-, α2,6-, or α2,8- linked sialic acid residue. In a further embodiment, the targeting moiety comprises an α2,3-siallylactose moiety or an α2,6-siallylactose moiety.

In one embodiment, the targeting moiety binds to a C-type lectin receptor, a galectin, a L-type lectin receptor or other carbohydrate receptors. In a further embodiment, the targeting moiety binds to DEC-205 (CD205; lymphocyte antigen 75), macrophage mannose receptor (MMR; CD206), Dectin-1, Dectin-2, macrophage-inducible C-type lectin (Mincle), dendritic cell-specific ICAM3-grabbing nonintegrin (DC-SIGN; CD209), DC NK lectin group receptor-1 (DNGR-1), Langerin (CD207), a lectican, an asialoglycoprotein receptor (ASGPR), C-lectin receptor dendritic cell immunoreceptor (CLEC4A; CLECSF6; DCIR), macrophage galactose-type lectin (MGL), a DC receptor, a collectin, a selectin, an NK-cell receptor, a multi-C-type lectin domain (CTLD) endocytic receptor, a Reg group (type VII) lectin, chondrolectin, tetranectin, polycystin, attractin (ATRN), eosinophil major basic protein (EMBP), DiGeorge Syndrome Critical Region Gene 2 (DGCR2), Thrombomodulin, Bimlec, a group XVI lectin (SEEC), or a group XVII lectin (CBCP/Frem1/QBRICK).

In one embodiment, Q is O.

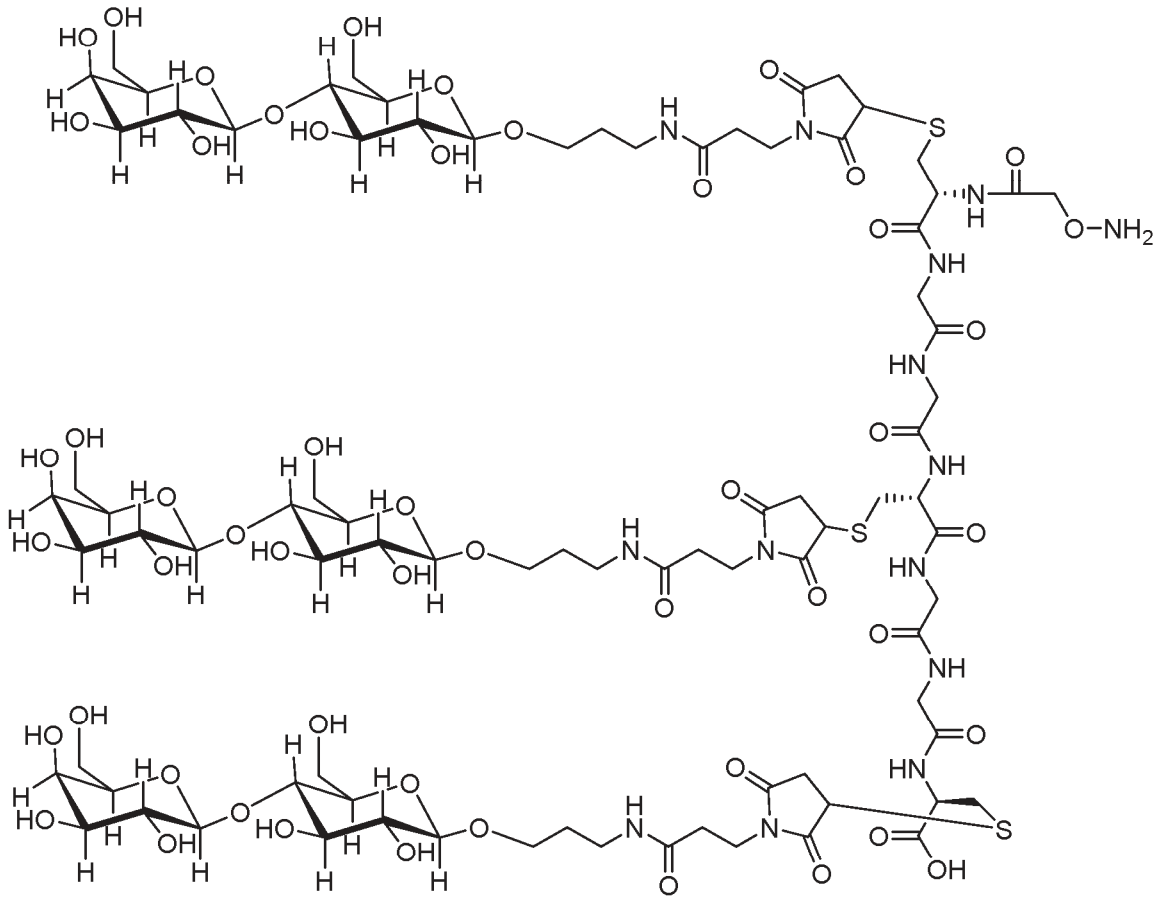
In one embodiment, the glycan comprises at least one moiety of the following structural formula:



In an embodiment, the targeting moiety is a trivalent GalNAc glycan moiety.

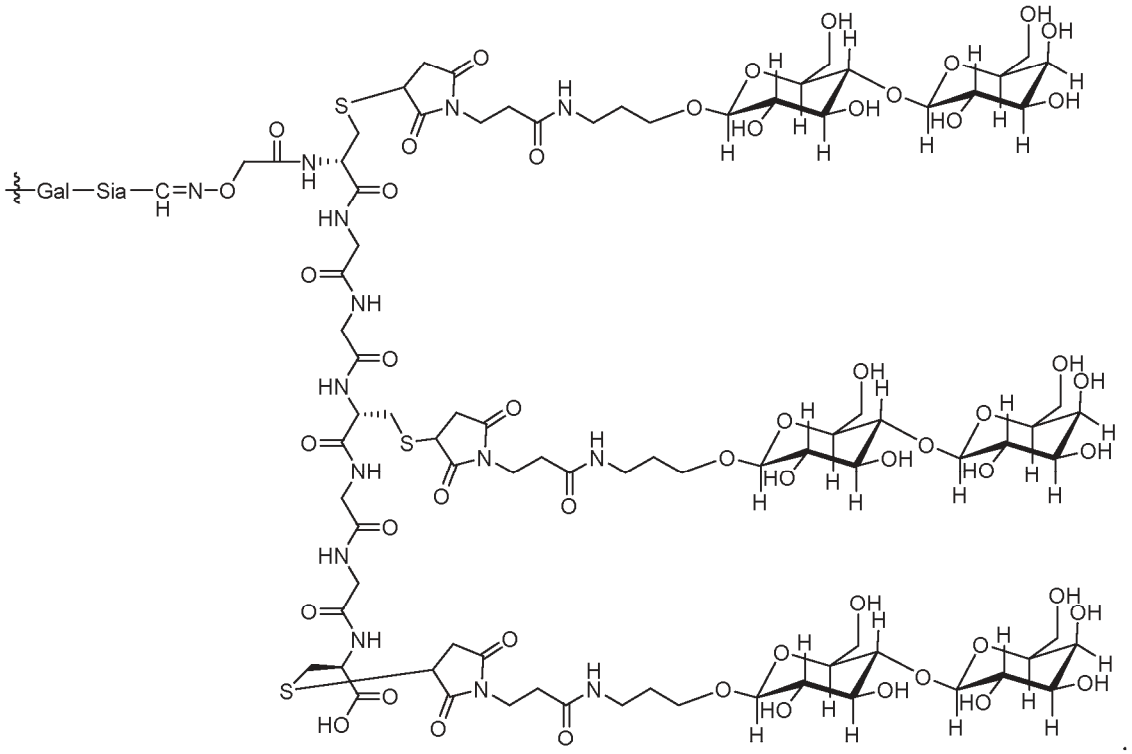
In one embodiment, the targeting moiety is a glycopeptide. In a further embodiment, the targeting moiety is a tri-galactosylated glycopeptide, e.g., lactose$_3$-Cys$_3$Gly$_4$.

In an embodiment, Sia is present and the lactose$_3$-Cys$_3$Gly$_4$ moiety is represented by Formula V:

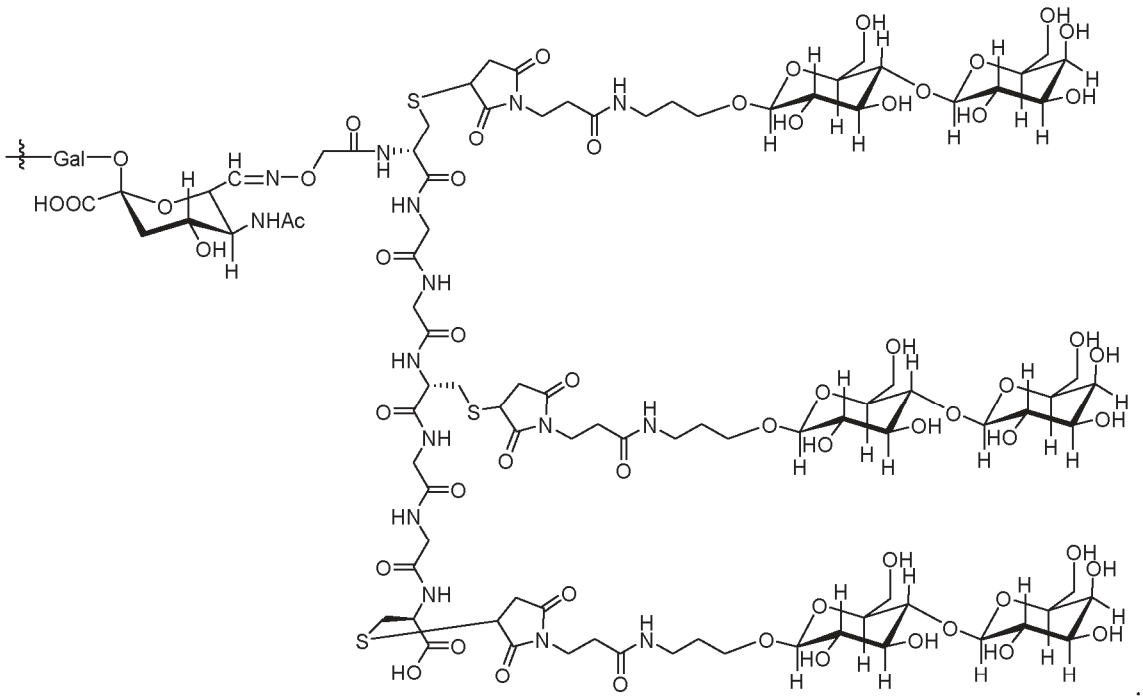


[Formula V].

In one embodiment, the glycan comprises at least one moiety of the following structural formula:

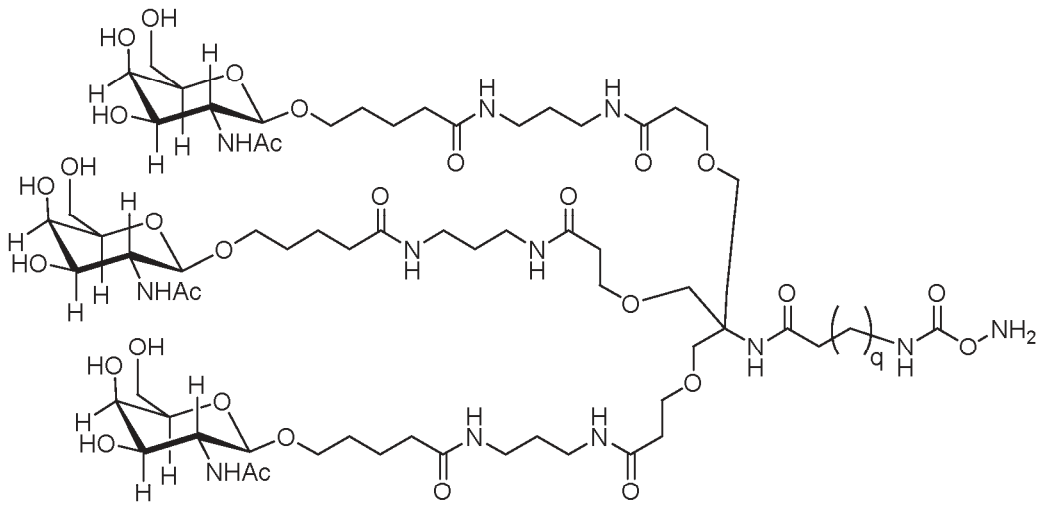


In one embodiment, the glycan comprises at least one moiety of the following structural formula:



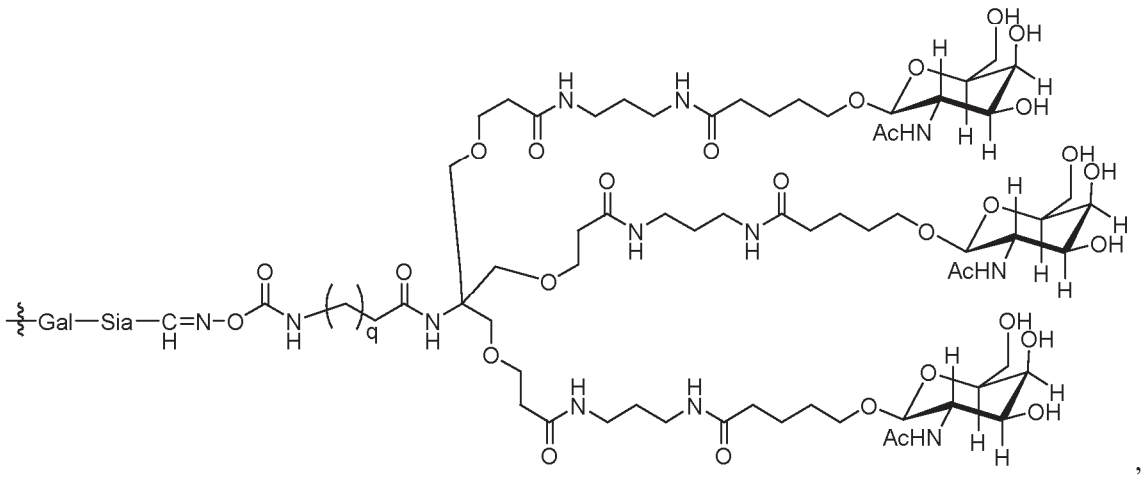
In one embodiment, the targeting moiety is a trivalent GalNAc glycan moiety.

In one embodiment, Sia is present and the trivalent GalNAc glycan moiety is represented by Formula VIII:



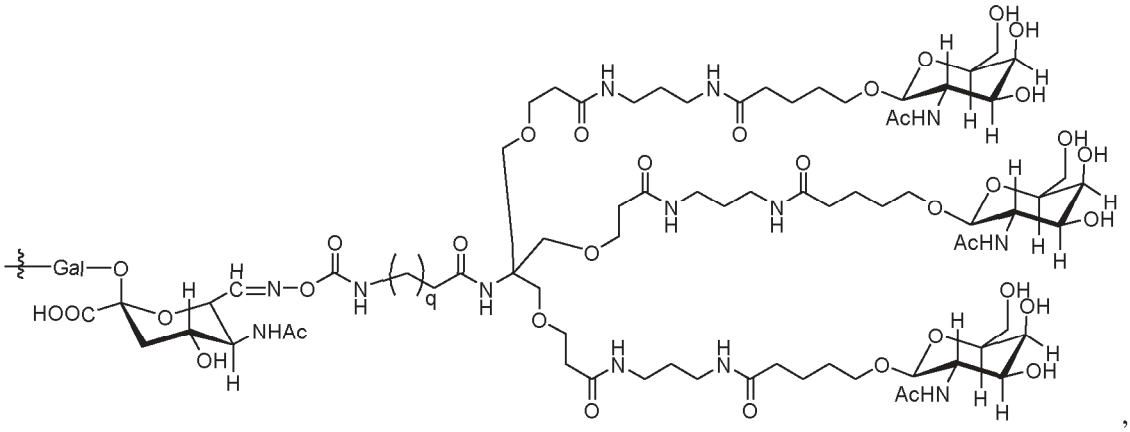
[Formula VIII], wherein q is an integer between 1 and 29 inclusive.

In another embodiment, the glycan comprises at least one moiety having the following structural formula:



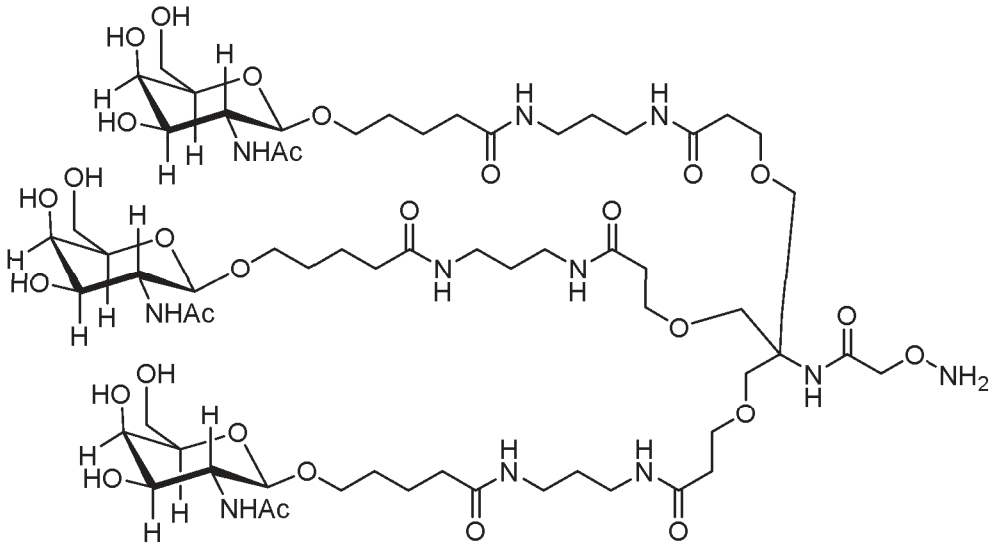
wherein q is an integer between 1 and 29 inclusive.

In another embodiment, the glycan comprises at least one moiety having the following structural formula:

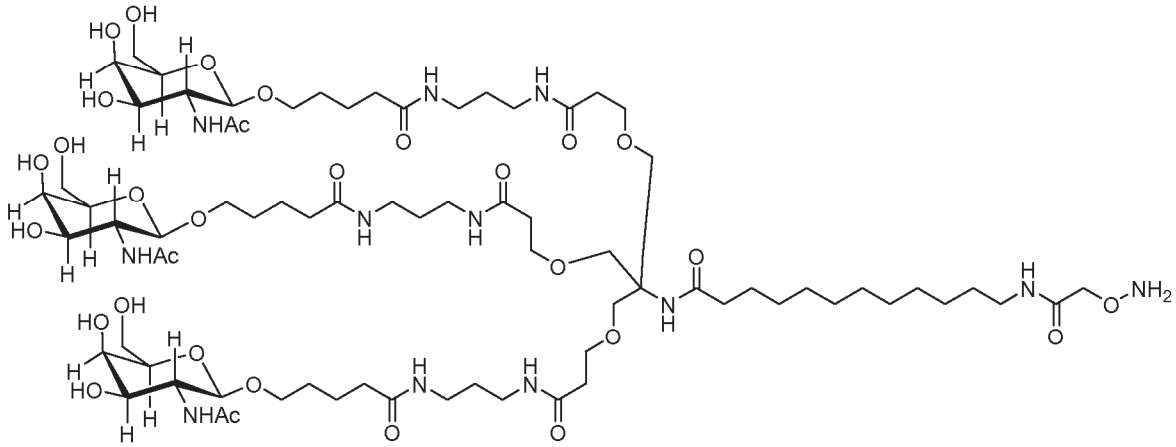


wherein q is an integer between 1 and 29 inclusive.

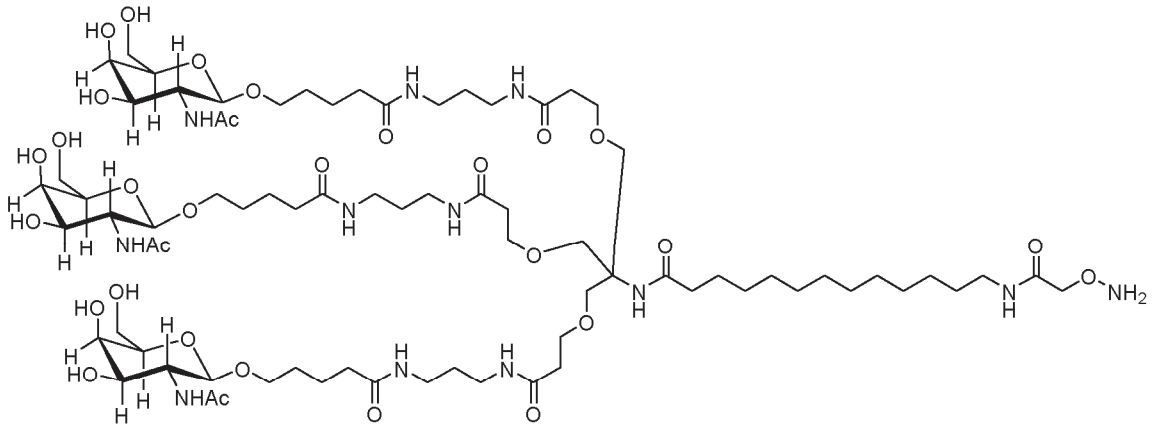
In an embodiment, the trivalent GalNAc glycan moiety is represented by Formula VII, Formula XIII, or Formula XIV:



[Formula VII];

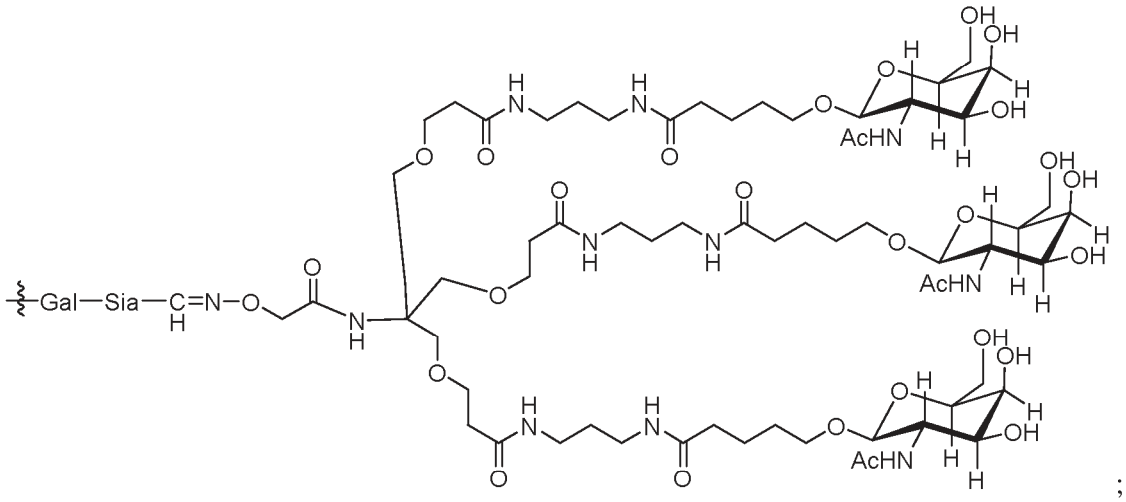


[Formula XIII]; or



[Formula XIV].

In one embodiment, the glycan comprises at least one moiety selected from the following structural formulae:



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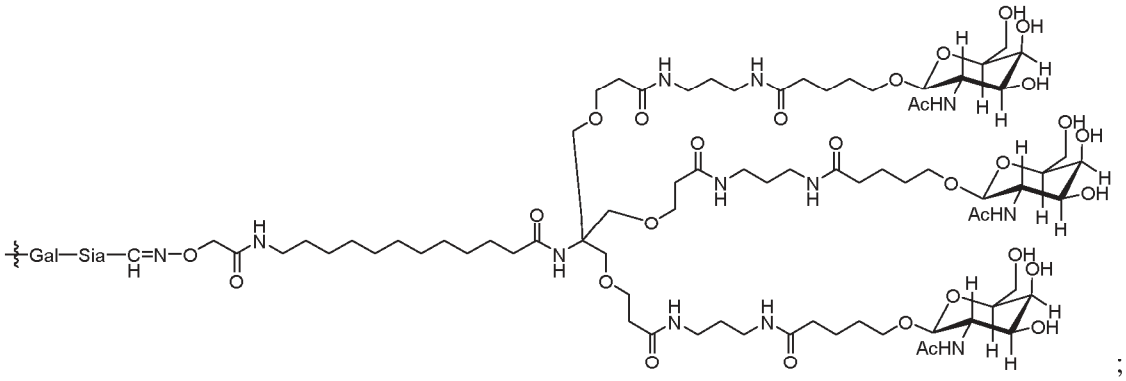
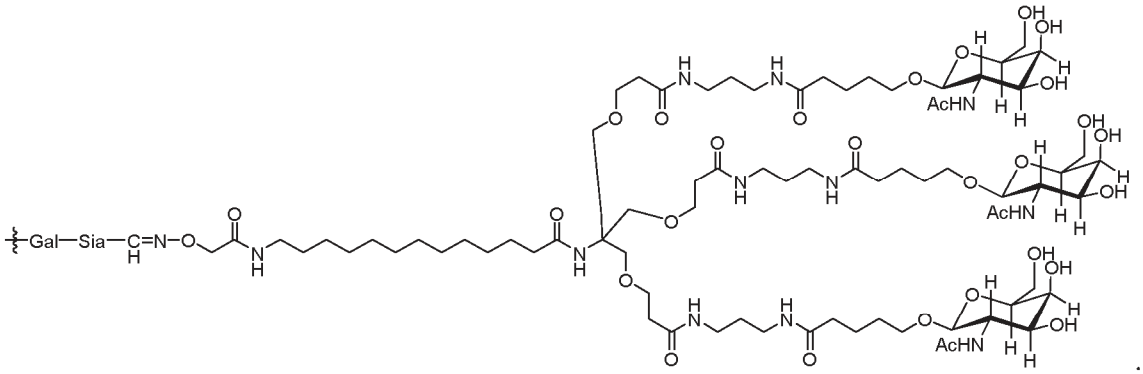


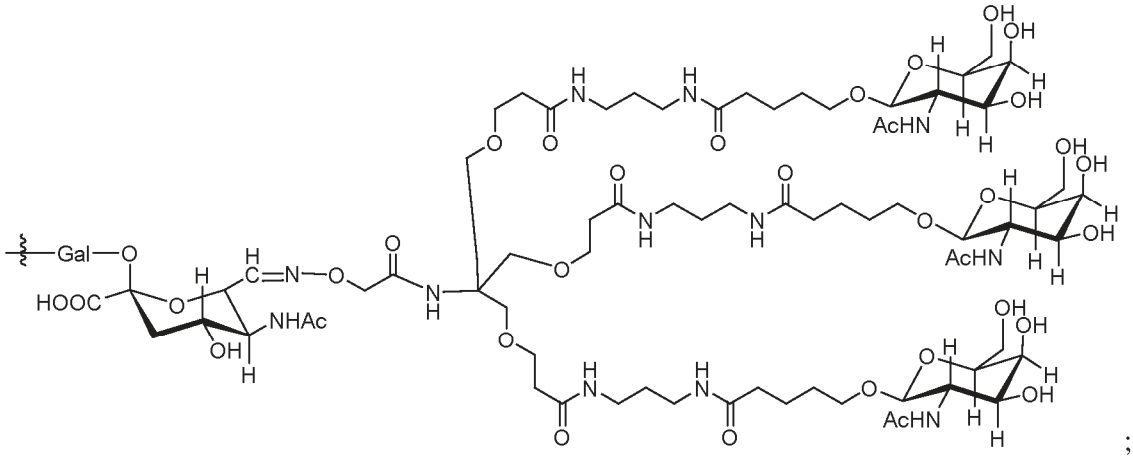
;

or

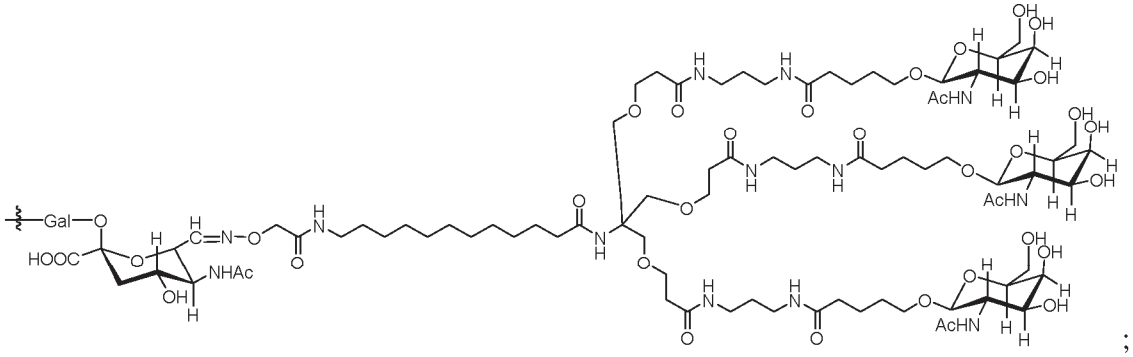


.

In one embodiment, the glycan comprises at least one moiety selected from the following structural formulae:

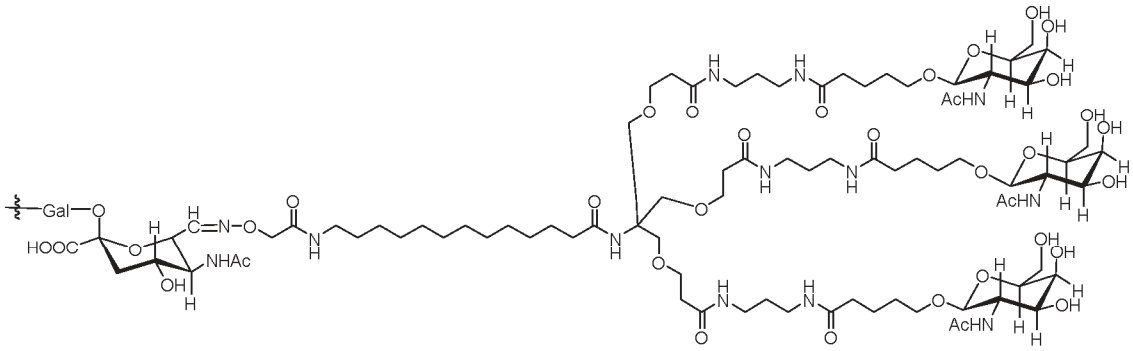


;



;

or



.

In an embodiment, the binding polypeptide comprises an Fc domain. In a further embodiment, the modified glycan is N-linked to the binding polypeptide via an asparagine residue at amino acid position 297 of the Fc domain, according to EU numbering. In another embodiment, the modified glycan is N-linked to the binding polypeptide via an asparagine residue at amino acid position 298 of the Fc domain, according to EU numbering.

In one embodiment, the Fc domain is human. In another embodiment, the binding polypeptide comprises a CH1 domain. In a further embodiment, the modified glycan is N-linked to the binding polypeptide via an asparagine residue at amino acid position 114 of the CH1domain, according to Kabat numbering.

In one embodiment, the connector moiety comprises a pH-sensitive linker, disulfide linker, enzyme-sensitive linker or other cleavable linker moiety. In an embodiment, the connector moiety comprising a linker moiety selected from the group of linker moieties depicted in Table 2 or 14.

In one embodiment, the binding polypeptide is an antibody or immunoadhesin.

In one aspect, the present invention provides a method of making the binding polypeptide of any one of the preceding claims, the method comprising reacting an effector moiety of Formula (I):

$$NH_2\text{-}Q\text{-}CON\text{-}X$$

Formula (I),

wherein:

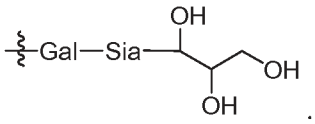
A) Q is NH or O;

B) CON is a connector moiety; and

C) X is a targeting moiety,

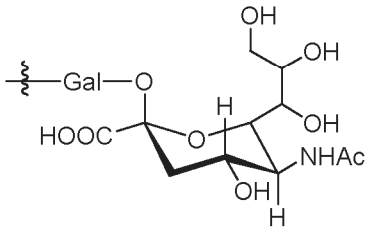
with an precursor binding polypeptide comprising an oxidized glycan.

In one embodiment, the initial binding polypeptide comprises at least one moiety of the following structural formula:

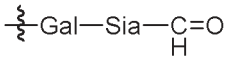


.

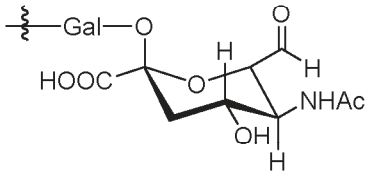
In one embodiment, the initial binding polypeptide comprises at least one moiety of the following structural formula:



In one embodiment, the precursor binding polypeptide comprises an oxidized glycan comprises at least one moiety of the following structural formula:



In one embodiment, the precursor binding polypeptide comprises an oxidized glycan comprises at least one moiety of the following structural formula:

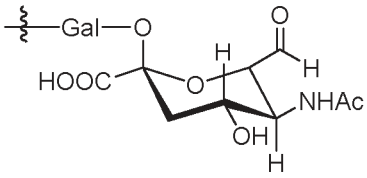


In one embodiment, the precursor binding polypeptide comprising an oxidized glycan is generated by reacting an initial binding polypeptide comprising a glycan with a mildly oxidizing agent. In a further embodiment, the mildly oxidizing agent is sodium periodate. In another embodiment, no more than 1mM sodium periodate is employed. In another embodiment, the mildly oxidizing agent is galactose oxidase.

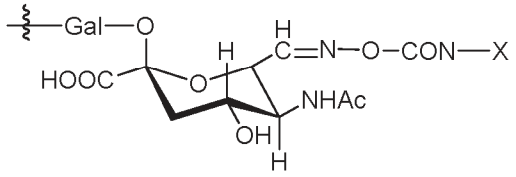
In one embodiment, the method of marking a binding protein comprises reacting an effector moiety of the following structural formula:

NH_2-O-CON-X

with the precursor binding polypeptide comprising an oxidized glycan comprising at least one moiety of the following structural formula:



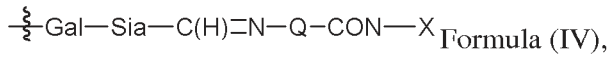
to form a binding polypeptide of the following structural formula:



In an embodiment, the targeting moiety is a trivalent GalNAc glycan moiety. In an embodiment, the reacting step is conducted in the presence of a salt comprising a metal ion. In a further embodiment, wherein the metal ion is a copper ion. In one embodiment, the salt is copper acetate. In another embodiment, the salt comprising a metal ion is present at a concentration of at least 0.1 mM.

In another embodiment, the binding polypeptide comprising the glycan comprises one or two terminal sialic acid residues. In a further embodiment, the terminal sialic acid residues are introduced by treatment of the binding polypeptide with a sialyltransferase or combination of sialyltransferase and galactosyltransferase. In one embodiment, an initial binding polypeptide is contacted with a mildly oxidizing agent in order to produce a precursor binding polypeptide or precursor binding protein. In one embodiment, the precursor binding polypeptide or precursor binding protein comprises an oxidized sialic acid moiety comprising a terminal aldehyde.

In one aspect, the present invention provides a binding polypeptide comprising at least one modified glycan comprising at least one moiety of Formula (IV):



Formula (IV),

wherein:

A) Q is NH or O;

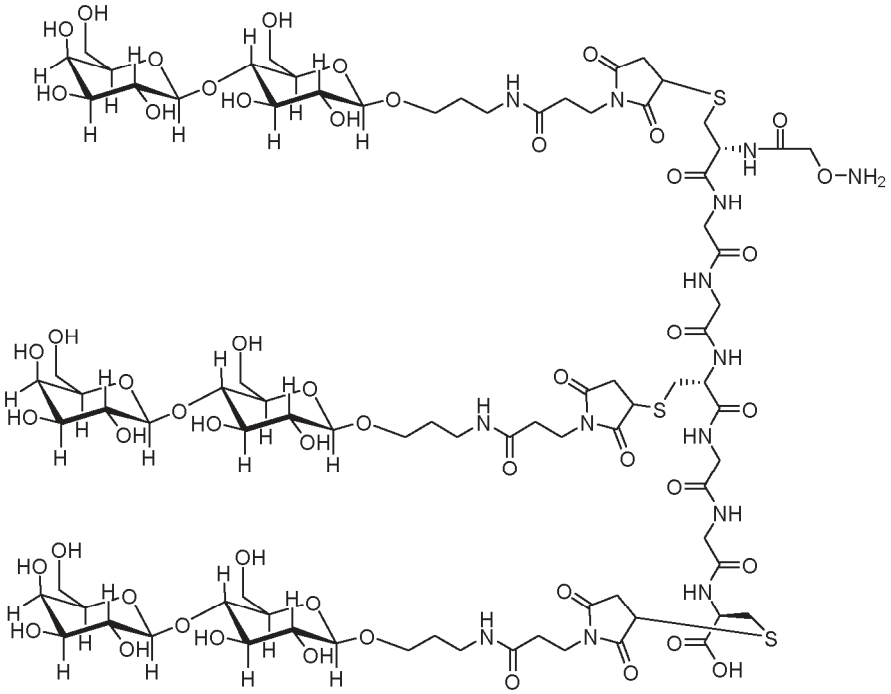
B) CON is a connector moiety; and

C) X is a lactose$_3$-Cys$_3$Gly$_4$ moiety;

D) Gal is a component derived from galactose;

E) Sia is a component derived from sialic acid; and

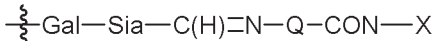
wherein Sia is present and the lactose$_3$-Cys$_3$Gly$_4$ moiety is represented by Formula V:



[Formula V].

In another embodiment, the present invention provides a composition comprising a binding polypeptide described supra and a pharmaceutically acceptable carrier or excipient. In one embodiment, the ratio of targeting moiety to binding polypeptide is equal to or more than about 4. In another embodiment, the ratio of targeting moiety to binding polypeptide is at least about 2. In a further embodiment, the present invention provides a method for treating a patient in need thereof comprising administering an effective amount of the composition.

In one aspect, the present invention provides a binding polypeptide comprising at least one modified glycan comprising at least one moiety of Formula (IV):



Formula (IV),

wherein:

A) Q is NH or O;

B) CON is a connector moiety; and

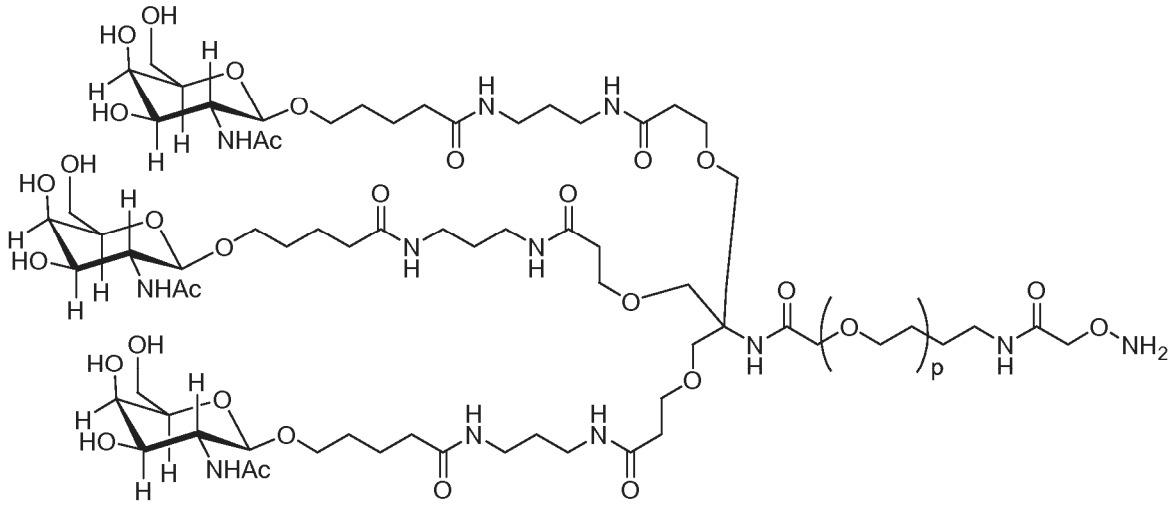
C) X is a moiety comprising PEG;

D) Gal is a component derived from galactose; and

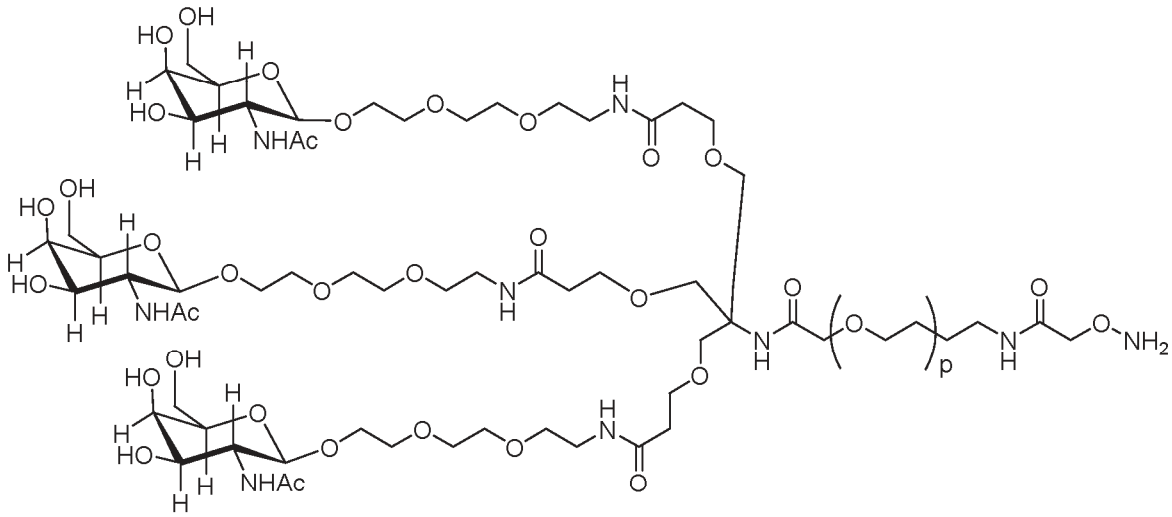
E) Sia is a component derived from sialic acid;

wherein Sia is present or absent.

In one embodiment, the glycan comprises at least one moiety represented by Formula IX or Formula XI:

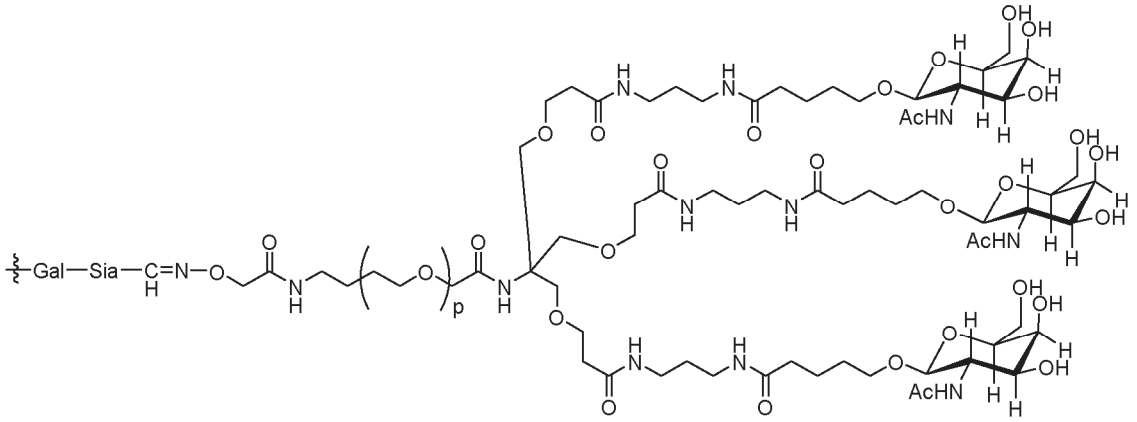


[Formula IX], wherein p has a value of 1 to 32; or

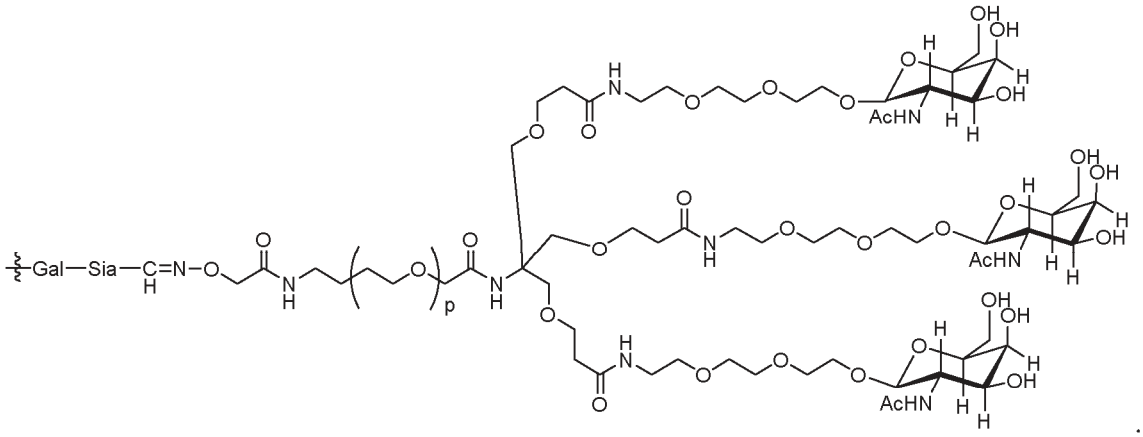


[Formula XI], wherein p has a value of 1 to 32.

In an embodiment, the glycan comprises at least one moiety selected from the following structural formulae:



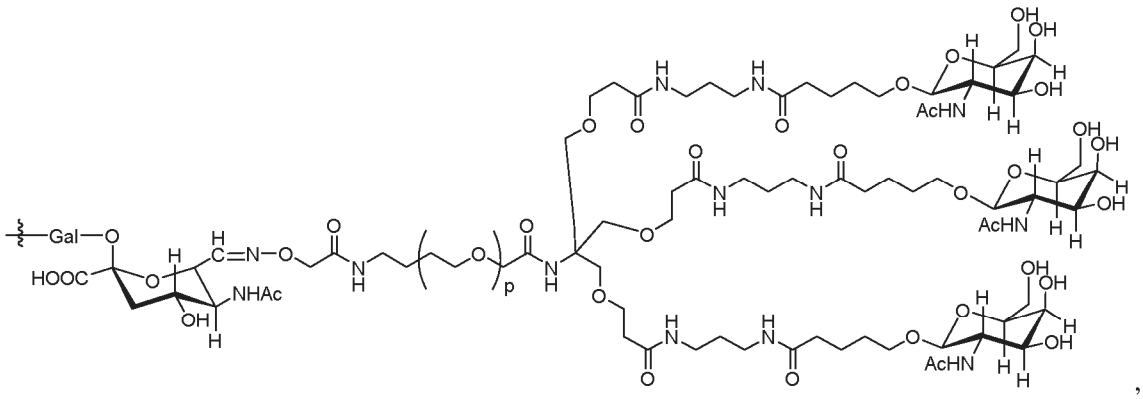
wherein p has a value of 1 to 32; or



.

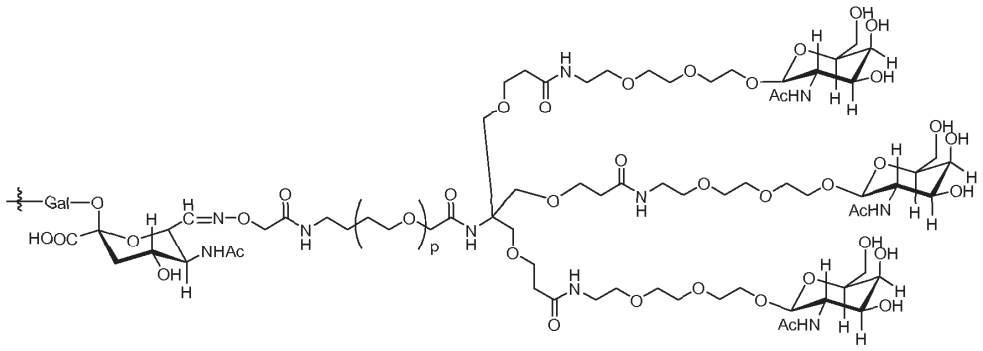
wherein p has a value of 1 to 32.

In one embodiment, the glycan comprises at least one moiety selected from the following structural formulae:



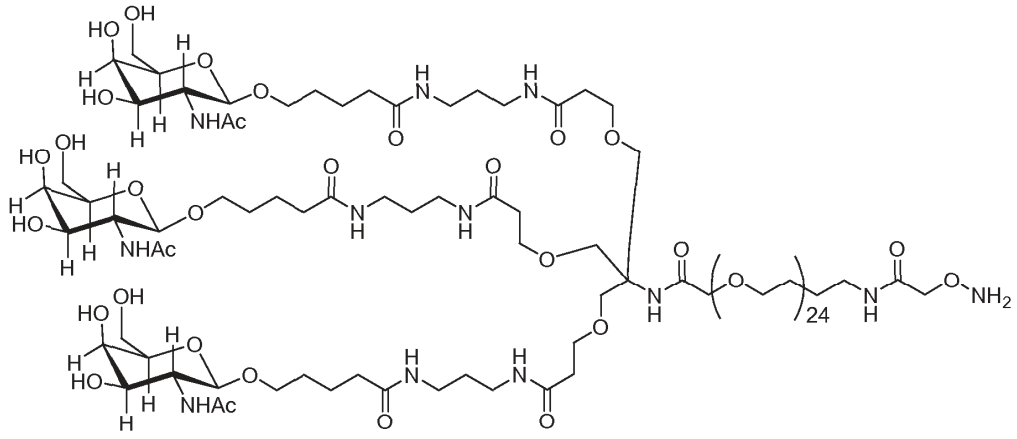
,

wherein p has a value of 1 to 32; or

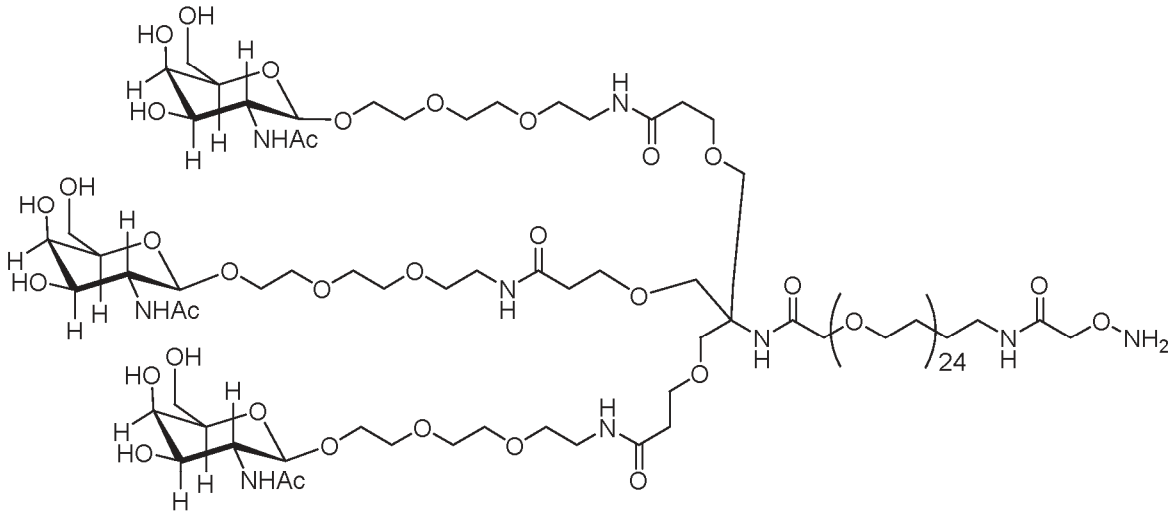


wherein p has a value of 1 to 32.

In an embodiment, the targeting moiety comprises PEG and is represented by Formula X or Formula XII:

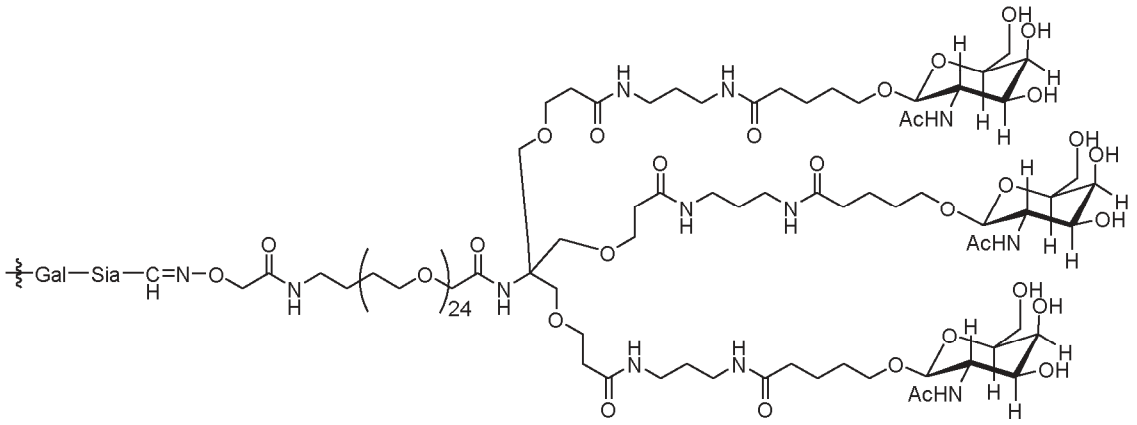


[Formula X]; or

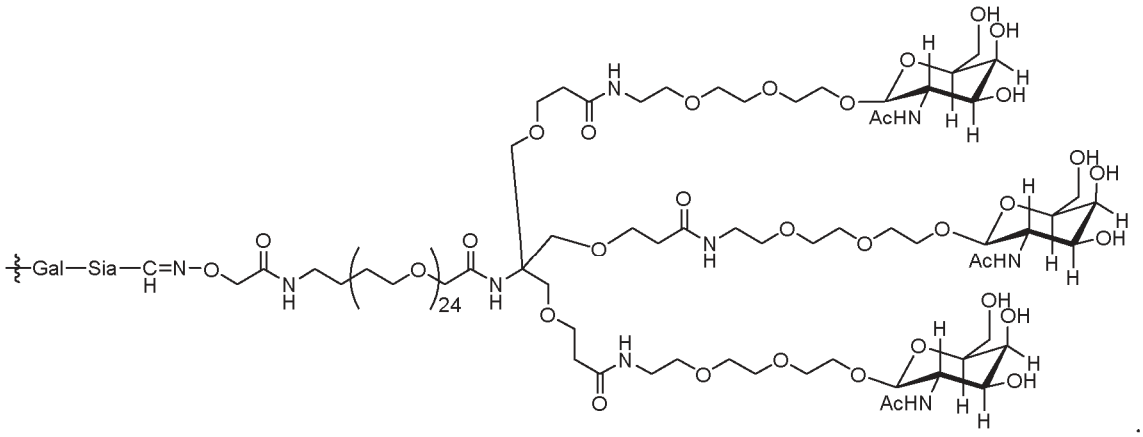


[Formula XII].

In one embodiment, the glycan comprises at least one moiety selected from the following structural formulae:



or



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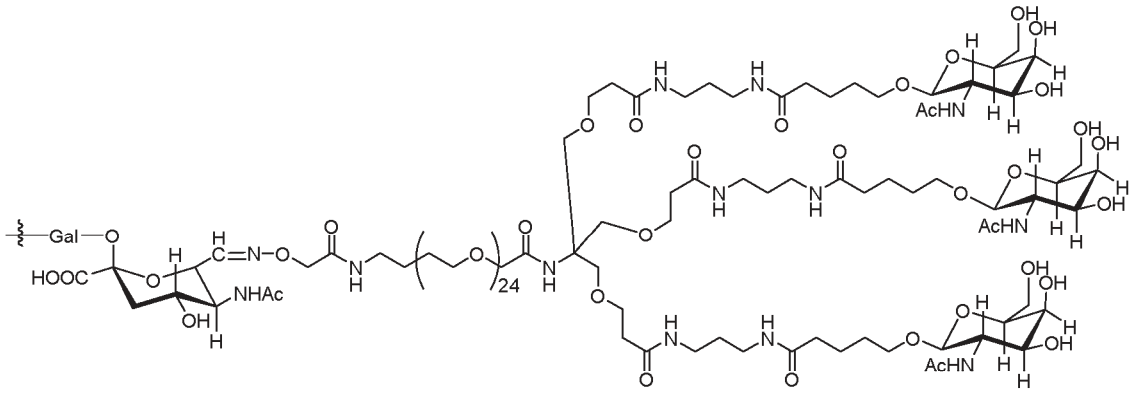
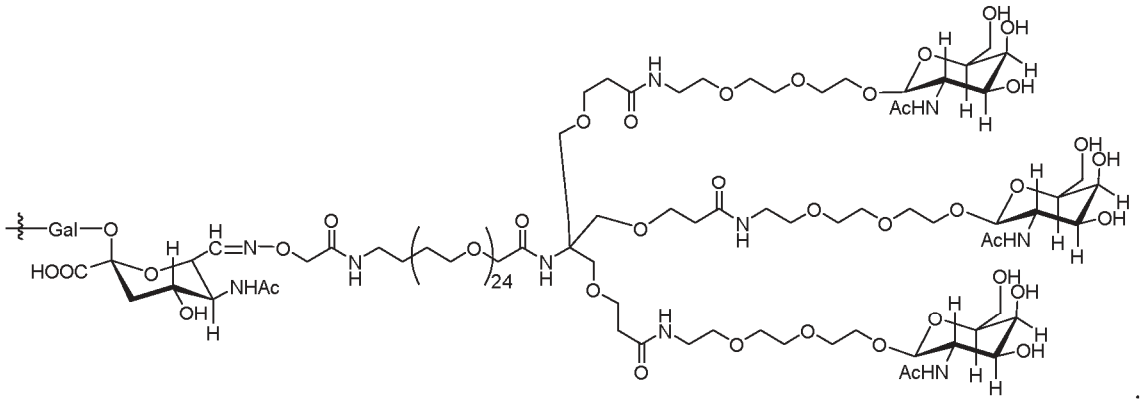
In an embodiment, the glycan comprises at least one moiety selected from the following structural formulae:



or



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In one embodiment, the PEG moiety comprises mono-PEG, bi-PEG, or tri-PEG. In another embodiment, the PEG moiety comprises 3 to 3.5 PEG. In another embodiment, the binding polypeptide is an antibody or immunoadhesin.

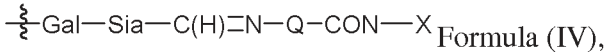
In another aspect, the present invention provides a method of making a PEGylated binding polypeptide comprising at least one oxidized glycan, wherein the method comprises: (a) reacting a binding polypeptide comprising at least one glycan with a mildly oxidizing agent in the presence of a salt comprising a metal ion, and (b) conjugating the oxidized binding polypeptide with at least one moiety comprising PEG.

In one embodiment, the metal ion is a copper ion. In another embodiment, the salt is copper acetate. In another embodiment, the salt comprising a metal ion is present at a concentration of at least 0.1 mM. In another embodiment, the mildly oxidizing agent is

periodate or galactose oxidase. In another embodiment, the at least one glycan is a modified glycan.

In one embodiment, the method of making a binding polypeptide comprises: a) reacting a binding polypeptide comprising at least one modified glycan with a mildly oxidizing agent in the presence of a salt comprising a metal ion; and b) conjugating the oxidized binding polypeptide with the moiety comprising PEG. In a further embodiment, the PEG moiety comprises mono-PEG, bi-PEG, or tri-PEG.

In another aspect, the present invention provides a binding polypeptide comprising at least one modified glycan comprising at least one moiety of Formula (IV):



Formula (IV),

wherein:

A) Q is NH or O;

B) CON is a connector moiety; and

C) X is a trivalent GalNAc glycan;

D) Gal is a component derived from galactose; and

E) Sia is a component derived from sialic acid;

wherein Sia is present and the trivalent GalNAc glycan moiety is represented by Formula VI.

In one embodiment, the binding protein comprises an N-glycan glycoform selected from the group consisting of: a G0 glycoform, a G1 glycoform, and a G2 glycoform. In a further embodiment, the N-glycan glycoform is selected from the group consisting of: a G1S1 glycoform, a G2S1 glycoform, a G2S2 glycoform, a G1F glycoform, a G2F glycoform, a G1S1F glycoform, a G2S1F glycoform, and a G2S2F glycoform.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other features and advantages of the present invention will be more fully understood from the following detailed description of illustrative embodiments taken in conjunction with the accompanying drawings.

Figures 1 (A-C) are a schematic illustration of the synthesis of an antibody drug conjugate where a toxin moiety is linked to an oxidized sialic acid residue of the antibody

glycan using an oxime linkage. **Figure 1A** depicts antibodies with wild type (existing) carbohydrates at position Asn297 and with engineered glycosylation sites at positions 114 (according to the Kabat numbering) and 298 (according to the EU numbering system). **Figure 1B** depicts both the canonical glycan structure G1F (left, seen at position Asn297 on the antibodies shown in **Figure 1A**) and the G2S1F structure (right, seen at positions Ala114 and Asn298 on the antibodies shown in **Figure 1A**). The glycan structures shown in **Figure 1B** have several different subunits: the white square boxes represent GlcNAc, the upside-down white triangle represents fucose, the white circles represent mannose, the shaded circles represent galactose, and the shaded upright triangle represents sialic acid. **Figure 1C** depicts the preparation of a conjugate of the instant invention. On the left is an initial binding protein with a Gal and Sia moiety. Following oxidation with NaIO4, the sialic moiety is oxidized to form a precursor binding protein and then reacted with a toxin-containing moiety to form a binding peptide comprising at least one moiety of Formula IV.

Figure 2 is a Coomassie-blue stained gel showing the expression and purification of glycosylation mutants.

Figure 3 depicts the results of surface plasmon resonance experiments used to assess the binding of αβTCR HEBE1 IgG antibody mutants to recombinant human FcγRIIIa (V158 & F158).

Figure 4 depicts the results of surface plasmon resonance experiments used to assess the binding of αβTCR HEBE1 IgG antibody mutants to recombinant human FcγRI.

Figure 5 depicts the cytokine release profile from PBMCs for TNFa, GM-CSF, IFNy and IL10 in the presence of mutant anti-αβTCR antibodies (day 2).

Figure 6 depicts the cytokine release profile from PBMCs for IL6, IL4 and IL2 in the presence of mutant anti-αβTCR antibodies (day 2).

Figure 7 depicts the cytokine release profile from PBMCs for TNFa, GM-CSF, IFNy and IL10 in the presence of mutant anti-αβTCR antibodies (day 4).

Figure 8 depicts the cytokine release profile from PBMCs for IL6, IL4 and IL2 in the presence of mutant anti-αβTCR antibodies (day 4).

Figures 9 (A-B) depict the results of experiments investigating the expression level of 2C3 mutants by Western blotting (**Figure 9A**) and surface plasmon resonance (**Figure 9B**).

Figure 10 depicts the results of experiments investigating glycosylation of 2C3 mutants pre- and post- PNGase F treatment.

Figure 11 depicts the results of SDS-PAGE experiments investigating glycosylation sites on 2C3 mutants isolated from cell culture.

Figures 12 (A-C) depict the results of surface plasmon resonance experiments used to assess the binding of modified anti-CD52 to recombinant human FcγRIIIa (V158). Anti-CD52 comprising S298N/Y300S mutations in the Fc domain were used to assess the effector function of the modified molecule. binding to CD52 peptide (**Figure 12A**), binding to FcγRIIIa (V158, **Figure 12B**), andcontrol binding to mouse FcRn (**Figure 12C**).

Figure 13 depicts the results of surface plasmon resonance experiments investigating the Fc binding properties of 2C3 mutants.

Figures 14 (A-B) depict the results of surface plasmon resonance experiments investigating the binding of modified anti-CD52 to both FcγRIIIa (Val158) (as above) and FcγRIIIa (Phe158). Anti-CD52 antibodies comprising S298N/Y300S mutations in the Fc domain were used to assess the effector function of the modified molecule binding to FcγRIIIa (Val158, Figure 14A) and FcγRIIIa (Phe58, Figure 14B).

Figures 15 (A-B) depict the analysis of C1q binding in the S298N/Y300S mutant and the WT 2C3 control (**Figure 15A**) and the results of an Eliza analysis confirming equivalent coating of the wells (**Figure 15B**).

Figure 16 depicts the results of plasmon resonance experiments experiments measuring the binding kinetics of 2C3 mutants to CD-52 peptide 741.

Figure 17 depicts the results of plasmon resonance experiments experiments comparing the antigen binding affinity of WT anti-CD-52 2C3 and the A114N hyperglycosylation mutant.

Figures 18 (A-D) depict the results of isoelectric focusing and mass spectrometry charge characterization experiments to determine the glycan content of 2C3 mutants.

Figures 19 (A-B) depict the results of concentration (Octet) and plamon resonance experiments comparing the antigen binding affinity of WT anti-CD52 2C3 and mutants.

Figure 20 depicts the results of SDS-PAGE experiments to demonstrate the additional glycosylation of the anti-TEM1 A114N mutant.

Figure 21 depicts the results of SDS-PAGE and hydrophobic interaction chromatography analysis of the A114N anti-Her2 mutant.

Figure 22 depicts the results of SDS-PAGE experiments to demonstrate the conjugation of PEG to the 2C3 A114N mutant through an aminooxy linkage.

Figure 23 depicts the results of LC-MS experiments to determine the glycan contents of anti-TEM1 A114N hyperglycosylation mutant.

Figure 24 depicts the results of LC-MS experiments to determine the glycan contents of a wild-type HER2 antibody and an A114N anti-Her2 hyperglycosylation mutant.

Figures 25 (A-C) depict an exemplary method for performing site-specific conjugation of an antibody.

Figure 26 depicts a synthesis of exemplary effector moieties: aminooxy-Cys-MC-VC-PABC-MMAE and aminooxy-Cys-MC-VC-PABC-PEG8-Dol10.

Figures 27 (A-C) depict characterization information for a sialylated HER2 antibody.

Figures 28 (A-D) depict characterization information for oxidized sialylated anti-HER 2 antibody.

Figure 29 depicts hydrophobic interaction chromatographs of glycoconjugates prepared with three different sialylated antibodies with two different aminooxy groups.

Figure 30 shows a HIC chromatograph of antiHer2 A114 glycosylation mutant conjugate with AO-MMAE prepared using GAM(+) chemistry.

Figures 31 (A-D) depict a comparison of the *in vitro* potency of an anti-HER2 glycoconjugate and thiol conjugate.

Figure 32 depicts a comparison of the *in vitro* potency of an anti FAP B11 glycoconjugate and thiol conjugate.

Figures 33 (A-D) depict a comparison of *in vivo* efficacy of anti-HER2 glycoconjugates and thiol conjugates in a Her2+ tumor cell xenograft model.

Figure 34 depicts the results of LC-MS experiments to determine the glycan content of a mutant anti-αβTCR antibody containing the S298N/Y300S mutation.

Figure 35 depicts the results of circular dichroism experiments to determine the relative thermal stability of a wild-type anti-αβTCR antibody and mutant anti-αβTCR antibody containing the S298N/Y300S mutation.

Figure 36 depicts the results of a cell proliferation assay for ADC prepared with the anti-HER antibody bearing the A114N hyperglycosylation mutation and AO-MMAE.

Figure 37 is a schematic illustration of the synthesis of an antibody drug conjugate where a targeting moiety is linked to an oxidized sialic acid residue of the antibody glycan using an oxime linkage.

Figure 38 is a schematic illustration depicting an exemplary method for performing site-specific conjugation of an antibody to a glycopeptide through an oxime linkage according to the described methods.

Figure 39 is a schematic illustration depicting site-specific conjugation of neoglycans to antibody through sialic acid in native Fc glycans.

Figure 40 is a series of exemplary glycans that may be used for conjugation including lactose aminooxy and bis Man-6-P (or bisM6P) hexamannose aminooxy (for aminooxy conjugation).

Figure 41 is a schematic depiction of the preparation of Man-6-P hexamannose maleimide.

Figure 42 depicts SDS-PAGE and MALDI-TOF characterization of Man-6-P hexamannose aminooxy conjugates made with rabbit polyclonal antibody.

Figure 43 depicts the results of surface plasmon resonance experiments used to assess the binding of control and Man-6-P hexamannose conjugated rabbit IgG antibodies to Man-6-P receptor.

Figure 44 depicts the uptake of Man-6-P conjugated rabbit IgG antibody in HepG2 and RAW cells.

Figure 45 depicts the characterization of control, Man-6-P conjugated, and lactose conjugated antibodies through SDS-PAGE and lectin blotting.

Figure 46 depicts the results of MALDI-TOF intact protein analyses for control, Man-6-P conjugated, and lactose conjugated antibodies.

Figure 47 depicts the characterization of polyclonal antibody conjugated to Man-6-P hexamannose maleimide (thiol conjugation at hinge cysteines) through SDS-PAGE (non-reducing and reducing), lectin blot (reducing), and Man-6-P quantitation.

Figure 48 depicts the characterization of polyclonal antibody conjugated to lactose maleimide (thio conjugation at hinge cysteines) through SDS-PAGE and galactose quantitation.

Figure 49 depicts the characterization of monoclonal antibody conjugated to Man-6-P hexamannose maleimide (thiol conjugation at hinge cysteines) through SDS-PAGE (non-reducing and reducing), and glycan (bis Man-6-P) quantitation.

Figure 50 depicts the results of size exclusion chromatography (SEC) analysis of a hinge cysteine polyclonal antibody conjugate.

Figure 51 depicts the results of size exclusion chromatography (SEC) analysis of a hinge cysteine monoclonal antibody conjugate.

Figure 52 depicts the results of sialidase titration and sialic acid quantitation to determine the amount of sialic acid release from NNAS, sialylated NNAS, and desialylated and galatosylated NNAS antibodies.

Figure 53 depicts the results of MALDI-TOF MS analysis to determine the glycan structures of a mouse NNAS antibody and a desialylated and galactosylated NNAS antibody.

Figure 54 depicts the results of MALDI-TOF MS analysis to determine the glycan structures of a mouse NNAS antibody and a sialylated NNAS antibody.

Figure 55 depicts the characterization of Man-6-P receptor (CI-MPR) bound to bis Man-6-P glycan-conjugated polyclonal and monoclonal antibodies through native Fc glycan or hinge disulfides using native PAGE.

Figure 56 depicts the characterization of enzyme modified and glycopeptide conjugated NNAS antibodies by SDS-PAGE (4-12% NuPAGE; reducing and non-reducing) and ECL lectin blotting (reducing).

Figure 57 depicts the results of terminal galactose quantitation in an NNAS antibody, a desialylated/galactosylated NNAS antibody, and a conjugated NNAS antibody in mol galactose or mol glycopeptide per mol antibody.

Figure 58 depicts the examination of lactose maleimide that had been modified with alpha-2,3-sialyltransferase and eluted from QAE purification columns with 20 mM NaCl. The resultant eluate was characterized using MALDI-TOF MS and Dionex HPLC.

Figures 59 (A-B) depict the characterization of rabbit polyclonal antibody conjugated with sialyllactose maleimide (thiol reaction) using SDS-PAGE and Dionex HPLC (sialic acid quantitation).

Figures 60 (A-D) depict the characterization of lactose maleimide sialylated with alpha-2,6-sialyltransferase and purified using a QAE-sepharose column. Analysis using Dionex HPLC is shown for (**Figure 60A**) a lactose standard; (**Figure 60B**) an alpha-2,6-sialyllactose standard; (**Figure 60C**) a lactose maleimide standard; and (**Figure 60D**) a fraction of alpha-2,6-sialyllactose maleimide eluted from a QAE-sepharose column.

Figure 61 depicts the characterization of a fraction of alpha-2,6-sialyllactose maleimide eluted from a QAE-sepharose column using MALDI-TOF MS.

Figures 62 (A-B) depict the characterization of a control antibody, an alpha-2,3-sialyllactose glycan conjugated polyclonal antibody, and an alpha-2,6-sialyllactose glycan conjugated polyclonal antibody through SDS-PAGE and Dionex HPLC (graph of sialic acid analysis shown).

Figure 63 depicts the characterization of control and enzyme modified (desialylated/galactosylated) NNAS mutant antibodies using SDS-PAGE and lectin blotting.

Figure 64 depicts the characterization through reducing and non-reducing SDS-PAGE of the PEGylated control antibody and Gal NNAS with various amounts of galactose oxidase.

Figure 65 depicts the results of estimated PEGylation of an antibody heavy chain from previous galactose oxidase titration using ProteinSimple.

Figure 66 depicts the characterization through reducing and non-reducing SDS-PAGE of the PEGylated control antibody and Gal NNAS with various molar excess of PEG over antibody.

Figure 67 depicts the results of estimated PEGylation of an antibody heavy chain from previous PEG titration using ProteinSimple.

Figure 68 is a structural drawing of aminooxy glycopeptide, lactose$_3$-Cys$_3$Gly$_4$.

Figures 69 (A-B) depict the characterization through reducing SDS-PAGE of the PEGylated control antibody and Gal NNAS with galactose oxidase in the absence of copper

acetate (**Figure 69A**) and in the presence of varying amounts of copper acetate (**Figure 69A and Figure 69B**).

Figure 70 depicts the characterization of enzyme modified wild-type, A114N, NNAS, and A114N/NNAS Herceptin by SDS-PAGE (4-12% NuPAGE; reducing and non-reducing) and ECL lectin blotting (reducing) along with the results of terminal galactose quantitation in mol galactose per mol antibody.

Figure 71 is a table depicting the sialic acid content (in mol/mol) of wild-type and mutant antibodies as measured using Dionex HPLC.

Figure 72 depicts the characterization of the PEGylation of wild-type and mutant antibodies through reducing and non-reducing SDS-PAGE.

Figure 73 is a table depicting the PEGylation (in mol/mol) of wild-type and mutant antibodies estimated using ProteinSimple.

Figure 74 is a series of photos depicting immunofluorescence staining of HepG2 cell uptake of control, enzyme modified (with galactosyltransferase), or conjugated (with lactose aminoxy or lactose maleimide) antibodies.

Figure 75 is a depiction of an exemplary trivalent GalNAc glycan.

Figure 76 depicts the results of surface plasmon resonance experiments used to assess the binding of trivalent GalNAc glycan-conjugated antibodies to ASGPR subunit H1.

Figure 77 is a depiction of a trivalent GalNAc-containing glycan and a trivalent galactose-containing glycopeptide used for conjugation.

Figure 78 depicts the results of surface plasmon resonance experiments used to assess the binding of trivalent GalNAc-conjugated and trivalent galactose containing glycopeptide-conjugated recombinant lysosomal enzymes to ASGPR subunit H1.

Figures 79 (A-D) are depiction of additional trivalent GalNAc glycans.

Figure 80 is a depiction of the results of conjugation of periodate oxidized recombinant lysosomal enzyme rhGAA with an excess of trivalent GalNAc glycan C12 (at 20, 40, 80, and 200-fold molar excess glycan over rhGAA). The resulting glycan conjugated per rhGAA is depicted.

Figure 81 depicts ASGPR binding of a recombinant lysosomal enzymes conjugated with trivalent GalNAc glycan C12 on Biacore. Enzymes conjugated with 20 (conjugate 1), 40, 80, and 200-fold (conjugate 4) excess of glycan all show strong binding to ASGPR subunit 1. There is no significant difference in binding among the conjugates (conjugates 1 to 4).

DETAILED DESCRIPTION

The current disclosure provides binding polypeptides (e.g., antibodies), and effector moiety conjugates (e.g., targeting moiety conjugates) thereof. In certain embodiments, the conjugates comprise a site-specifically engineered drug-glycan linkage within native or modified glycans of an antigen binding polypeptide such as an IgG molecule. The current disclosure also provides nucleic acids encoding antigen-binding polypeptides, recombinant expression vectors and host cells for making antigen-binding polypeptides. Methods of using the antigen-binding polypeptides disclosed herein to treat disease are also provided.

I. Definitions

Unless otherwise defined herein, scientific and technical terms used herein have the meanings that are commonly understood by those of ordinary skill in the art. In the event of any latent ambiguity, definitions provided herein take precedent over any dictionary or extrinsic definition. Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The use of "or" means "and/or" unless stated otherwise. The use of the term "including", as well as other forms, such as "includes" and "included", is not limiting.

Generally, nomenclatures used in connection with 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 provided herein 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. Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein.

The nomenclatures 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 are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

That the disclosure may be more readily understood, select terms are defined below.

[001] The term "polypeptide" refer to any polymeric chain of amino acids and encompasses native or artificial proteins, polypeptide analogs or variants of a protein sequence, or fragments thereof, unless otherwise contradicted by context. A polypeptide may be monomeric or polymeric. For an antigenic polypeptide, a fragment of a polypeptide optionally contains at least one contiguous or nonlinear epitope of a polypeptide. The precise boundaries of the at least one epitope fragment can be confirmed using ordinary skill in the art. A polypeptide fragment comprises at least about 5 contiguous amino acids, at least about 10 contiguous amino acids, at least about 15 contiguous amino acids, or at least about 20 contiguous amino acids, for example.

[002] The term "isolated protein" or "isolated polypeptide" refer to a protein or polypeptide that by virtue of its origin or source of derivation is not associated with naturally associated components that accompany it in its native state; is substantially free of other proteins from the same species; is expressed by a cell from a different species; or does not occur in nature. Thus, a protein or polypeptide that is chemically synthesized or synthesized in a cellular system different from the cell from which it naturally originates will be "isolated" from its naturally associated components. A protein or polypeptide may also be rendered substantially free of naturally associated components by isolation using protein purification techniques well known in the art.

As used herein, the term "binding protein" or "binding polypeptide" shall refer to a protein or polypeptide (e.g., an antibody or fragment thereof) that contains at least one binding site which is responsible for selectively binding to a target antigen of interest (e.g., a human antigen). Exemplary binding sites include an antibody variable domain, a ligand binding site of a receptor, or a receptor binding site of a ligand. In certain aspects, the binding proteins or binding polypeptides comprise multiple (e.g., two, three, four, or more)

binding sites. In certain aspects, the binding protein or binding polypeptide is not a therapeutic enzyme.

As used herein, the term "native residue" shall refer to an amino acid residue that occurs naturally at a particular amino acid position of a binding polypeptide (e.g., an antibody or fragment thereof) and which has not been modified, introduced, or altered by the hand of man. As used herein, the term "altered binding protein," "altered binding polypeptide," "modified binding protein" or "modified binding polypeptide" shall refer to binding polypeptides and/or binding proteins (e.g., an antibody or fragment thereof) comprising at least one amino acid substitution, deletion and/or addition relative to the native (i.e., wild-type) amino acid sequence, and/or a mutation that results in altered glycosylation (e.g., hyperglycosylation, hypoglycosylation and/or aglycosylation) at one or more amino acid positions relative to the native (i.e., wild-type) amino acid sequence.

As used herein, the term "initial binding polypeptide" or "initial binding protein" shall refer to a binding polypeptide or binding protein that is contacted with a mildly oxidizing agent to produce a "precursor binding polypeptide" or a "precursor binding protein," respectively (see **Figures 1A-C**). As used herein, the term "precursor binding polypeptide" or "precursor binding protein" shall refer to a mildly oxidized polypeptide or protein that can be reacted with one or more of the effector moieties described herein. In certain embodiments, an initial binding polypeptide, initial binding protein, precursor binding polypeptide, and/or precursor binding protein (e.g., an antibody or fragment thereof) contains at least one binding site which is responsible for selectively binding to a target antigen of interest (e.g., a human antigen). Exemplary binding sites include an antibody variable domain, a ligand binding site of a receptor, or a receptor binding site of a ligand. In certain aspects, an initial binding polypeptide, initial binding protein, precursor binding polypeptide, and/or precursor binding protein comprises multiple (e.g., two, three, four, or more) binding sites. In certain aspects, the initial binding polypeptide, initial binding protein, precursor binding polypeptide, and/or precursor binding protein is not a therapeutic enzyme. An initial binding polypeptide, initial binding protein, precursor binding polypeptide, and/or precursor binding protein may have a wild-type sequence or they may comprise at least one amino acid substitution, deletion and/or addition relative to the native (i.e., wild-type) amino acid sequence, and/or a mutation that results in altered glycosylation (e.g., hyperglycosylation,

hypoglycosylation and/or aglycosylation) at one or more amino acid positions relative to the native (i.e., wild-type) amino acid sequence.

The term “ligand” refers to any substance capable of binding, or of being bound, to another substance. Similarly, the term “antigen” refers to any substance to which an antibody may be generated. Although “antigen” is commonly used in reference to an antibody binding substrate, and “ligand” is often used when referring to receptor binding substrates, these terms are not distinguishing, one from the other, and encompass a wide range of overlapping chemical entities. For the avoidance of doubt, antigen and ligand are used interchangeably throughout herein. Antigens/ligands may be a peptide, a polypeptide, a protein, an aptamer, a polysaccharide, a sugar molecule, a carbohydrate, a lipid, an oligonucleotide, a polynucleotide, a synthetic molecule, an inorganic molecule, an organic molecule, and any combination thereof.

The term "specifically binds" as used herein, refers to the ability of an antibody or an antigen-binding fragment thereof to bind to an antigen with a dissociation constant (Kd) of at most about 1×10^{-6} M, 1×10^{-7} M, 1×10^{-8} M, 1×10^{-9} M, 1×10^{-10} M, 1×10^{-11} M, 1×10^{-12} M, or less, and/or to bind to an antigen with an affinity that is at least two-fold greater than its affinity for a nonspecific antigen.

As used herein, the term "antibody" refers to such assemblies (e.g., intact antibody molecules, antibody fragments, or variants thereof) which have significant known specific immunoreactive activity to an antigen of interest (e.g. a tumor associated antigen). Antibodies and immunoglobulins comprise light and heavy chains, with or without an interchain covalent linkage between them. Basic immunoglobulin structures in vertebrate systems are relatively well understood.

As will be discussed in more detail below, the generic term "antibody" comprises five distinct classes of antibody that can be distinguished biochemically. While all five classes of antibodies are clearly within the scope of the current disclosure, the following discussion will generally be directed to the IgG class of immunoglobulin molecules. With regard to IgG, immunoglobulins comprise two identical light chains of molecular weight approximately 23,000 Daltons, and two identical heavy chains of molecular weight 53,000-70,000. The four chains are joined by disulfide bonds in a "Y" configuration wherein the light chains bracket the heavy chains starting at the mouth of the "Y" and continuing through the variable region.

Light chains of immunoglobulin are classified as either kappa or lambda (κ, λ). Each heavy chain class may be bound with either a kappa or lambda light chain. In general, the light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages or non-covalent linkages when the immunoglobulins are generated either by hybridomas, B cells, or genetically engineered host cells. In the heavy chain, the amino acid sequences run from an N-terminus at the forked ends of the Y configuration to the C-terminus at the bottom of each chain. Those skilled in the art will appreciate that heavy chains are classified as gamma, mu, alpha, delta, or epsilon, (γ, μ, α, δ, ε) with some subclasses among them (e.g., γl-γ4). It is the nature of this chain that determines the "class" of the antibody as IgG, IgM, IgA IgG, or IgE, respectively. The immunoglobulin isotype subclasses (e.g., IgG1, IgG2, IgG3, IgG4, IgA1, etc.) are well characterized and are known to confer functional specialization. Modified versions of each of these classes and isotypes are readily discernable to the skilled artisan in view of the instant disclosure and, accordingly, are within the scope of the current disclosure.

Both the light and heavy chains are divided into regions of structural and functional homology. The term "region" refers to a part or portion of an immunoglobulin or antibody chain and includes constant region or variable regions, as well as more discrete parts or portions of said regions. For example, light chain variable regions include "complementarity determining regions" or "CDRs" interspersed among "framework regions" or "FRs", as defined herein.

The regions of an immunoglobulin heavy or light chain may be defined as "constant" (C) region or "variable" (V) regions, based on the relative lack of sequence variation within the regions of various class members in the case of a "constant region", or the significant variation within the regions of various class members in the case of a "variable regions". The terms "constant region" and "variable region" may also be used functionally. In this regard, it will be appreciated that the variable regions of an immunoglobulin or antibody determine antigen recognition and specificity. Conversely, the constant regions of an immunoglobulin or antibody confer important effector functions such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. The subunit structures and three dimensional configurations of the constant regions of the various immunoglobulin classes are well known.

The constant and variable regions of immunoglobulin heavy and light chains are folded into domains. The term "domain" refers to a globular region of a heavy or light chain comprising peptide loops (e.g., comprising 3 to 4 peptide loops) stabilized, for example, by β-pleated sheet and/or intrachain disulfide bond. Constant region domains on the light chain of an immunoglobulin are referred to interchangeably as "light chain constant region domains", "CL regions" or "CL domains". Constant domains on the heavy chain (e.g. hinge, CH1, CH2 or CH3 domains) are referred to interchangeably as "heavy chain constant region domains", "CH" region domains or "CH domains". Variable domains on the light chain are referred to interchangeably as "light chain variable region domains", "VL region domains or "VL domains". Variable domains on the heavy chain are referred to interchangeably as "heavy chain variable region domains", "VH region domains" or “VH domains".

By convention the numbering of the variable constant region domains increases as they become more distal from the antigen binding site or amino-terminus of the immunoglobulin or antibody. The N-terminus of each heavy and light immunoglobulin chain is a variable region and at the C-terminus is a constant region; the CH3 and CL domains actually comprise the carboxy-terminus of the heavy and light chain, respectively. Accordingly, the domains of a light chain immunoglobulin are arranged in a VL-CL orientation, while the domains of the heavy chain are arranged in the VH-CH1-hinge-CH2-CH3 orientation.

Amino acid positions in a heavy chain constant region, including amino acid positions in the CH1, hinge, CH2, CH3, and CL domains, may be numbered according to the Kabat index numbering system (see Kabat et al, in "Sequences of Proteins of Immunological Interest", U.S. Dept. Health and Human Services, 5th edition, 1991). Alternatively, antibody amino acid positions may be numbered according to the EU index numbering system (see Kabat et al, ibid).

As used herein, the term "VH domain" includes the amino terminal variable domain of an immunoglobulin heavy chain, and the term "VL domain" includes the amino terminal variable domain of an immunoglobulin light chain.

As used herein, the term "CH1 domain" includes the first (most amino terminal) constant region domain of an immunoglobulin heavy chain that extends, e.g., from about positions 114-223 in the Kabat numbering system (EU positions 118-215). The CH1 domain is adjacent to the VH domain and amino terminal to the hinge region of an immunoglobulin

heavy chain molecule, and does not form a part of the Fc region of an immunoglobulin heavy chain.

As used herein, the term "hinge region" includes the portion of a heavy chain molecule that joins the CH1 domain to the CH2 domain. This hinge region comprises approximately 25 residues and is flexible, thus allowing the two N-terminal antigen binding regions to move independently. Hinge regions can be subdivided into three distinct domains: upper, middle, and lower hinge domains (Roux et al. J. Immunol. 1998, 161 :4083).

As used herein, the term "CH2 domain" includes the portion of a heavy chain immunoglobulin molecule that extends, e.g., from about positions 244-360 in the Kabat numbering system (EU positions 231-340). The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. In one embodiment, a binding polypeptide of the current disclosure comprises a CH2 domain derived from an IgG1 molecule (e.g. a human IgG1 molecule).

As used herein, the term "CH3 domain" includes the portion of a heavy chain immunoglobulin molecule that extends approximately 110 residues from N-terminus of the CH2 domain, e.g., from about positions 361-476 of the Kabat numbering system (EU positions 341 -445). The CH3 domain typically forms the C-terminal portion of the antibody. In some immunoglobulins, however, additional domains may extend from CH3 domain to form the C-terminal portion of the molecule (e.g. the CH4 domain in the μ chain of IgM and the e chain of IgE). In one embodiment, a binding polypeptide of the current disclosure comprises a CH3 domain derived from an IgG1 molecule (e.g. a human IgG1 molecule).

As used herein, the term "CL domain" includes the constant region domain of an immunoglobulin light chain that extends, e.g., from about Kabat position 107A-216. The CL domain is adjacent to the VL domain. In one embodiment, a binding polypeptide of the current disclosure comprises a CL domain derived from a kappa light chain (e.g., a human kappa light chain).

As used herein, the term "Fc region" is defined as the portion of a heavy chain constant region beginning in the hinge region just upstream of the papain cleavage site (i.e. residue 216 in IgG, taking the first residue of heavy chain constant region to be 114) and ending at the C-terminus of the antibody. Accordingly, a complete Fc region comprises at least a hinge domain, a CH2 domain, and a CH3 domain.

The term "native Fc" as used herein refers to a molecule comprising the sequence of a non-antigen-binding fragment resulting from digestion of an antibody or produced by other means, whether in monomeric or multimeric form, and can contain the hinge region. The original immunoglobulin source of the native Fc is typically of human origin and can be any of the immunoglobulins, such as IgG1 and IgG2. Native Fc molecules are made up of monomeric polypeptides that can be linked into dimeric or multimeric forms by covalent (i.e., disulfide bonds) and non-covalent association. The number of intermolecular disulfide bonds between monomeric subunits of native Fc molecules ranges from 1 to 4 depending on class (e.g., IgG, IgA, and IgE) or subclass (e.g., IgG1, IgG2, IgG3, IgA1, and IgGA2). One example of a native Fc is a disulfide-bonded dimer resulting from papain digestion of an IgG. The term "native Fc" as used herein is generic to the monomeric, dimeric, and multimeric forms.

The term "Fc variant" as used herein refers to a molecule or sequence that is modified from a native Fc but still comprises a binding site for the salvage receptor, FcRn (neonatal Fc receptor). Exemplary Fc variants, and their interaction with the salvage receptor, are known in the art. Thus, the term "Fc variant" can comprise a molecule or sequence that is humanized from a non-human native Fc. Furthermore, a native Fc comprises regions that can be removed because they provide structural features or biological activity that are not required for the antibody-like binding polypeptides featured in the invention. Thus, the term "Fc variant" comprises a molecule or sequence that lacks one or more native Fc sites or residues, or in which one or more Fc sites or residues has be modified, that affect or are involved in: (1) disulfide bond formation, (2) incompatibility with a selected host cell, (3) N-terminal heterogeneity upon expression in a selected host cell, (4) glycosylation, (5) interaction with complement, (6) binding to an Fc receptor other than a salvage receptor, or (7) antibody-dependent cellular cytotoxicity (ADCC).

The term "Fc domain" as used herein encompasses native Fc and Fc variants and sequences as defined above. As with Fc variants and native Fc molecules, the term "Fc domain" includes molecules in monomeric or multimeric form, whether digested from whole antibody or produced by other means.

As indicated above, the variable regions of an antibody allow it to selectively recognize and specifically bind epitopes on antigens. That is, the VL domain and VH domain of an antibody combine to form the variable region (Fv) that defines a three dimensional

antigen binding site. This quaternary antibody structure forms the antigen binding site present at the end of each arm of the Y. More specifically, the antigen binding site is defined by three complementary determining regions (CDRs) on each of the heavy and light chain variable regions. As used herein, the term "antigen binding site" includes a site that specifically binds (immunoreacts with) an antigen (e.g., a cell surface or soluble antigen). The antigen binding site includes an immunoglobulin heavy chain and light chain variable region and the binding site formed by these variable regions determines the specificity of the antibody. An antigen binding site is formed by variable regions that vary from one antibody to another. The altered antibodies of the current disclosure comprise at least one antigen binding site.

In certain embodiments, binding polypeptides of the current disclosure comprise at least two antigen binding domains that provide for the association of the binding polypeptide with the selected antigen. The antigen binding domains need not be derived from the same immunoglobulin molecule. In this regard, the variable region may or be derived from any type of animal that can be induced to mount a humoral response and generate immunoglobulins against the desired antigen. As such, the variable region of the a binding polypeptide may be, for example, of mammalian origin e.g., may be human, murine, rat, goat, sheep, non-human primate (such as cynomolgus monkeys, macaques, etc.), lupine, or camelid (e.g., from camels, llamas and related species).

In naturally occurring antibodies, the six CDRs present on each monomeric antibody are short, non-contiguous sequences of amino acids that are specifically positioned to form the antigen binding site as the antibody assumes its three dimensional configuration in an aqueous environment. The remainder of the heavy and light variable domains show less inter-molecular variability in amino acid sequence and are termed the framework regions. The framework regions largely adopt a β-sheet conformation and the CDRs form loops which connect, and in some cases form part of, the β-sheet structure. Thus, these framework regions act to form a scaffold that provides for positioning the six CDRs in correct orientation by inter-chain, non-covalent interactions. The antigen binding domain formed by the positioned CDRs defines a surface complementary to the epitope on the immunoreactive antigen. This complementary surface promotes the non-covalent binding of the antibody to the immunoreactive antigen epitope.

Exemplary binding polypeptides include antibody variants. As used herein, the term “antibody variant” includes synthetic and engineered forms of antibodies which are altered

such that they are not naturally occurring, e.g., antibodies that comprise at least two heavy chain portions but not two complete heavy chains (such as, domain deleted antibodies or minibodies); multispecific forms of antibodies (e.g., bispecific, trispecific, etc.) altered to bind to two or more different antigens or to different epitopes on a single antigen); heavy chain molecules joined to scFv molecules and the like. In addition, the term "antibody variant" includes multivalent forms of antibodies (e.g., trivalent, tetravalent, etc., antibodies that bind to three, four or more copies of the same antigen.

As used herein the term "valency" refers to the number of potential target binding sites in a polypeptide. Each target binding site specifically binds one target molecule or specific site on a target molecule. When a polypeptide comprises more than one target binding site, each target binding site may specifically bind the same or different molecules (e.g., may bind to different ligands or different antigens, or different epitopes on the same antigen). The subject binding polypeptides typically has at least one binding site specific for a human antigen molecule.

The term "specificity" refers to the ability to specifically bind (e.g., immunoreact with) a given target antigen (e.g., a human target antigen). A binding polypeptide may be monospecific and contain one or more binding sites which specifically bind a target or a polypeptide may be multispecific and contain two or more binding sites which specifically bind the same or different targets. In certain embodiments, a binding polypeptide is specific for two different (e.g., non-overlapping) portions of the same target. In certain embodiments, a binding polypeptide is specific for more than one target. Exemplary binding polypeptides (e.g., antibodies) which comprise antigen binding sites that bind to antigens expressed on tumor cells are known in the art and one or more CDRs from such antibodies can be included in an antibody featured in the invention.

The term "linking moiety" includes moieties which are capable of linking the effector moiety to the binding polypeptides disclosed herein. The linking moiety may be selected such that it is cleavable (e.g., enzymatically cleavable or pH-sensitive) or non-cleavable. Exemplary linking moieties are set forth in Table 2 herein.

As used herein, the term "effector moiety" comprises agents (e.g. proteins, nucleic acids, lipids, carbohydrates, glycopeptides, and fragments thereof) with biological or other functional activity. For example, a modified binding polypeptide comprising an effector moiety conjugated to a binding polypeptide has at least one additional function or property as

compared to the unconjugated antibody. For example, the conjugation of a cytotoxic drug (e.g., an effector moiety) to binding polypeptide results in the formation of a binding polypeptide with drug cytotoxicity as second function (i.e. in addition to antigen binding). In another example, the conjugation of a second binding polypeptide to the binding polypeptide may confer additional binding properties. In certain embodiments, where the effector moiety is a genetically encoded therapeutic or diagnostic protein or nucleic acid, the effector moiety may be synthesized or expressed by either peptide synthesis or recombinant DNA methods that are well known in the art. In another aspect, where the effector moiety is a non-genetically encoded peptide, or a drug moiety, the effector moiety may be synthesized artificially or purified from a natural source. As used herein, the term "drug moiety" includes anti-inflammatory, anticancer, anti-infective (e.g., anti-fungal, antibacterial, anti-parasitic, anti-viral, etc.), and anesthetic therapeutic agents. In a further embodiment, the drug moiety is an anticancer or cytotoxic agent. Compatible drug moieties may also comprise prodrugs. Exemplary effector moieties are set forth in Table 1 herein.

As used herein the term “mildly oxidizing” refers to a reagent that accepts an electron from another species (and is therefore itself reduced), but does not attract those electrons very strongly. Examples of mildly oxidizing agents include, but are not limited to, periodate oxidase and galactose oxidase.

In certain embodiments, an “effector moiety” comprises a “targeting moiety.” As used herein, the term "targeting moiety" refers to an effector moiety that binds to a target molecule. Targeting moieties can comprise, without limitation, proteins, nucleic acids, lipids, carbohydrates (e.g., glycans), and combinations thereof (e.g., glycoproteins, glycopeptides, and glycolipids).

As used herein, the term "prodrug" refers to a precursor or derivative form of a pharmaceutically active agent that is less active, reactive or prone to side effects as compared to the parent drug and is capable of being enzymatically activated or otherwise converted into a more active form in vivo. Prodrugs compatible with the compositions of the current disclosure include, but are not limited to, phosphate-containing prodrugs, amino acid-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs that can be converted to the

more active cytotoxic free drug. One skilled in the art may make chemical modifications to the desired drug moiety or its prodrug in order to make reactions of that compound more convenient for purposes of preparing modified binding polypeptides of the current disclosure. The drug moieties also include derivatives, pharmaceutically acceptable salts, esters, amides, and ethers of the drug moieties described herein. Derivatives include modifications to drugs identified herein which may improve or not significantly reduce a particular drug's desired therapeutic activity.

As used herein, the term "anticancer agent" includes agents which are detrimental to the growth and/or proliferation of neoplastic or tumor cells and may act to reduce, inhibit or destroy malignancy. Examples of such agents include, but are not limited to, cytostatic agents, alkylating agents, antibiotics, cytotoxic nucleosides, tubulin binding agents, hormones, hormone antagonists, cytotoxic agents, and the like. Cytotoxic agents include tomaymycin derivatives, maytansine derivatives, cryptophycine derivatives, anthracycline derivatives, bisphosphonate derivatives, leptomycin derivatives, streptonigrin derivatives, auristatine derivatives, and duocarmycin derivatives. Any agent that acts to retard or slow the growth of immunoreactive cells or malignant cells is within the scope of the current disclosure.

The term "antigen" or "target antigen" as used herein refers to a molecule or a portion of a molecule that is capable of being bound by the binding site of a binding polypetpide. A target antigen may have one or more epitopes.

The term “salt” comprises a metal ion. For example, a metal ion includes but is not limited to an alkali metal (Group Ia), e.g. lithium, sodium, and potassium, an alkaline earth metal (Group IIa), e.g., magnesium and calcium, a transition metal, e.g., copper, zinc, nickel, iron, and manganese in usual valences. Exemplary usual valences of metals include, for example, sodium(I), calcium(II), magnesium(II), zinc(II), copper(I), and copper(II). Example salts comprising a metal ion, include but are not limited to, copper(II) acetate ($Cu_2(OAc)_4$), zinc(II) acetate ($Zn_2(OAc)_4$), iron chloride (Fe_3Cl_3) and calcium chloride ($CaCl_2$).

II. Binding Polypeptides

In one aspect, the current disclosure provides binding polypeptides (e.g., antibodies, antibody fragments, antibody variants, and fusion proteins) comprising a glycosylated domain, e.g, a glycosylated constant domain. The binding polypeptides disclosed herein encompass any binding polypeptide that comprises a domain having an N-linked

glycosylation site. In certain embodiments, the binding polypeptide is an antibody, or fragment or derivative thereof. Any antibody from any source or species can be employed in the binding polypeptides disclosed herein. Suitable antibodies include without limitation, human antibodies, humanized antibodies or chimeric antibodies.

In certain embodiments, the glycosylated domain is an Fc domain. In certain embodiments, the glycosylation domain is a native glycosylation domain at N297, according to EU numbering.

In other embodiments, the glycosylation domain is an engineered glycosylation domain. Exemplary engineered glycosylation domains in Fc domain comprise an asparagine residue at amino acid position 298, according to EU numbering, and/or a serine or threonine residue at amino acid position 300, according to EU numbering.

Fc domains from any immunoglobulin class (e.g., IgM, IgG, IgD, IgA and IgE) and species can be used in the binding polypeptides disclosed herein. Chimeric Fc domains comprising portions of Fc domains from different species or Ig classes can also be employed. In certain embodiments, the Fc domain is a human IgG1 Fc domain. In the case of a human IgG1 Fc domain, mutation of the wild-type amino acid at Kabat position 298 to an asparagine and Kabat position 300 to a serine or threonine results in the formation of an N-linked glycosylation consensus site (i.e., the N-X-T/S sequon, where X is any amino acid except proline). However, in the case of Fc domains of other species and/or Ig classes or isotypes, the skill artisan will appreciate that it may be necessary to mutate Kabat position 299 of the Fc domain if a proline residue is present to recreate an N-X-T/S sequon.

In other embodiments, the current disclosure provides binding polypeptides (e.g., antibodies, antibody fragments, antibody variants, and fusion proteins) comprising at least one CH1 domain having an N-linked glycosylation site. Such exemplary binding polypeptides may comprise, for example, and engineered glycosylation site at position 114, according to Kabat numbering.

CH1 domains from any immunoglobulin class (e.g., IgM, IgG, IgD, IgA and IgE) and species can be used in the binding polypeptides disclosed herein. Chimeric CHI domains comprising portions of CHI domains from different species or Ig classes can also be employed. In certain embodiments, the CH1 domain is a human IgG1 CH1 domain. In the case of a human IgG1 domain, mutation of the wild-type amino acid at position 114 to an asparagine results in the formation of an N-linked glycosylation consensus site (i.e., the N-X-

T/S sequon, where X is any amino acid except proline). However, in the case of other CH1 domains of other species and/or Ig classes or isotypes, the skilled artisan will appreciate that it may be necessary to mutate positions 115 and/or 116 of the CH1 domain to create an N-X-T/S sequon.

In certain embodiments, the binding polypeptide of the current disclosure may comprise an antigen binding fragment of an antibody. The term "antigen-binding fragment" refers to a polypeptide fragment of an immunoglobulin or antibody which binds antigen or competes with intact antibody (i.e., with the intact antibody from which they were derived) for antigen binding (i.e., specific binding). Antigen binding fragments can be produced by recombinant or biochemical methods that are well known in the art. Exemplary antigen-binding fragments include Fv, Fab, Fab', and (Fab')2. In exemplary embodiments, the antigen-binding fragment of the current disclosure is an altered antigen-binding fragment comprising at least one engineered glycosylation site. In one exemplary embodiment, an altered antigen binding fragment of the current disclosure comprises an altered VH domain described supra. In another exemplary embodiment, an altered antigen binding fragment of the current disclosure comprises an altered CH1 domain described.

In exemplary embodiments, the binding polypeptide comprises a single chain variable region sequence (ScFv). Single chain variable region sequences comprise a single polypeptide having one or more antigen binding sites, e.g., a VL domain linked by a flexible linker to a VH domain. ScFv molecules can be constructed in a VH-linker-VL orientation or VL-linker-VH orientation. The flexible hinge that links the VL and VH domains that make up the antigen binding site includes from about 10 to about 50 amino acid residues. Connecting peptides are known in the art. Binding polypeptides may comprise at least one scFv and/or at least one constant region. In one embodiment, a binding polypeptide of the current disclosure may comprise at least one scFv linked or fused to an antibody or fragment comprising a CH1 domain (e.g. a CH1 domain comprising an asparagine residue at Kabat position 114/EU position 118) and/or a CH2 domain (e.g. a CH2 domain comprising an asparagine residue at EU position 298, and a serine or threonine residue at EU position 300).

In certain exemplary embodiments, a binding polypeptide of the current disclosure is a multivalent (e.g., tetravalent) antibody which is produced by fusing a DNA sequence encoding an antibody with a ScFv molecule (e.g., an altered ScFv molecule). For example, in one embodiment, these sequences are combined such that the ScFv molecule (e.g., an altered

ScFv molecule) is linked at its N-terminus or C-terminus to an Fc fragment of an antibody via a flexible linker (e.g., a gly/ser linker). In another embodiment a tetravalent antibody of the current disclosure can be made by fusing an ScFv molecule to a connecting peptide, which is fused to a CH1 domain (e.g. a CH1 domain comprising an asparagine residue at Kabat position 114/EU position 118) to construct an ScFv-Fab tetravalent molecule.

In another embodiment, a binding polypeptide of the current disclosure is an altered minibody. An altered minibody of the current disclosure is a dimeric molecule made up of two polypeptide chains each comprising an ScFv molecule (e.g., an altered ScFv molecule comprising an altered VH domain described supra) which is fused to a CH3 domain or portion thereof via a connecting peptide. Minibodies can be made by constructing an ScFv component and connecting peptide-CH3 components using methods described in the art (see, e.g., US patent 5,837,821 or WO 94/09817A1). In another embodiment, a tetravalent minibody can be constructed. Tetravalent minibodies can be constructed in the same manner as minibodies, except that two ScFv molecules are linked using a flexible linker. The linked scFv-scFv construct is then joined to a CH3 domain.

In another embodiment, a binding polypeptide of the current disclosure comprises a diabody. Diabodies are dimeric, tetravalent molecules each having a polypeptide similar to scFv molecules, but usually having a short (less than 10, e.g.,1-5) amino acid residue linker connecting both variable domains, such that the VL and VH domains on the same polypeptide chain cannot interact. Instead, the VL and VH domain of one polypeptide chain interact with the VH and VL domain (respectively) on a second polypeptide chain (see, for example, WO 02/02781). Diabodies of the current disclosure comprise an scFv molecule fused to a CH3 domain.

In other embodiments, the binding polypeptides comprise multispecific or multivalent antibodies comprising one or more variable domain in series on the same polypeptide chain, e.g., tandem variable domain (TVD) polypeptides. Exemplary TVD polypeptides include the "double head" or "Dual-Fv" configuration described in U.S. Patent No. 5,989,830. In the Dual-Fv configuration, the variable domains of two different antibodies are expressed in a tandem orientation on two separate chains (one heavy chain and one light chain), wherein one polypeptide chain has two VH domains in series separated by a peptide linker (VH1-linker-VH2) and the other polypeptide chain consists of complementary VL domains connected in series by a peptide linker (VL1-linker-VL2). In the cross-over double head configuration, the

variable domains of two different antibodies are expressed in a tandem orientation on two separate polypeptide chains (one heavy chain and one light chain), wherein one polypeptide chain has two VH domains in series separated by a peptide linker (VH1-linker-VH2) and the other polypeptide chain consists of complementary VL domains connected in series by a peptide linker in the opposite orientation (VL2-linker-VL1). Additional antibody variants based on the "Dual-Fv" format include the Dual-Variable-Domain IgG (DVD-IgG) bispecific antibody (see U.S. Patent No. 7,612,181 and the TBTI format (see US 2010/0226923 A1). The addition of constant domains to respective chains of the Dual-Fv (CH1-Fc to the heavy chain and kappa or lambda constant domain to the light chain) leads to functional bispecific antibodies without any need for additional modifications (i.e., obvious addition of constant domains to enhance stability).

In another exemplary embodiment, the binding polypeptide comprises a cross-over dual variable domain IgG (CODV-IgG) bispecific antibody based on a "double head" configuration (see US20120251541 A1, which is incorporated by reference herein in its entirety). CODV-IgG antibody variants have one polypeptide chain with VL domains connected in series to a CL domain (VL1-L1-VL2-L2-CL) and a second polypeptide chain with complementary VH domains connected in series in the opposite orientation to a CH1 domain (VH2-L3-VH1-L4-CH1), where the polypeptide chains form a cross-over light chain-heavy chain pair. In certain embodiment, the second polypeptide may be further connected to an Fc domain (VH2-L3-VH1-L4-CH1-Fc). In certain embodiments, linker L3 is at least twice the length of linker L1 and/or linker L4 is at least twice the length of linker L2. For example, L1 and L2 may be 1-3 amino acid residues in length, L3 may be 2 to 6 amino acid residues in length, and L4 may be 4 to 7 amino acid residues in length. Examples of suitable linkers include a single glycine (Gly) residue; a diglycine peptide (Gly-Gly); a tripeptide (Gly-Gly-Gly); a peptide with four glycine residues (Gly-Gly-Gly-Gly) (SEQ ID NO: 40); a peptide with five glycine residues (Gly-Gly-Gly-Gly-Gly) (SEQ ID NO: 41); a peptide with six glycine residues (Gly-Gly-Gly-Gly-Gly-Gly) (SEQ ID NO: 42); a peptide with seven glycine residues (Gly-Gly-Gly-Gly-Gly-Gly-Gly) (SEQ ID NO: 43); a peptide with eight glycine residues (Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly) (SEQ ID NO: 44). Other combinations of amino acid residues may be used such as the peptide Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 45) and the peptide Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 46).

In certain embodiments, the binding polypeptide comprises an immunoadhesin molecule comprising a non-antibody binding region (e.g., a receptor, ligand, or cell-adhesion molecule) fused to an antibody constant region (see e.g., Ashkenazi et al., Methods, 1995 8(2), 104–115, which is incorporated by reference herein in its entirety)

In certain embodiments, the binding polypeptide comprises immunoglobulin-like domains. Suitable immunoglobulin-like domains include, without limitation, fibronectin domains (see, for example, Koide et al. (2007), *Methods Mol. Biol.* 352: 95–109, which is incorporated by reference herein in its entirety), DARPin (see, for example, Stumpp et al. (2008) *Drug Discov. Today* 13 (15–16): 695–701, which is incorporated by reference herein in its entirety), Z domains of protein A (see, Nygren et al. (2008) *FEBS J.* 275 (11): 2668–76, which is incorporated by reference herein in its entirety), Lipocalins (see, for example, Skerra et al. (2008) *FEBS J.* 275 (11): 2677–83, which is incorporated by reference herein in its entirety), Affilins (see, for example, Ebersbach et al. (2007) *J. Mol. Biol.* 372 (1): 172–85, which is incorporated by reference herein in its entirety), Affitins (see, for example, Krehenbrink et al. (2008). *J. Mol. Biol.* 383 (5): 1058–68 , which is incorporated by reference herein in its entirety), Avimers (see, for example, Silverman et al. (2005) *Nat. Biotechnol.* 23 (12): 1556–61 , which is incorporated by reference herein in its entirety), Fynomers, (see, for example, Grabulovski et al. (2007) *J Biol Chem* 282 (5): 3196–3204 , which is incorporated by reference herein in its entirety), and Kunitz domain peptides (see, for example, Nixon et al. (2006) *Curr Opin Drug Discov Devel* 9 (2): 261–8, which is incorporated by reference herein in its entirety).

III. N-linked Glycans

In certain embodiments, the binding polypeptides featured in the invention employ glycans that are "N-linked" via an asparagine residue to a glycosylation site in the polypeptide backbone of the binding polypeptide. The glycosylation site may be a native or engineered glycosylation site. Additionally or alternatively, the glycan may be a native glycan or an engineered glycan (e.g., a modified glycan) containing one or more non-native linkages. . "N-glycans" or "N-linked glycans" are attached at an amide nitrogen of an asparagine or an arginine residue in a protein via an N-acetylglucosamine residue. These "N-linked glycosylation sites" occur in the peptide primary structure containing, for example, the amino acid sequence asparagine-X-serine/threonine, where X is any amino acid residue

except proline and aspartic acid. Such N-Glycans are fully described in, for example, Drickamer K, Taylor ME (2006). Introduction to Glycobiology, 2nd ed., which is incorporated herein by reference in its entirety.

In certain embodiments, glycoengineered binding proteins and/or binding polypeptides and methods of making glycoengineered binding proteins and/or binding polypeptides are provided. As used herein, the term "glycoengineering" refers to any art-recognized method for altering the glycoform profile of a binding protein composition to generate a "modified glycan."

As used herein the terms "G0 glycoform," "G1 glycoform," and "G2 glycoform" refer to N-Glycan glycoforms that have zero, one or two terminal galactose residues respectively. These terms include G0, G1, and G2 glycoforms that are fucosylated or comprise a bisecting N-acetylglucosamine residue.

In certain embodiments, the G1 and G2 glycoforms further comprise sialic acid residues linked to one or both of the terminal galactose residues to form G1S1, G2S1 and G2S2 glycoforms. As used herein the terms "G1S1 glycoform," "G2S1 glycoform," and "G2S2 glycoform" refer to N-Glycan glycoforms that have a sialic acid residue linked to the sole terminal galactose residue in a G1 glycoform, one of the terminal galactose residue in a G2 glycoform, or both of the terminal galactose residues in a G2 glycoform, respectively. These terms include G1S1, G2S1 and G2S2 glycoforms that are fucosylated or comprise a bisecting N-acetylglucosamine residue. In certain embodiments, the sialic acid residues of G1S1, G2S1 and G2S2 glycoforms are linked by alpha-2,6-sialic acid linkages to the terminal galactose residue of each glycoform in order to enhance the anti-inflammatory activity of the binding molecule (see e.g., Anthony et al., PNAS 105: 19571-19578, 2008).

As used herein the terms "G1F glycoform," "G2F glycoform," "G1S1F glycoform," "G2S1F glycoform," and "G2S2F glycoform" refer to "G1 glycoform," "G2 glycoform" "G1S1 glycoform," "G2S1 glycoform," and "G2S2 glycoform" that are fucosylated, respectively.

In certain exemplary embodiments, the binding polypeptide comprises the native glycosylation site of an antibody Fc domain. This native glycosylation site comprises a wild-type asparagine residue at position 297 of the Fc domain (N297), according to EU numbering. The native N-linked glycan that resides at this position is generally linked through a β-

glycosylamide linkage to the nitrogen group of the N297 side chain. However, other suitable art recognized linkages can also be employed. An N297 N-linked glycan may contain a terminal mannose, N-acetyl-glucosamine, galactose or sialic acid.

In other exemplary embodiments, the binding polypeptides comprise one or more engineered glycosylation sites. Such engineered glycosylation sites comprise the substitution of one or more wild-type amino acids in the polypeptide backbone of the binding polypeptide with an asparagine residue that is capable of being N-glycosylated by the glycosylation enzymes of a cell. Exemplary engineered glycosylation sites include the introduction of asparagine mutation at amino acid position 298 of the Fc domain (298N) according to EU numbering or amino acid position 114 of a CH1 domain (114N) according to Kabat numbering (position 118 of a CH1 domain according to EU numbering).

Any type of naturally occurring or synthetic (i.e., non-natural or modified) N-linked glycan can be linked to a glycosylation site of a binding polypeptide featured in the invention. In certain embodiments, the glycan comprises a saccharide (e.g., a saccharide residue located at terminus of an oligosaccharide) that can be oxidized (e.g., by periodate treatment or galactose oxidase) to produce a group suitable for conjugation to an effector moiety (e.g., a reactive aldehyde group). Suitable oxidizable saccharides include, without limitation, galactose and sialic acid (e.g., N-Acetylneuraminic acid). In certain embodiments, the glycan is a biantennary glycan. In certain embodiments, the glycan is a naturally occurring mammalian glycoform.

Glycosylation can be achieved through any means known in the art. In certain embodiments, the glycosylation is achieved by expression of the binding polypeptides in cells capable of N-linked glycosylation. Any natural or engineered cell (e.g., prokaryotic or eukaryotic (e.g., CHO or NS0 cells)) can be employed. In general, mammalian cells are employed to effect glycosylation. The N-glycans that are produced in mammalian cells are commonly referred to as complex, high mannose, hybrid-type N-glycans (see e.g., Drickamer (2006)). These complex N-glycans have a structure that typically has two to six outer branches with a sialyllactosamine sequence linked to an inner core structure $Man_3GlcNAc_2$. A complex N-glycan has at least one branch, and or at least two branches, of alternating GlcNAc and galactose (Gal) residues that terminate in oligosaccharides such as, for example: NeuNAc-; NeuAc α2,6 GalNAc α1-; NeuAc α2,3 Gal β1,3 GalNAc α1-; and NeuAc α2,3/6 Gal β1,4 GlcNAc β 1.; In addition, sulfate esters can occur on galactose, GalNAc, and

GlcNAc residues. NeuAc can be O-acetylated or replaced by NeuGl (N-glycolylneuraminic acid). Complex N-glycans may also have intrachain substitutions of bisecting GlcNAc and core fucose (Fuc).

Additionally or alternatively, glycosylation can be achieved or modified through enzymatic means, *in vitro*. For example, one or more glycosyltransferases may be employed to add specific saccharide residues to the native or engineered N-glycan of a binding polypeptide, and one or more glycosidases may be employed to remove unwanted saccharides from the N-linked glycan. Such enzymatic means are well known in the art (see. e.g., WO2007/005786, which is incorporated herein by reference in its entirety).

IV. Immunological Effector Functions and Fc Modifications

In certain embodiments, binding polypeptides may comprise an antibody constant region (e.g., an IgG constant region e.g., a human IgG constant region, e.g., a human IgG1 or IgG4 constant region) which mediates one or more effector functions. For example, binding of the C1-complex to an antibody constant region may activate the complement system. Activation of the complement system is important in the opsonisation and lysis of cell pathogens. The activation of the complement system also stimulates the inflammatory response and may also be involved in autoimmune hypersensitivity. Further, antibodies bind to receptors on various cells via the Fc region (Fc receptor binding sites on the antibody Fc region bind to Fc receptors (FcRs) on a cell). There are a number of Fc receptors which are specific for different classes of antibody, including IgG (gamma receptors), IgE (epsilon receptors), IgA (alpha receptors) and IgM (mu receptors). Binding of antibody to Fc receptors on cell surfaces triggers a number of important and diverse biological responses including engulfment and destruction of antibody-coated particles, clearance of immune complexes, lysis of antibody-coated target cells by killer cells (called antibody-dependent cell-mediated cytotoxicity, or ADCC), release of inflammatory mediators, placental transfer and control of immunoglobulin production. In some embodiments, the binding polypeptides (e.g., antibodies or antigen binding fragments thereof) bind to an Fc-gamma receptor. In alternative embodiments, binding polypeptides may comprise a constant region which is devoid of one or more effector functions (e.g., ADCC activity) and/or is unable to bind Fcγ receptor.

Certain embodiments include antibodies in which at least one amino acid in one or more of the constant region domains has been deleted or otherwise altered so as to provide

desired biochemical characteristics such as reduced or enhanced effector functions, the ability to non-covalently dimerize, increased ability to localize at the site of a tumor, reduced serum half-life, or increased serum half-life when compared with a whole, unaltered antibody of approximately the same immunogenicity. For example, certain antibodies for use in the diagnostic and treatment methods described herein are domain deleted antibodies which comprise a polypeptide chain similar to an immunoglobulin heavy chain, but which lack at least a portion of one or more heavy chain domains. For instance, in certain antibodies, one entire domain of the constant region of the modified antibody will be deleted, for example, all or part of the CH2 domain will be deleted.

In certain other embodiments, binding polypeptides comprise constant regions derived from different antibody isotypes (e.g., constant regions from two or more of a human IgG1, IgG2, IgG3, or IgG4). In other embodiments, binding polypeptides comprises a chimeric hinge (i.e., a hinge comprising hinge portions derived from hinge domains of different antibody isotypes, e.g., an upper hinge domain from an IgG4 molecule and an IgG1 middle hinge domain). In one embodiment, binding polypeptides comprise an Fc region or portion thereof from a human IgG4 molecule and a Ser228Pro mutation (EU numbering) in the core hinge region of the molecule.

In certain embodiments, the Fc portion may be mutated to increase or decrease effector function using techniques known in the art. For example, the deletion or inactivation (through point mutations or other means) of a constant region domain may reduce Fc receptor binding of the circulating modified antibody thereby increasing tumor localization. In other cases it may be that constant region modifications consistent with the instant invention moderate complement binding and thus reduce the serum half life and nonspecific association of a conjugated cytotoxin. Yet other modifications of the constant region may be used to modify disulfide linkages or oligosaccharide moieties that allow for enhanced localization due to increased antigen specificity or flexibility. The resulting physiological profile, bioavailability and other biochemical effects of the modifications, such as tumor localization, biodistribution and serum half-life, may easily be measured and quantified using well know immunological techniques without undue experimentation.

In certain embodiments, an Fc domain employed in an antibody is an Fc variant. As used herein, the term "Fc variant" refers to an Fc domain having at least one amino acid substitution relative to the wild-type Fc domain from which said Fc domain is derived. For

example, wherein the Fc domain is derived from a human IgG1 antibody, the Fc variant of said human IgG1 Fc domain comprises at least one amino acid substitution relative to said Fc domain.

The amino acid substitution(s) of an Fc variant may be located at any position (i.e., any EU convention amino acid position) within the Fc domain. In one embodiment, the Fc variant comprises a substitution at an amino acid position located in a hinge domain or portion thereof. In another embodiment, the Fc variant comprises a substitution at an amino acid position located in a CH2 domain or portion thereof. In another embodiment, the Fc variant comprises a substitution at an amino acid position located in a CH3 domain or portion thereof. In another embodiment, the Fc variant comprises a substitution at an amino acid position located in a CH4 domain or portion thereof.

The binding polypeptides may employ any art-recognized Fc variant which is known to impart an improvement (e.g., reduction or enhancement) in effector function and/or FcR binding. Said Fc variants may include, for example, any one of the amino acid substitutions disclosed in International PCT Publications WO88/07089A1, WO96/14339A1, WO98/05787A1, WO98/23289A1, WO99/51642A1, WO99/58572A1, WO00/09560A2, WO00/32767A1, WO00/42072A2, WO02/44215A2, WO02/060919A2, WO03/074569A2, WO04/016750A2, WO04/029207A2, WO04/035752A2, WO04/063351A2, WO04/074455A2, WO04/099249A2, WO05/040217A2, WO05/070963A1, WO05/077981A2, WO05/092925A2, WO05/123780A2, WO06/019447A1, WO06/047350A2, and WO06/085967A2 or U.S. Pat. Nos. 5,648,260; 5,739,277; 5,834,250; 5,869,046; 6,096,871; 6,121,022; 6,194,551; 6,242,195; 6,277,375; 6,528,624; 6,538,124; 6,737,056; 6,821,505; 6,998,253; and 7,083,784, each of which is incorporated in its entirety by reference herein. In one exemplary embodiment, a binding polypeptide may comprise an Fc variant comprising an amino acid substitution at EU position 268 (e.g., H268D or H268E). In another exemplary embodiment, a binding polypeptide may comprise an amino acid substitution at EU position 239 (e.g., S239D or S239E) and/or EU position 332 (e.g., I332D or I332Q).

In certain embodiments, a binding polypeptide may comprise an Fc variant comprising an amino acid substitution which alters the antigen-independent effector functions of the antibody, in particular the circulating half-life of the binding polypeptide. Such binding polypeptides exhibit either increased or decreased binding to FcRn when compared to

binding polypeptides lacking these substitutions, therefore, have an increased or decreased half-life in serum, respectively. Fc variants with improved affinity for FcRn are anticipated to have longer serum half-lives, and such molecules have useful applications in methods of treating mammals where long half-life of the administered antibody is desired, e.g., to treat a chronic disease or disorder. In contrast, Fc variants with decreased FcRn binding affinity are expected to have shorter half-lives, and such molecules are also useful, for example, for administration to a mammal where a shortened circulation time may be advantageous, e.g. for in vivo diagnostic imaging or in situations where the starting antibody has toxic side effects when present in the circulation for prolonged periods. Fc variants with decreased FcRn binding affinity are also less likely to cross the placenta and, thus, are also useful in the treatment of diseases or disorders in pregnant women. In addition, other applications in which reduced FcRn binding affinity may be desired include applications localized to the brain, kidney, and/or liver. In one exemplary embodiment, the altered binding polypeptides (e.g., antibodies or antigen binding fragments thereof) exhibit reduced transport across the epithelium of kidney glomeruli from the vasculature. In another embodiment, the altered binding polypeptides (e.g., antibodies or antigen binding fragments thereof) exhibit reduced transport across the blood brain barrier (BBB) from the brain into the vascular space. In one embodiment, an antibody with altered FcRn binding comprises an Fc domain having one or more amino acid substitutions within the "FcRn binding loop" of an Fc domain. The FcRn binding loop is comprised of amino acid residues 280-299 (according to EU numbering). Exemplary amino acid substitutions which alter FcRn binding activity are disclosed in International PCT Publication No. WO05/047327 which is incorporated in its entirety by reference herein. In certain exemplary embodiments, the binding polypeptides (e.g., antibodies or antigen binding fragments thereof) comprise an Fc domain having one or more of the following substitutions: V284E, H285E, N286D, K290E and S304D (EU numbering). In yet other exemplary embodiments, the binding molecules comprise a human Fc domain with the double mutation H433K/N434F (see, e.g., US Patent No. 8,163,881).

In other embodiments, binding polypeptides, for use in the diagnostic and treatment methods described herein have a constant region, e.g., an IgG1 or IgG4 heavy chain constant region, which is altered to reduce or eliminate glycosylation. For example, binding polypeptides (e.g., antibodies or antigen binding fragments thereof) may also comprise an Fc variant comprising an amino acid substitution which alters the glycosylation of the antibody

Fc. For example, said Fc variant may have reduced glycosylation (e.g., N- or O-linked glycosylation). In exemplary embodiments, the Fc variant comprises reduced glycosylation of the N-linked glycan normally found at amino acid position 297 (EU numbering). In another embodiment, the antibody has an amino acid substitution near or within a glycosylation motif, for example, an N-linked glycosylation motif that contains the amino acid sequence NXT or NXS. In a particular embodiment, the antibody comprises an Fc variant with an amino acid substitution at amino acid position 228 or 299 (EU numbering). In more particular embodiments, the antibody comprises an IgG1 or IgG4 constant region comprising an S228P and a T299A mutation (EU numbering).

Exemplary amino acid substitutions which confer reduced or altered glycosylation are disclosed in International PCT Publication No. WO05/018572, which is incorporated in its entirety by reference herein. In some embodiments, the binding polypeptides are modified to eliminate glycosylation. Such binding polypeptides may be referred to as "agly" binding polypeptides (e.g. "agly" antibodies). While not being bound by theory, it is believed that "agly" binding polypeptides may have an improved safety and stability profile *in vivo*. Agly binding polypeptides can be of any isotype or subclass thereof, e.g., IgG1, IgG2, IgG3, or IgG4. In certain embodiments, agly binding polypeptides comprise an aglycosylated Fc region of an IgG4 antibody which is devoid of Fc-effector function, thereby eliminating the potential for Fc mediated toxicity to the normal vital organs that express IL-6. In yet other embodiments, binding polypeptides comprise an altered glycan. For example, the antibody may have a reduced number of fucose residues on an N-glycan at Asn297 of the Fc region, i.e., is afucosylated. Afucosylation increases FcγRII binding on the NK cells and potently increases ADCC. It has been shown that a diabody comprising an anti-IL-6 scFv and an anti-CD3 scFv induces killing of IL-6 expressing cells by ADCC. Accordingly, in one embodiment, an afucosylated anti-IL-6 antibody is used to target and kill IL-6-expressing cells. In another embodiment, the binding polypeptide may have an altered number of sialic acid residues on the N-glycan at Asn297 of the Fc region. Numerous art-recognized methods are available for making "agly" antibodies or antibodies with altered glycans. For example, genetically engineered host cells (e.g., modified yeast, e.g., *Picchia*, or CHO cells) with modified glycosylation pathways (e.g., glycosyl-transferase deletions) can be used to produce such antibodies.

V. Effector Moieties

In certain embodiments, the binding polypeptides of the current disclosure comprise effector moieties (e.g., targeting moieties). In general these effector moieties are conjugated (either directly or through a linker moiety) to an N-linked glycan on the binding polypeptide, (e.g., an N-linked glycan linked to N298 (EU numbering) of the CH2 domain and/or N114 (Kabat numbering) of a CH1 domain). In certain embodiments, the binding polypeptide is full length antibody comprising two CH1 domains with a glycan at Kabat position 114 (EU position 118), wherein both of the glycans are conjugated to one or more effector moieties.

Any effector moiety can be added to the binding polypeptides disclosed herein. The effector moieties can add a non-natural function to an altered antibody or fragments thereof without significantly altering the intrinsic activity of the binding polypeptide. In certain exemplary embodiments, an effector moiety is a targeting moiety (e.g., a glycopeptide or neoglycan). A modified binding polypeptide (e.g., an antibody) of the current disclosure may comprise one or more effector moieties, which may be the same of different.

In one embodiment, the effector moiety can be of Formula (I):

H_2N-Q-CON-X

Formula (I),

wherein:

A) Q is NH or O; and

B) CON is a connector moiety; and

C) X is an effector moiety (e.g., a targeting moiety as defined herein).

The connector moiety connects the therapeutic agent to H_2N-Q- . The connector moiety can include at least one of any suitable components known to those skilled in the art, including, for example, an alkylenyl component, a polyethylene glycol component, a poly(glycine) component, a poly(oxazoline) component, a carbonyl component, a component derived from cysteinamide, a component derived from valine coupled with citruline, and a component derived from 4-aminobenzyl carbamate, or any combination thereof.

In another embodiment, the effector moiety of Formula (I) can be of Formula (Ia):

H_2N-Q-CH_2-C(O)-Z-X

Formula (Ia),

wherein:

A) Q is NH or O; and

B) Z is -Cys-$(MC)_a$-$(VC)_b$-$(PABC)_c$-$(C_{16}H_{32}O_8C_2H_4)_f$,

wherein

i. Cys is a component derived cysteinamide;

ii. MC is a component derived from maleimide;

iii. VC is a component derived from valine coupled with citruline;

iv. PABC is a component derived from 4-aminobenzyl carbamate;

v. X is an effector moiety (e.g., a targeting moiety as defined herein);

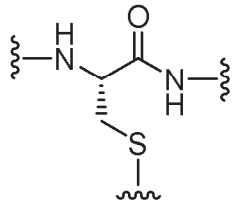
vi. a is 0 or 1;

vii. b is 0 or 1;

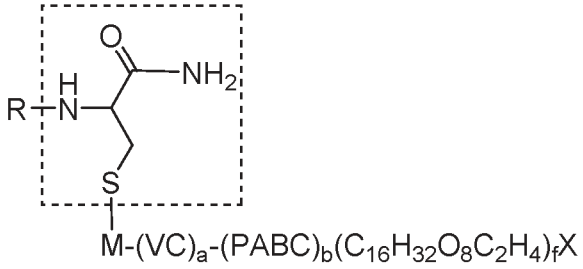
viii. c is 0 or 1; and

ix. f is 0 or 1

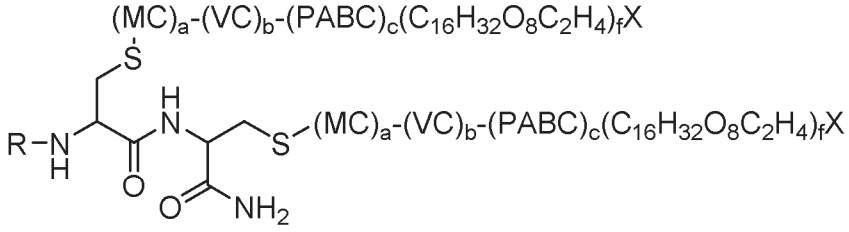
The "component derived from cysteinamide" is the point of attachment to H_2N-Q-CH_2-C(O)-. In one embodiment, the "component derived from cysteinamide" can refer to one or more portions of the effector moiety having the structure:



In one embodiment, the "Cys" component of an effector moiety may include one such portion. For example, the following structure shows an effector moiety with one such portion (wherein the "Cys" component is indicated with the dotted line box):

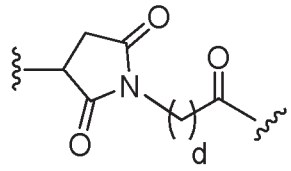


In another embodiment, the "Cys" component of an effector moiety may include two or more such portions. For example, the following moiety contains two such portions:



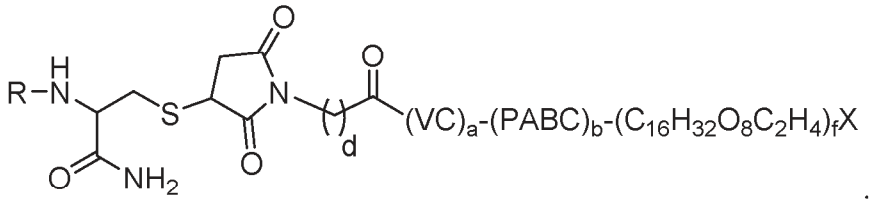
As can be seen from the structure, each "Cys" component bears an $-(MC)_a-(VC)_b-(PABC)_c-(C_{16}H_{32}O_8C_2H_4)_f$ -X group.

In one embodiment, the phrase "component derived from maleimide" can refer to any portion of the effector moiety having the structure:

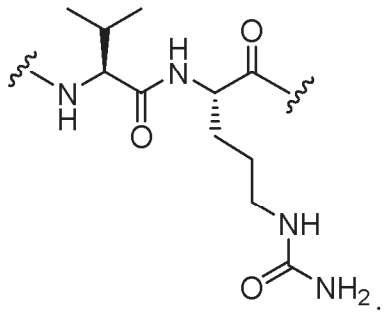


wherein d is an integer from 2 to 5. The number of MC components included in any Cys-$(MC)_a-(VC)_b-(PABC)_c-(C_{16}H_{32}O_8C_2H_4)_f$ -X group in the effector moiety is indicated by subscript "a," and can be 0 or 1 In one embodiment, a is 1. In another embodiment, b is 0.

In one embodiment, the "Cys" component can be connected to the "MC" component via the sulfur atom in the "Cys" component, as indicated with the dotted line box in the structure below:

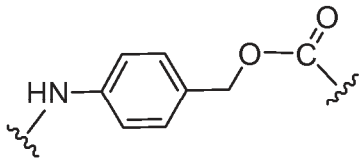


In one embodiment, the phrase "component derived from valine coupled with citruline" can refer to any portion of the effector moiety with the following structure:



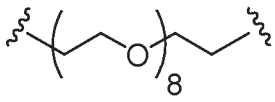
The number of VC components included in any Cys-$(MC)_a$-$(VC)_b$-$(PABC)_c$-$(C_{16}H_{32}O_8C_2H_4)_f$ -X group in the effector moiety is indicated by subscript "b," and can be 0 or 1. In one embodiment, b is 1. In another embodiment, b is 0.

In one embodiment, the phrase "component derived from 4-aminobenzyl carbamate" can refer to any portion of the effector moiety with the following structure:



The number of PABC components included in any Cys-$(MC)_a$-$(VC)_b$-$(PABC)_c$-$(C_{16}H_{32}O_8C_2H_4)_f$ -X group in the effector moiety is indicated by subscript "c," and can be 0 or 1. In one embodiment, c is 1. In another embodiment, c is 0.

In one embodiment, "$C_{16}H_{32}O_8C_2H_4$" refers to the following structure:



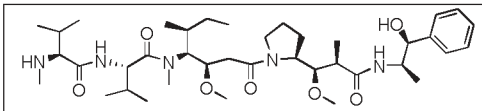
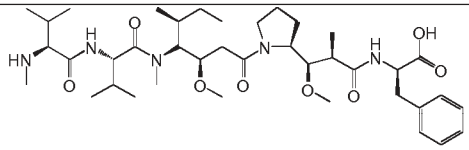
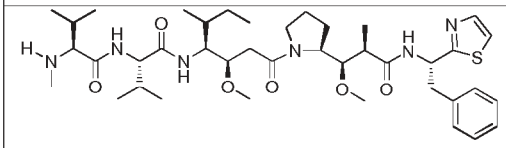
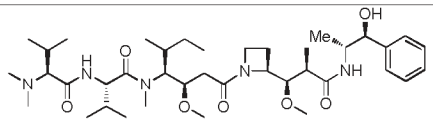
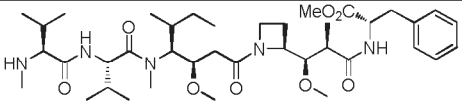
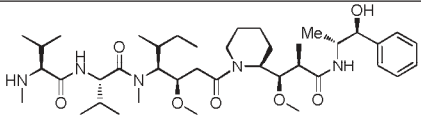
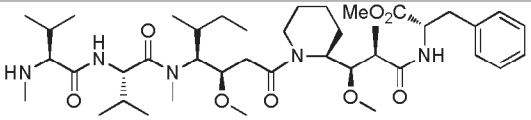
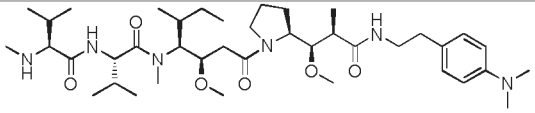
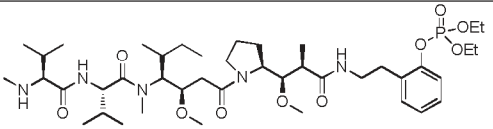
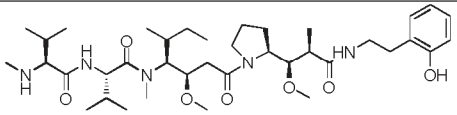
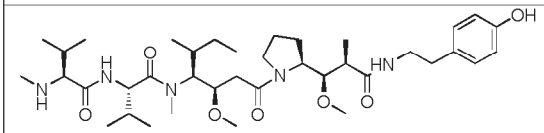
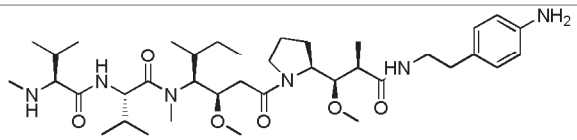
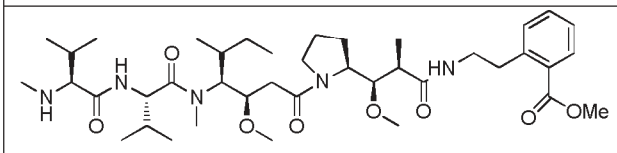
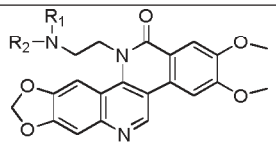
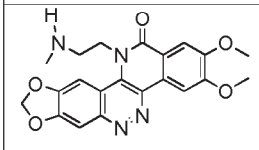
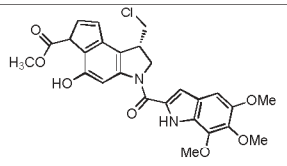
The number of $C_{16}H_{32}O_8$ units included in any Cys-$(MC)_a$-$(VC)_b$-$(PABC)_c$-$(C_{16}H_{32}O_8C_2H_4)_f$ -X group in the effector moiety is indicated by subscript "f," In one embodiment, f is 1. In another embodiment, f is 0.

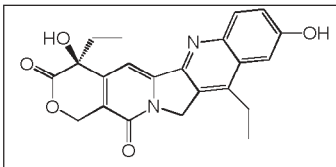
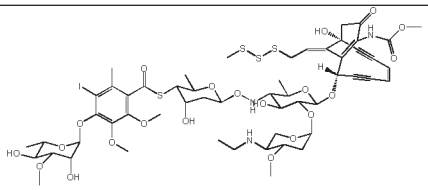
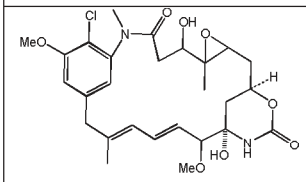
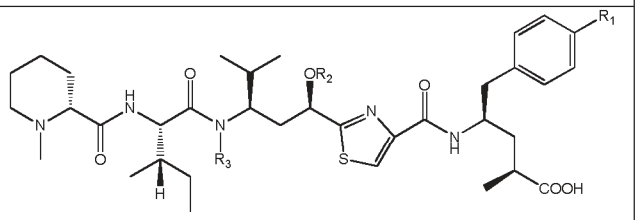
In one embodiment, a is 1, b is 1, c is 1, and f is 0.

a) Therapeutic Effector Moieties

In certain embodiments, the binding polypeptides of the current disclosure are conjugated to an effector moiety comprising a therapeutic agent, e.g. a drug moiety (or prodrug thereof) or radiolabeled compound. In one embodiment the therapeutic agent is a cytotoxin. Exemplary cytotoxic therapeutic agents are set forth in Table 1 herein.

Table 1. Exemplary cytotoxic therapeutic agents

	
	 R_1= alkyl, aryl, alkoxy, aryloxy, R_2, R_3= alkyl, aryl

Further exemplary drug moieties include anti-inflammatory, anti-cancer, anti-infective (e.g., anti-fungal, antibacterial, anti-parasitic, anti-viral, etc.), and anesthetic therapeutic agents. In a further embodiment, the drug moiety is an anti-cancer agent. Exemplary anti-cancer agents include, but are not limited to, cytostatics, enzyme inhibitors, gene regulators, cytotoxic nucleosides, tubulin binding agents or tubulin inhibitors, proteasome inhibitors, hormones and hormone antagonists, anti-angiogenesis agents, and the like. Exemplary cytostatic anti-cancer agents include alkylating agents such as the anthracycline family of drugs (e.g. adriamycin, carminomycin, cyclosporin-A, chloroquine, methopterin, mithramycin, porfiromycin, streptonigrin, porfiromycin, anthracenediones, and aziridines). Other cytostatic anti-cancer agents include DNA synthesis inhibitors (e.g., methotrexate and dichloromethotrexate, 3-amino-l,2,4-benzotriazine 1,4-dioxide, aminopterin, cytosine β-D-arabinofuranoside, 5-fluoro-5'-deoxyuridine, 5-fluorouracil, ganciclovir, hydroxyurea, actinomycin-D, and mitomycin C), DNA-intercalators or cross-linkers (e.g., bleomycin, carboplatin, carmustine, chlorambucil, cyclophosphamide, cis-diammineplatinum(II) dichloride (cisplatin), melphalan, mitoxantrone, and oxaliplatin), and DNA-RNA transcription regulators (e.g., actinomycin D, daunorubicin, doxorubicin, homoharringtonine, and idarubicin). Other exemplary cytostatic agents that are compatible with the present disclosure include ansamycin benzoquinones, quinonoid derivatives (e.g. quinolones, genistein, bactacyclin), busulfan, ifosfamide, mechlorethamine, triaziquone, diaziquone, carbazilquinone, indoloquinone EO9, diaziridinyl-benzoquinone methyl DZQ,

triethylenephosphoramide, and nitrosourea compounds (e.g. carmustine, lomustine, semustine).

Exemplary cytotoxic nucleoside anti-cancer agents include, but are not limited to: adenosine arabinoside, cytarabine, cytosine arabinoside, 5-fluorouracil, fludarabine, floxuridine, ftorafur, and 6-mercaptopurine. Exemplary anti-cancer tubulin binding agents include, but are not limited to: taxoids (e.g. paclitaxel, docetaxel, taxane), nocodazole, rhizoxin, dolastatins (e.g. Dolastatin-10, -11, or -15), colchicine and colchicinoids (e.g. ZD6126), combretastatins (e.g. Combretastatin A-4, AVE-6032), and vinca alkaloids (e.g. vinblastine, vincristine, vindesine, and vinorelbine (navelbine)). Exemplary anti-cancer hormones and hormone antagonists include, but are not limited to: corticosteroids (e.g. prednisone), progestins (e.g. hydroxyprogesterone or medroprogesterone), estrogens, (e.g. diethylstilbestrol), antiestrogens (e.g. tamoxifen), androgens (e.g. testosterone), aromatase inhibitors (e.g. aminogluthetimide), 17-(allylamino)-17-demethoxygeldanamycin, 4-amino-1 ,8-naphthalimide, apigenin, brefeldin A, cimetidine, dichloromethylene-diphosphonic acid, leuprolide (leuprorelin), luteinizing hormone-releasing hormone, pifithrin-a, rapamycin, sex hormone-binding globulin, and thapsigargin. Exemplary anti-cancer, anti-angiogenesis compounds include, but are not limited to: Angiostatin Kl -3, DL-a-difluoromethyl-ornithine, endostatin, fumagillin, genistein, minocycline, staurosporine, and (±)-thalidomide.

Exemplary anti-cancer enzyme inhibitors include, but are not limited to: S(+)-camptothecin, curcumin, (-)-deguelin, 5,6-diCH1orobenz-imidazole 1-β-D-ribofuranoside, etoposide, formestane, fostriecin, hispidin, 2-imino-l-imidazolidineacetic acid (cyclocreatine), mevinolin, trichostatin A, tyrphostin AG 34, and tyrphostin AG 879.

Examplary anti-cancer gene regulators include, but are not limited to: 5-aza-2'-deoxycytidine, 5-azacytidine, cholecalciferol (vitamin D3), 4-hydroxytamoxifen, melatonin, mifepristone, raloxifene, trans-retinal (vitamin A aldehydes), retinoic acid, vitamin A acid, 9-cis-retinoic acid, 13-cis-retinoic acid, retinol (vitamin A), tamoxifen, and troglitazone.

Other classes of anti-cancer agents include, but are not limited to: the pteridine family of drugs, diynenes, and the podophyllotoxins. Particularly useful members of those classes include, for example, methopterin, podophyllotoxin, or podophyllotoxin derivatives such as etoposide or etoposide phosphate, leurosidine, vindesine, leurosine and the like.

Still other anti-cancer agents that are compatible with the teachings herein include auristatins (e.g. auristatin E and monomethylauristan E), geldanamycin, calicheamicin,

gramicidin D, maytansanoids (e.g. maytansine), neocarzinostatin, topotecan, taxanes, cytochalasin B, ethidium bromide, emetine, tenoposide, colchicin, dihydroxy anthracindione, mitoxantrone, procaine, tetracaine, lidocaine, propranolol, puromycin, and analogs or homologs thereof.

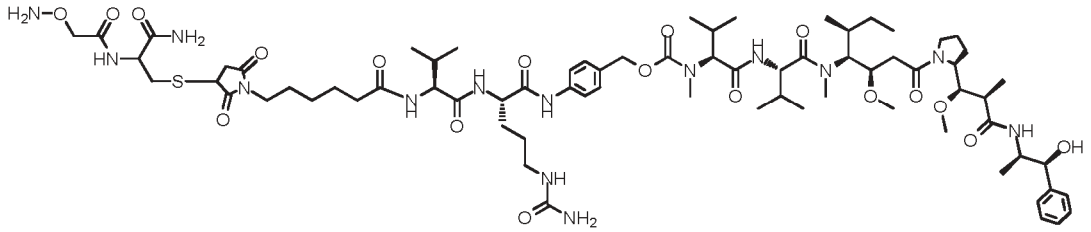
Still other anti-cancer agents that are compatible with the teachings herein include tomaymycin derivatives, maytansine derivatives, cryptophycine derivatives, anthracycline derivatives, bisphosphonate derivatives, leptomycin derivatives, streptonigrin derivatives, auristatine derivatives, and duocarmycin derivatives.

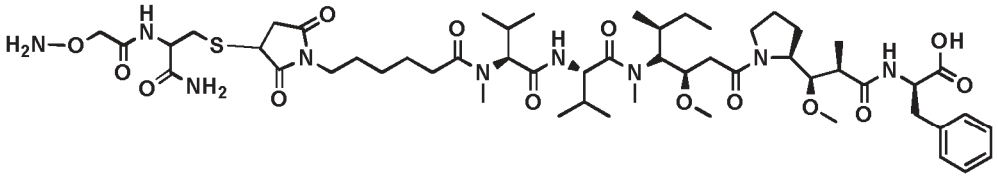
Another class of compatible anti-cancer agents that may be used as drug moieties are radiosensitizing drugs that may be effectively directed to tumor or immunoreactive cells. Such drug moeities enhance the sensitivity to ionizing radiation, thereby increasing the efficacy of radiotherapy. Not to be limited by theory, an antibody modified with a radiosensitizing drug moiety and internalized by the tumor cell would deliver the radiosensitizer nearer the nucleus where radiosensitization would be maximal. Antibodies which lose the radiosensitizer moiety would be cleared quickly from the blood, localizing the remaining radiosensitization agent in the target tumor and providing minimal uptake in normal tissues. After clearance from the blood, adjunct radiotherapy could be administered by external beam radiation directed specifically to the tumor, radioactivity directly implanted in the tumor, or systemic radioimmunotherapy with the same modified antibody.

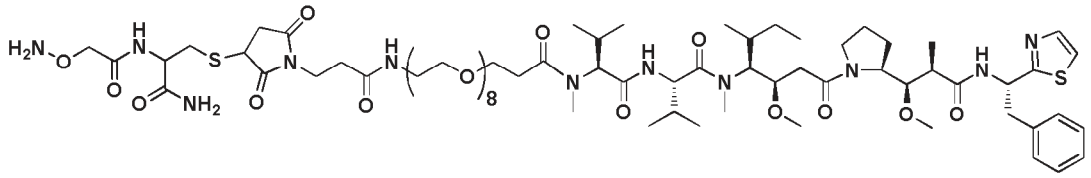
In one embodiment, the therapeutic agent comprises radionuclides or radiolabels with high-energy ionizing radiation that are capable of causing multiple strand breaks in nuclear DNA, leading to cell death. Exemplary high-energy radionuclides include: ^{90}Y, ^{125}I, ^{131}I, ^{123}I, ^{111}In, ^{105}Rh, ^{153}Sm, ^{67}Cu, ^{67}Ga, ^{166}Ho, ^{177}Lu, ^{186}Re and ^{188}Re. These isotopes typically produce high energy α-or β-particles which have a short path length. Such radionuclides kill cells to which they are in close proximity, for example neoplastic cells to which the conjugate has attached or has entered. They have little or no effect on non-localized cells and are essentially non-immunogenic. Alternatively, high-energy isotopes may be generated by thermal irradiation of an otherwise stable isotope, for example as in boron neutron -capture therapy (Guan et al., PNAS, 95: 13206-10, 1998).

In one embodiment, the therapeutic agent is selected from MMAE, MMAF, and PEG8-Do110.

Exemplary therapeutic effector moieties include the structures:







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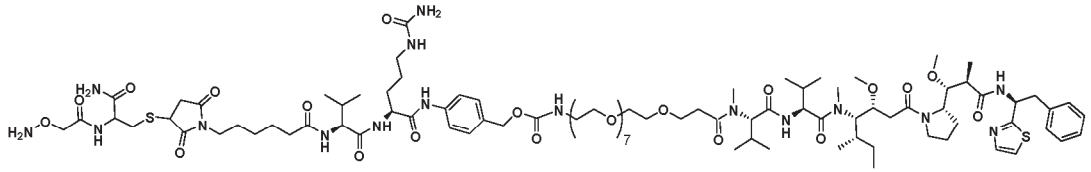
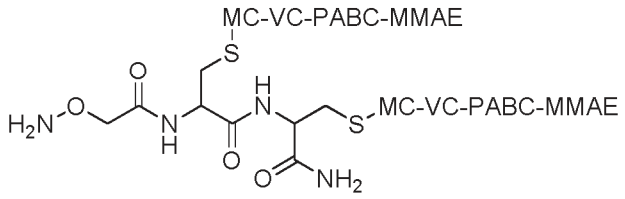
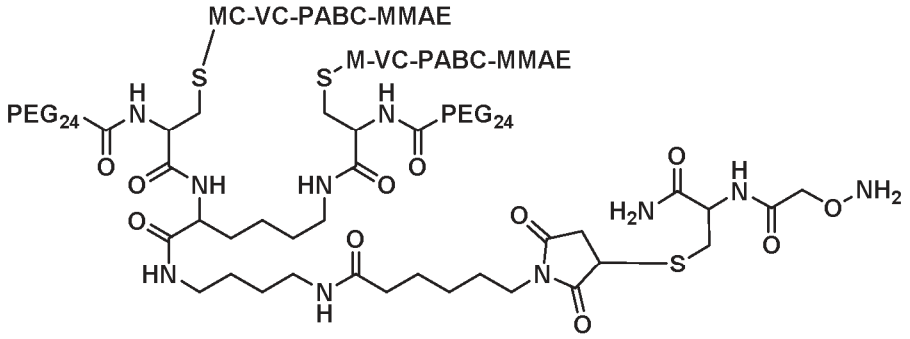
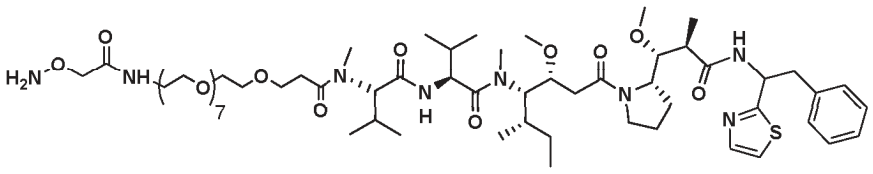
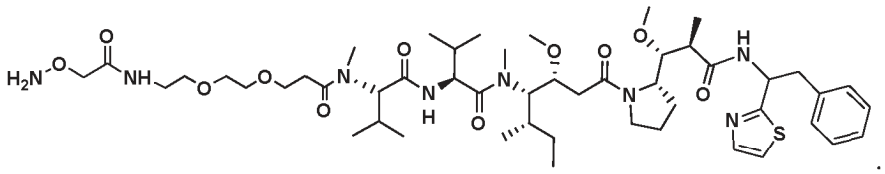






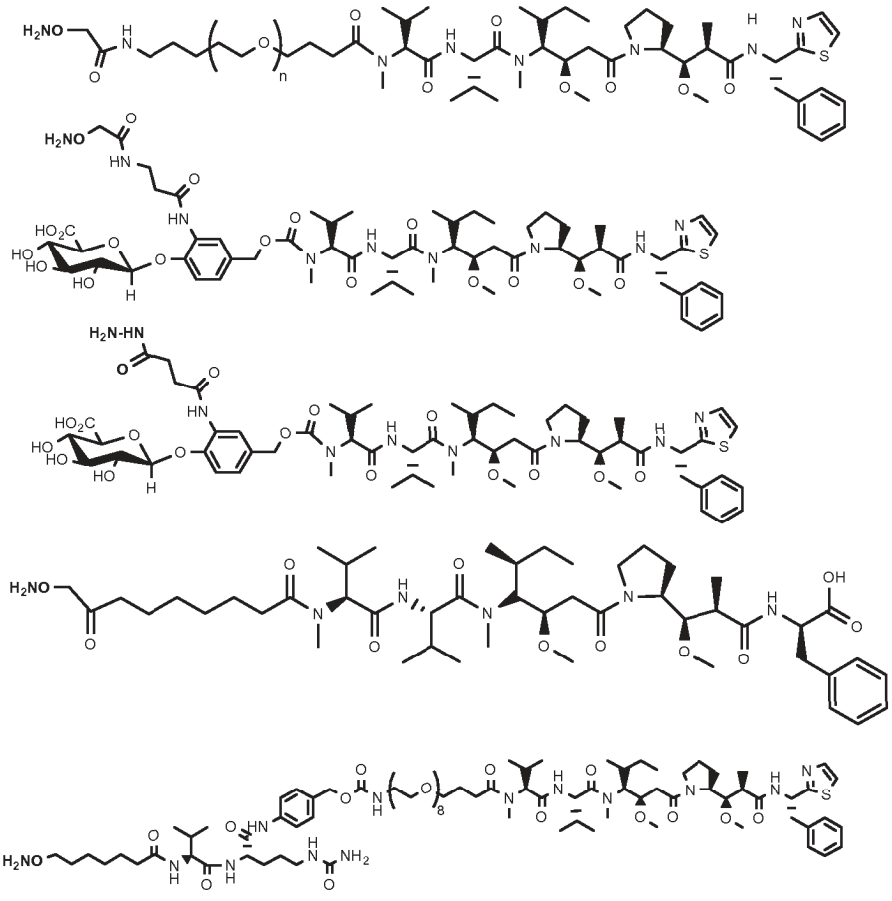


and



.

In one embodiment, the effector moiety is selected from:



In certain embodiments, the effector moiety contains more than one therapeutic agent. These multiple therapeutic agents can be the same or different.

b) Diagnostic Effector Moieties

In certain embodiments, the binding polypeptides of the current disclosure are conjugated to an effector moiety comprising a diagnostic agent. In one embodiment, the diagnostic agent is a detectable small molecule label e.g. biotin, fluorophores, chromophores, spin resonance probes, or radiolabels. Exemplary fluorophores include fluorescent dyes (e.g. fluorescein, rhodamine, and the like) and other luminescent molecules (e.g. luminal). A fluorophore may be environmentally-sensitive such that its fluorescence changes if it is located close to one or more residues in the modified binding polypeptide that undergo structural changes upon binding a substrate (e.g. dansyl probes). Exemplary radiolabels include small molecules containing atoms with one or more low sensitivity nuclei (^{13}C, ^{15}N, ^{2}H, ^{125}I, ^{124}I, ^{123}I, ^{99}Tc, ^{43}K, ^{52}Fe, ^{64}Cu, ^{68}Ga, ^{111}In and the like). The radionuclide can be, e.g., a gamma, photon, or positron-emitting radionuclide with a half-life suitable to permit activity or detection after the elapsed time between administration and localization to the imaging site.

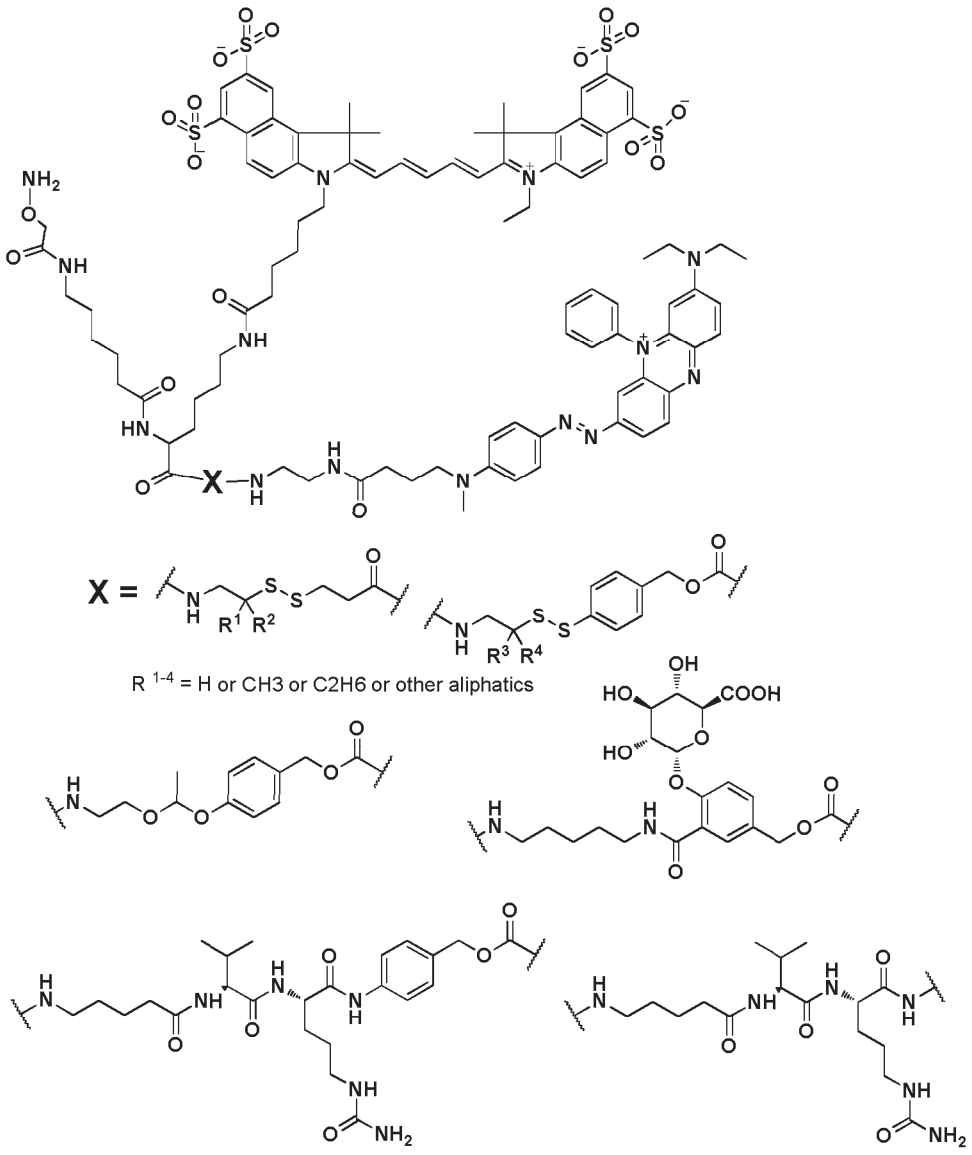
In one embodiment, the diagnostic agent is a polypeptide. Exemplary diagnostic polypeptides include enzymes with fluorogenic or chromogenic activity, e.g. the ability to cleave a substrate which forms a fluorophore or chromophore as a product (i.e. reporter proteins such as luciferase). Other diagnostic proteins may have intrinsic fluorogenic or chromogenic activity (e.g., green, red, and yellow fluorescent bioluminescent aequorin proteins from bioluminescent marine organisms) or they may comprise a protein containing one or more low-energy radioactive nuclei (^{13}C, ^{15}N, ^{2}H, ^{125}I, ^{124}I, ^{123}I, ^{99}Tc, ^{43}K, ^{52}Fe, ^{64}Cu, ^{68}Ga, ^{111}In and the like).

With respect to the use of radiolabeled conjugates in conjunction with the present disclosure, binding polypeptides of the current disclosure may be directly labeled (such as through iodination) or may be labeled indirectly through the use of a chelating agent. As used herein, the phrases "indirect labeling" and "indirect labeling approach" both mean that a chelating agent is covalently attached to a binding polypeptide and at least one radionuclide is associated with the chelating agent. Such chelating agents are typically referred to as bifunctional chelating agents as they bind both the polypeptide and the radioisotope. Exemplary chelating agents comprise 1-isothiocycmatobenzyl-3-methyldiothelene triaminepentaacetic acid ("MX-DTPA") and cyclohexyl diethylenetriamine pentaacetic acid ("CHX-DTPA") derivatives. Other chelating agents comprise P-DOTA and EDTA derivatives. Exemplary radionuclides for indirect labeling include 111In and 90Y. Most

imaging studies utilize 5 mCi 111In-labeled antibody, because this dose is both safe and has increased imaging efficiency compared with lower doses, with optimal imaging occurring at three to six days after antibody administration. See, for example, Murray, (1985), J. Nuc. Med. 26: 3328 and Carraguillo et al, (1985), J. Nuc. Med. 26: 67. An exemplary radionuclide for direct labeling is 131I. Those skilled in the art will appreciate that non-radioactive conjugates may also be assembled depending on the selected agent to be conjugated.

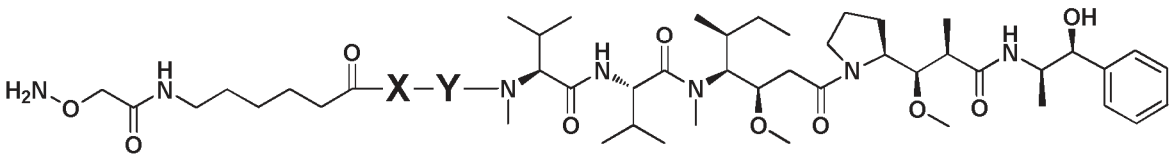
In certain embodiments, the diagnostic effector moiety is a FRET (Fluorescence Resonance Energy Transfer) probe. FRET has been used for a variety of diagnostic applications including cancer diagnostics. A FRET probe may include a cleavable linker (enzyme sensitive or pH linker) connecting the donor and acceptor moieties of the FRET probe, wherein cleavage results in enhanced fluorescence (including near Infrared) (see, e.g., A. Cobos-Correa et. al. *Membrane-bound FRET probe visualizes MMP12 activity in pulmonary inflammation*, Nature Chemical Biology (2009), 5(9), 628-63; S. Gehrig et.al. *Spatially Resolved Monitoring of Neutrophil Elastase Activity with Ratiometric Fluorescent Reporters* (2012) Angew. Chem. Int. Ed. , 51, 6258 –6261).

In one embodiment, the effector moiety is selected from:

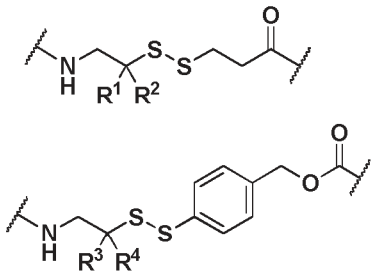

X =
R 1-4 = H or CH3 or C2H6 or other aliphatics

c) Functionalized Effector Moieties

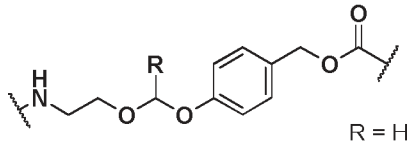
In certain embodiments, effector moieties may be functionalized to contain one or more additional groups in addition to the effector moiety itself. For example, the effector moiety may contain cleavable linkers which release the effector moiety from the binding polypeptide under particular conditions. In exemplary embodiments, the effector moiety may include a linker that is cleavable by cellular enzymes and/or is pH sensitive. Additionally or alternatively, the effector moiety may contain a disulfide bond that cleaved by intracellular glutathione upon uptake into the cell. Exemplary disulfide and pH senstitive linkers are provided below:



X =

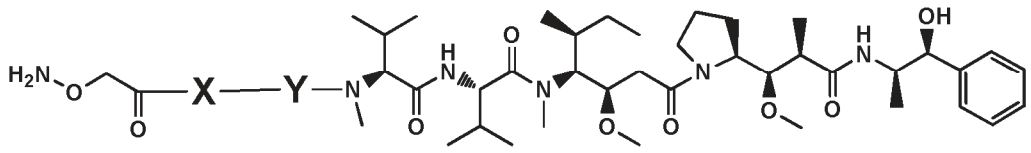


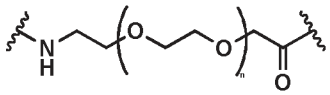
R^{1-4} = H or CH_3 or C_2H_6 or other aliphatics

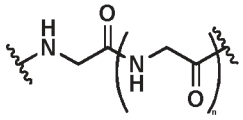


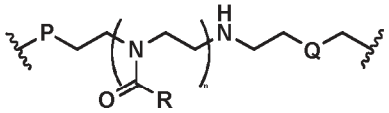
R = H or substituted or unsunstituted alkyl, alkylaryl groups

In yet other embodiments, the effector moiety may include hydrophilic and biocompatible moieties such as poly(glycine), poly(oxazoline), and/or PEG moieties. Exemplary structures ("Y") are provided below:



Y =  PEG

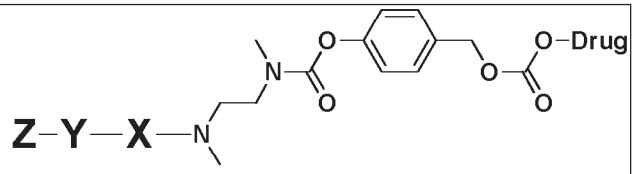
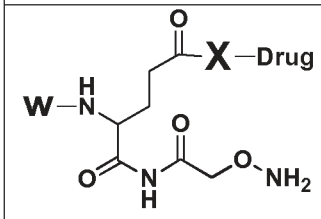
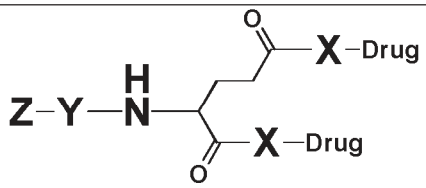
 poly(glycine)

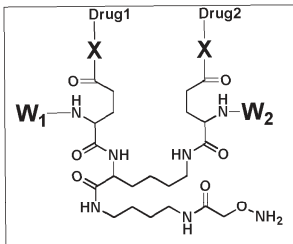
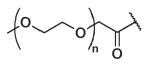
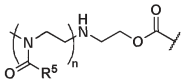
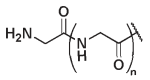
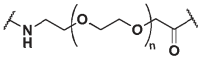
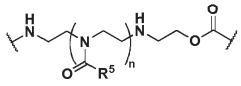
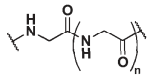
 poly(oxazoline)

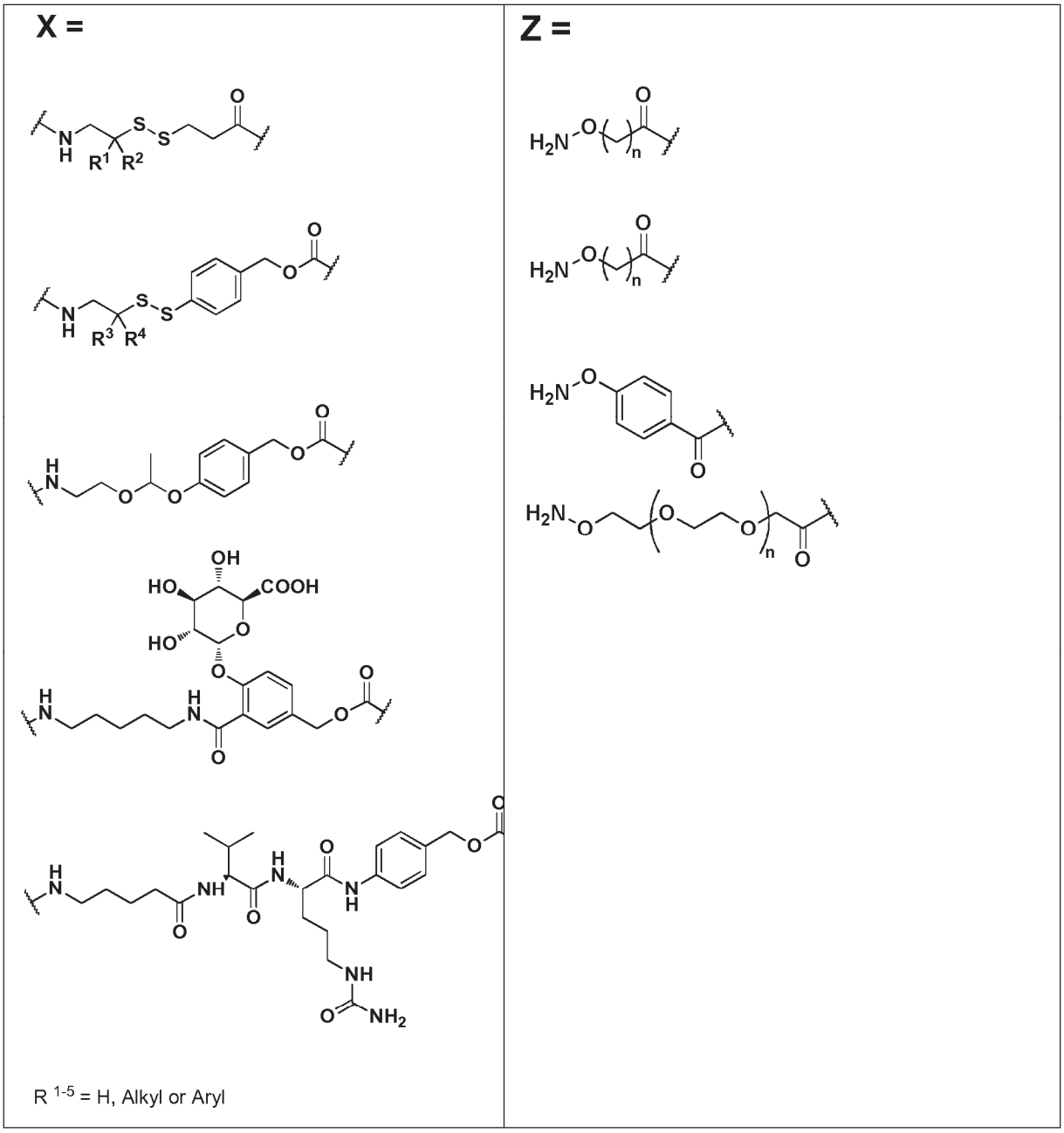
R = H, unsubstituted or functional group containing alkyl groups
P and Q = same or different functional groups for linking drugs, reporter molecules and protein

In certain embodiments, the effector moiety contains an aminooxy group which facilitates conjugation to a binding polypeptide via a stable oxime linkage. Exemplary effector moieties containing aminooxy groups are set forth in Table 2 herein.

Table 2. Exemplary aminoxy effector moieties (wherein X can be any linker, Y is any spacer, and wherein X and/or Y are optional)

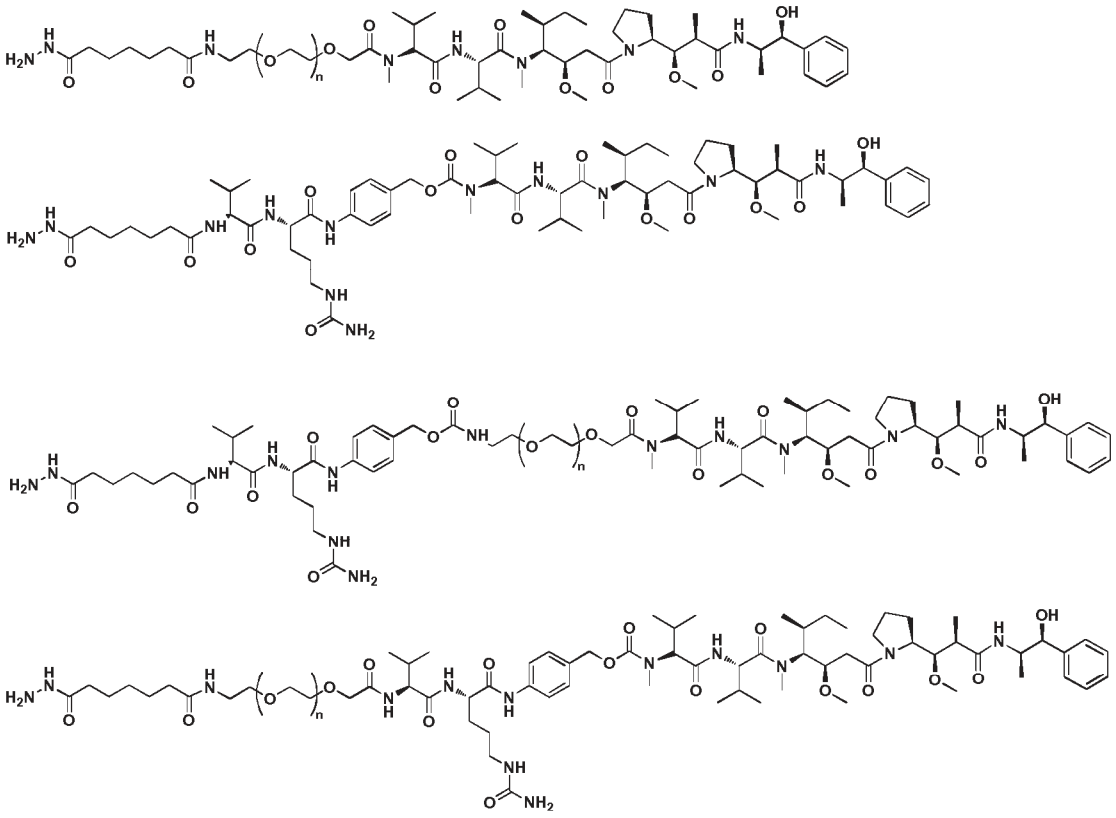
Z–Y—X—Drug	
	

	W, W1 and W2 =   
	Y =   



In other embodiments, the effector moiety contains a hydrazide and/or N-alkylated hydrazine group to facilitate conjugation to a binding polypeptide via a stable hydrazone linkage. Exemplary effector moieties containing aminooxy groups are set forth in **Table 14** herein.

Table 14. Exemplary hydrazine and/or hydrazide effector moieties



d) Targeting Moieties

In certain embodiments, effector moieties comprise targeting moieties that specifically bind to one or more target molecules. Any type of targeting moiety can be employed including, without limitation, proteins, nucleic acids, lipids, carbohydrates (e.g., glycans), and combinations thereof (e.g., glycoproteins, glycopeptides, and glycolipids). In certain embodiments, the targeting moiety is a carbohydrate or glycopeptide. In certain embodiments, the targeting moiety is a glycan. Targeting moieties can be naturally or non-naturally occurring molecules. Targeting moieties suitable for conjugation may include those containing aminooxy linkers (see, e.g., **Figure 40 and 41**).

The targeting moieties described in the present invention may bind to any type of cell, including animal (e.g., mammalian), plant, or insect cells either *in vitro* or *in vivo*, without limitation. The cells may be of endodermal, mesodermal, or ectodermal origins, and may include any cell type. In certain embodiments, the targeting moiety binds to a cell, e.g., a mammalian cell, a facilitates delivery of a binding polypeptide to the targeted cell, e.g., to improve cell-targeting and/or uptake. Exemplary target cells include, without limitation, immune cells (e.g., lymphocytes such as B cells, T cells, natural killer (NK) cells, basophils, macrophages, or dendritic cells), liver cells (e.g., hepatocytes or non-parenchymal cells such as liver sinusoidal endothelial cells, Kupffer cells, or hepatic stellate cells), tumor cells (e.g., any malignant or benign cell including hepatoma cells, lung cancer cells, sarcoma cells, leukemia cells, or lymphoma cells), vascular cells (e.g., aortic endothelial cells or pulmonary artery endothelial cells), epithelial cells (e.g., simple squamous epithelial cells, simple columnar epithelial cells, pseudostratified columnar epithelial cells, or stratified squamous epithelial cells), or mesenchymal cells (e.g., cells of the lymphatic and circulatory systems, bone, and cartilage cells).

In one embodiment, the binding polypeptide comprising one or more targeting moieties is internalized by the cell. In another embodiment, the amount of the binding polypeptide comprising one or more targeting moieties internalized by the cell is greater than the amount of a reference binding polypeptide lacking a targeting moiety internalized by the cell.

In one embodiment, the targeting moiety binds to a receptor on the target cell. For example, the targeting moiety may comprise a mannose 6 phosphate moiety that binds to a mannose 6 phosphate receptor on the cell. In other exemplary embodiments, the targeting moiety binds to a Siglec on a target cell. Exemplary Siglecs include sialoadhesin (Siglec-1), CD22 (Siglec-2), CD33 (Siglec-3), MAG (Siglec-4), Siglec-5, Siglec-6, Siglec-7, Siglec-8, Siglec-9, Siglec-10, Siglec-11, Siglec-12, Siglec-14, or Siglec-15. In yet other embodiments, the targeting moiety comprises an α2,3-, α2,6-, or α2,8- linked sialic acid residue. In a further embodiment, the targeting moiety comprises an α2,3-siallylactose moiety or an α2,6-siallylactose moiety. Other exemplary receptors include lectin receptors, including but not limited to C-type lectin receptors, galectins, and L-type lectin receptors. Exemplary lectin receptors include: DEC-205 (CD205; lymphocyte antigen 75), macrophage mannose receptor (MMR; CD206), Dectin-1, Dectin-2, macrophage-inducible C-type lectin (Mincle), dendritic

cell-specific ICAM3-grabbing nonintegrin (DC-SIGN, CD209), DC NK lectin group receptor-1 (DNGR-1), Langerin (CD207), a lectican, an asialoglycoprotein receptor, C-lectin receptor dendritic cell immunoreceptor (CLEC4A; CLECSF6; DCIR), macrophage galactose-type lectin (MGL), a DC receptor, a collectin, a selectin, an NK-cell receptor, a multi- C-type lectin domain (CTLD) endocytic receptor, a Reg group (type VII) lectin, chondrolectin, tetranectin, polycystin, attractin (ATRN), eosinophil major basic protein (EMBP), DiGeorge Syndrome Critical Region Gene 2 (DGCR2), Thrombomodulin, Bimlec, a group XVI lectin (SEEC), and a group XVII lectin (CBCP/Frem1/QBRICK).

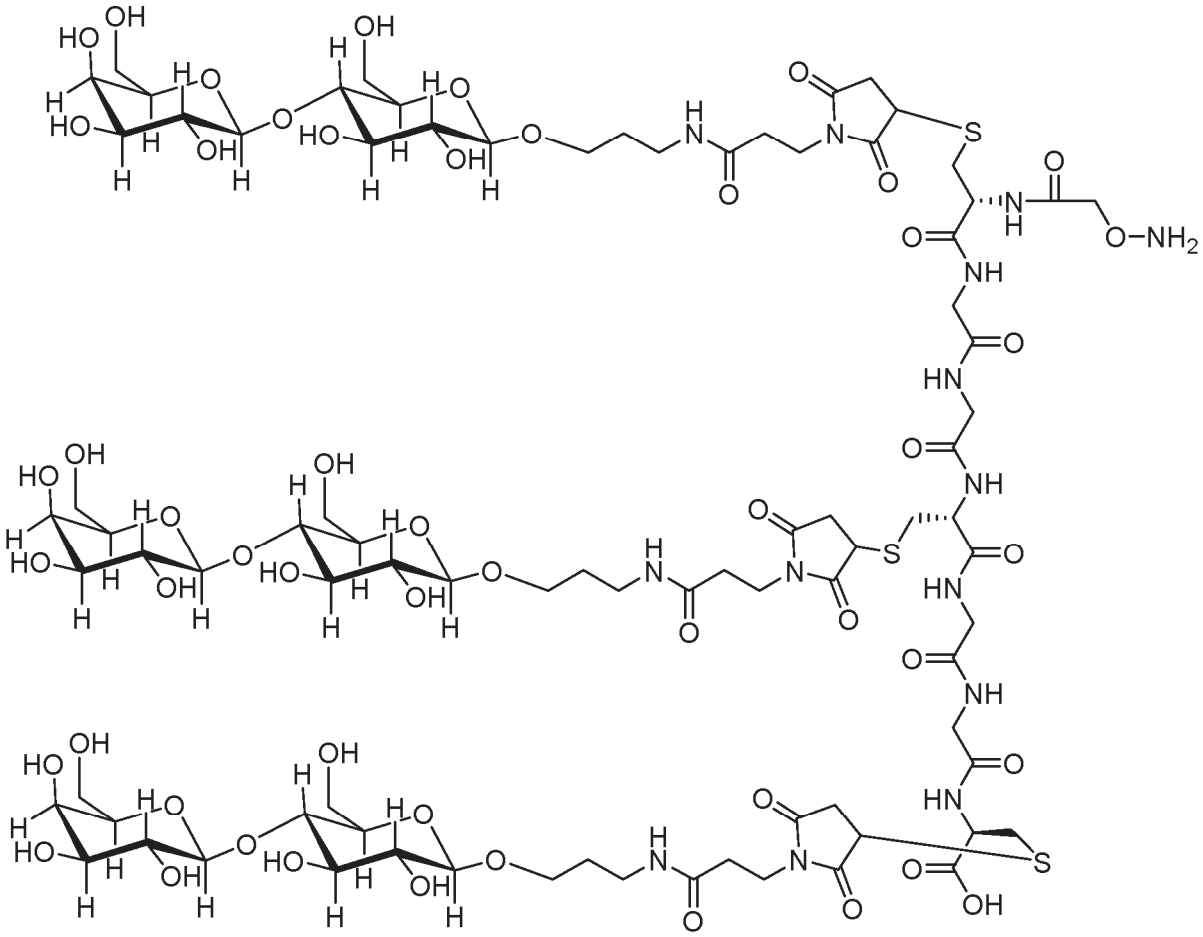
The binding polypeptides of the present invention may be used to remove toxic compounds and harmful substances from the liver in multiple diseases by targeting carbohydrate receptors (e.g., mannose 6-phosphate receptor, mannose receptor, and asialoglycoprotein receptor). Please see: Ganesan, L.P. et al: Rapid and Efficient Clearance of Blood-borne Virus by Liver Sinusoidal Endothelium. PLoS Pathogens 2011, 9: 1; and Monnier, V.M. et al: Glucosepane: a poorly understood advanced glycation end product of growing importance for diabetes and its complications. Clin Chem Lab Med 2014; 52: 21.

The binding polypeptides of the present invention may also be used to target tumor cells through targeting one or more different cell receptors including, but not limited to: carbohydrate receptors, asialoglycoprotein receptors, and/or Siglecs. Please see: Chen, W.C. et al: In vivo targeting of B-cell lymphoma with glycan ligands of CD22. Blood 2010, 115: 4778; Chen, W.C. et al: Targeting B lymphoma with nanoparticles bearing glycan ligands of CD22. Leuk Lymphoma 2012, 53: 208; Hatakeyama, S. et al: Targeted drug delivery to tumor vasculature by a carbohydrate mimetic peptide. PNAS, 2011, 108: 19587; Hong, F. et al: β-Glucan Functions as an Adjuvant for Monoclonal Antibody Immunotherapy by Recruiting Tumoricidal Granulocytes as Killer Cells. Cancer Res. 2003, 23: 9023; Kawasakia, N. et al: Targeted delivery of lipid antigen to macrophages via the CD169/sialoadhesin endocytic pathway induces robust invariant natural killer T cell activation. PNAS 2013, 110: 7826; and
Medina, S.H. et al: N-acetylgalactosamine-functionalized dendrimers as hepatic cancer cell-targeted carriers. Biomaterials 2011, 32: 4118.

The binding peptides of the present invention may also be used to regulate immune response through various receptors including, but not limited to, carbohydrate receptors, DC-SIGNs, and/or Siglecs. Please see: Anthony, R.M. et al: Recapitulation of IVIG Anti-

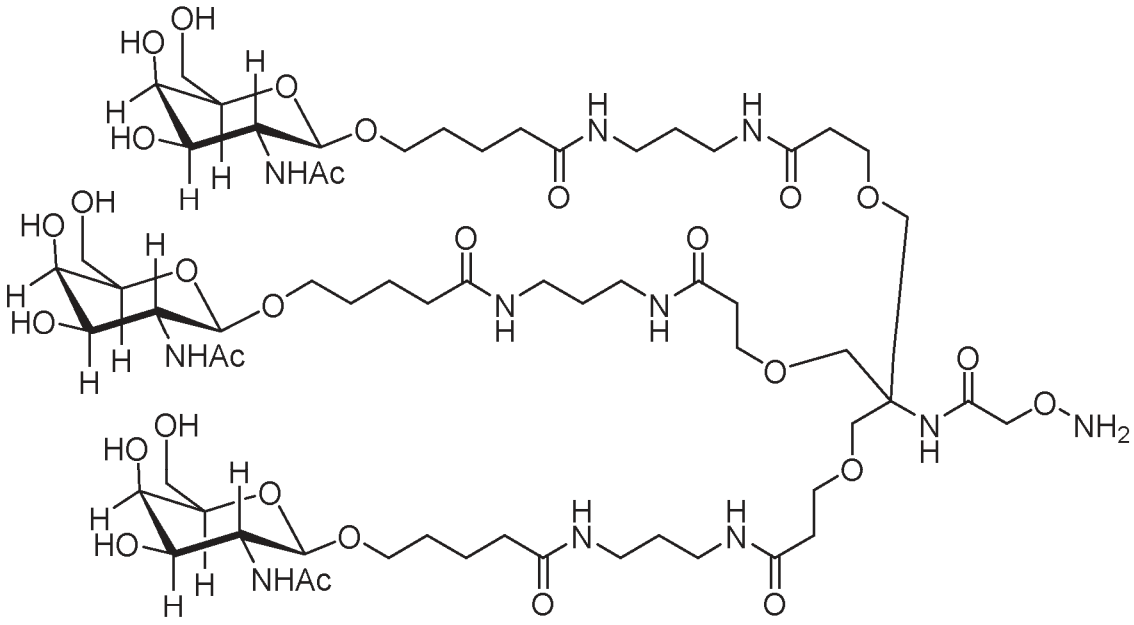
Inflammatory Activity with a Recombinant IgG Fc. Science 2008, 320: 373; Anthony, R.M. et al: Identification of a receptor required for the anti-inflammatory activity of IVIG. PNAS 2008, 105: 19571; Kaneko, Y. et al: Anti-Inflammatory Activity of Immunoglobulin G Resulting from Fc Sialylation. Science 2006, 313: 670; and Mattner, J. et al: Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. Nature 2005, 434: 525.

In one embodiment, the targeting moiety is a glycopeptide. In a further embodiment, the targeting moiety is a tri-galactosylated glycopeptide, e.g., lactose$_3$-Cys$_3$Gly$_4$ (shown in Formula V, below):



[Formula V].

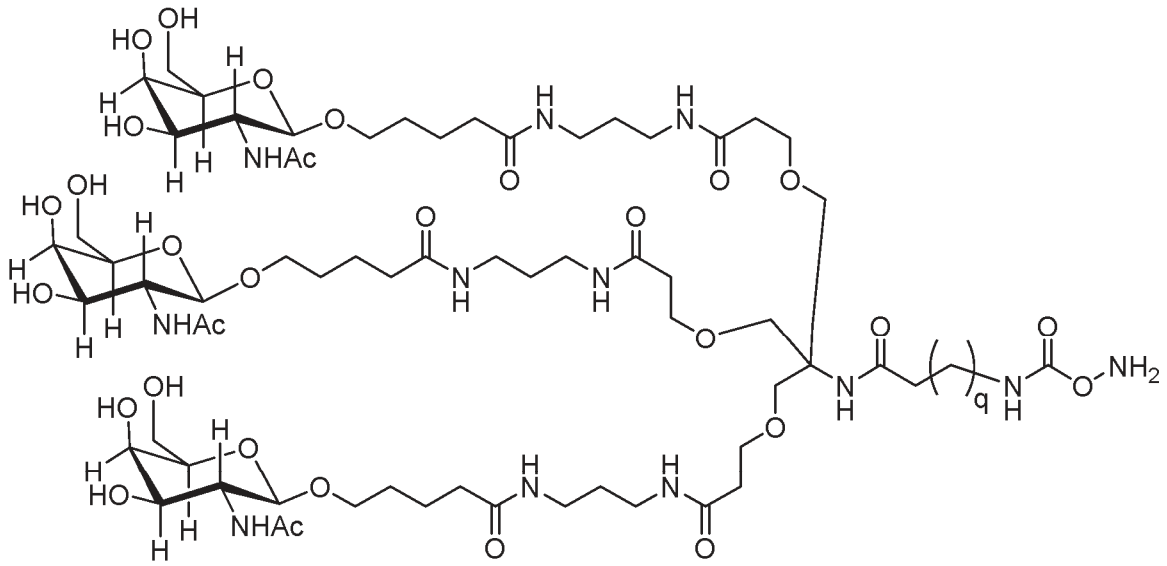
In some embodiments, the targeting moiety may be represented by Formula VII:



[Formula VII].

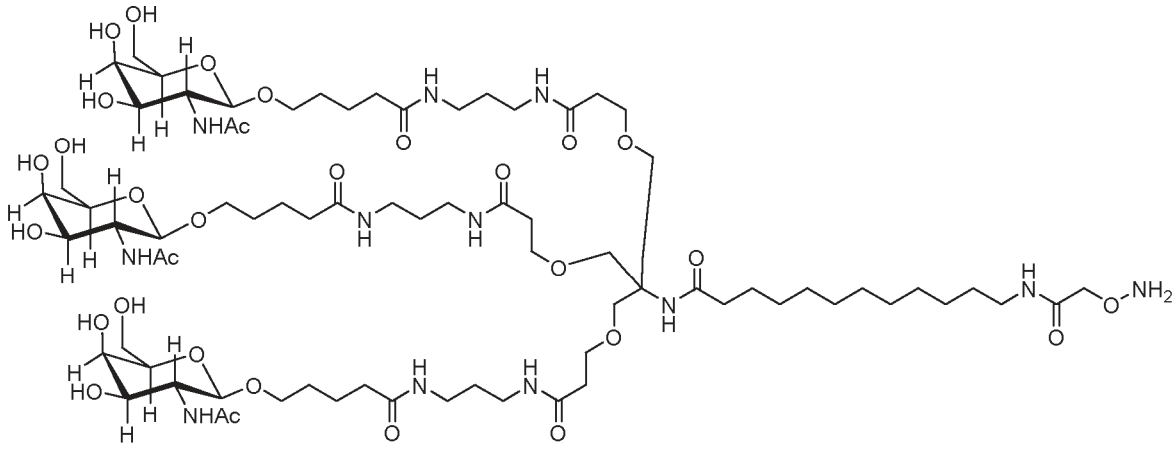
In some embodiments, the connector moiety of the effector moiety of Formula (I) comprises a spacer, including but not limited to a C_{2-30} alkyl or 1 to 32 PEG. In some embodiments, the connector moiety of the effector moiety is chosen based on approximate length (**Figure 79**). Appropriate spacer lengths include but are not limited to ~12 Å, ~16 Å, ~20 Å, ~31 Å, ~45 Å, ~60 Å, ~80 Å, and ~88 Å.

In certain embodiments, the effector moiety of Formula (I) may be represented by Formula VIII:

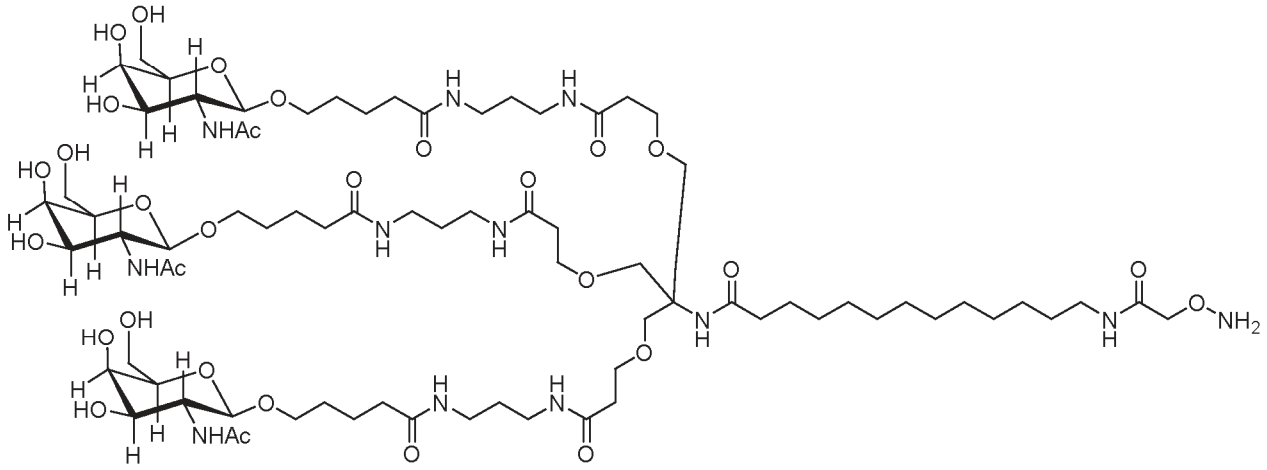


[Formula VIII],

wherein, q is an integer between 1 and 29 inclusive. For example, q may be 6, 8, 10, 11, 12, 16, 18, or 22. In example embodiments, the effector moiety of Formula VIII may be represented by:

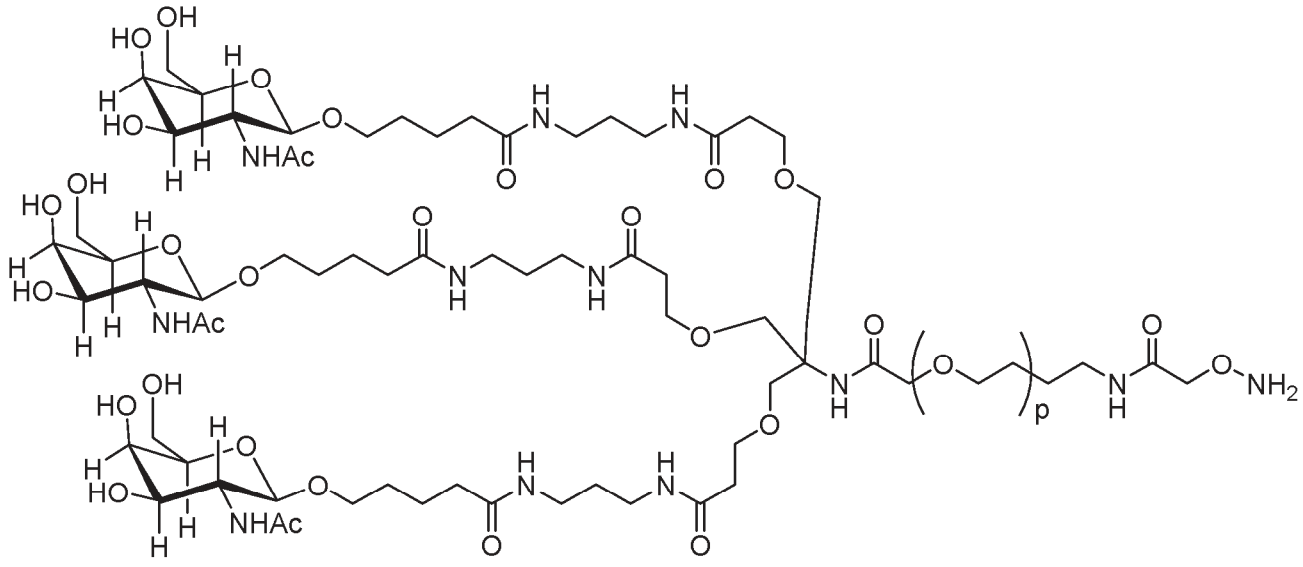


[Formula XIII] or



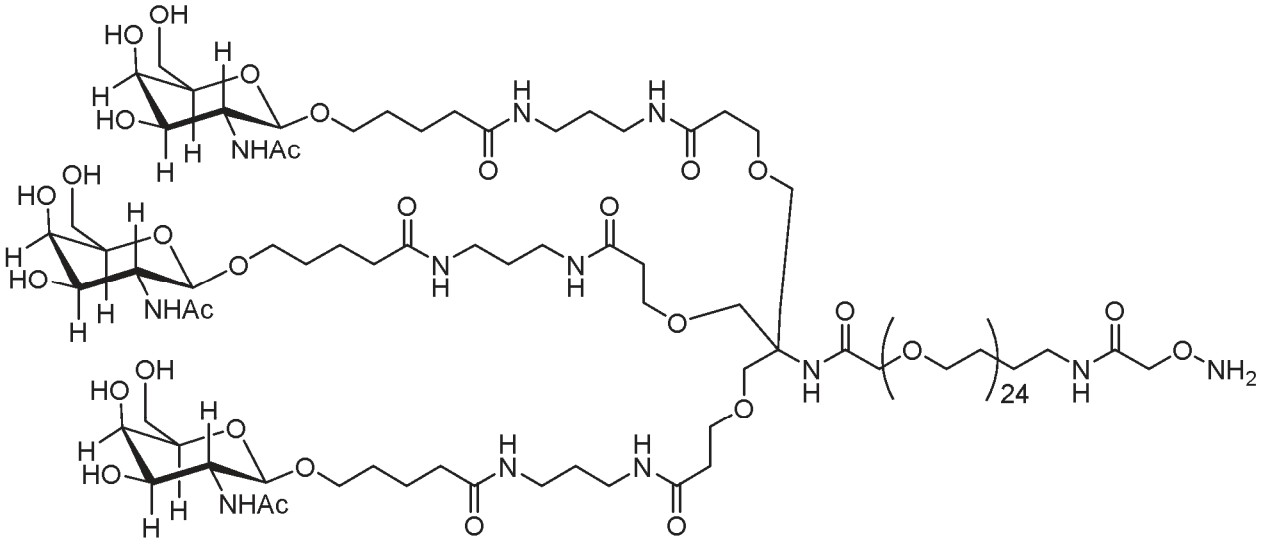
[Formula XIV].

In other embodiments, the effector moiety of Formula (I) may be represented by Formula IX:



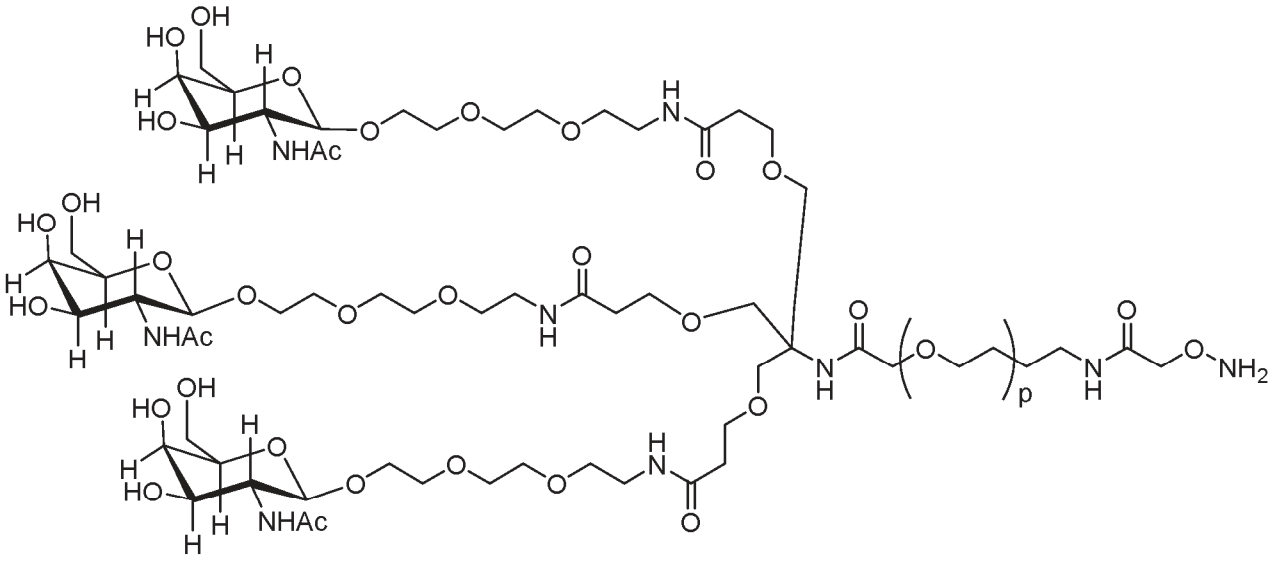
[Formula IX],

wherein, p has a value of 1 to 32. For example, p may be 2, 4, 6, 8, 11, 12, or 24. In example embodiments, the effector moiety of Formula IX may be represented by Formula X:



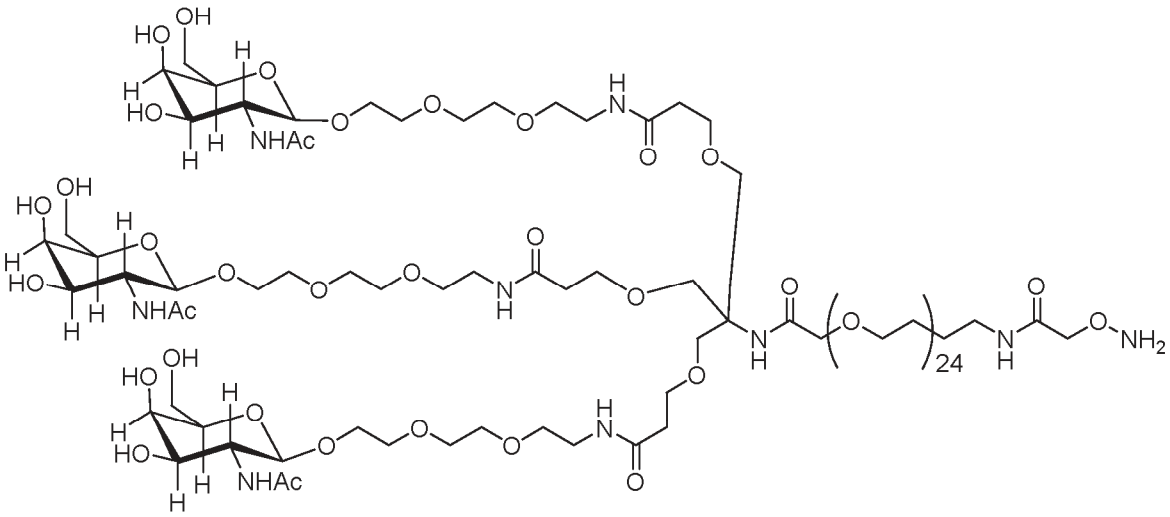
[Formula X].

In other embodiments, the effector moiety of Formula (I) may be represented by Formula XI:



[Formula XI],

wherein p has a value of 1 to 32. For example, p may be 2, 4, 6, 8, 11, 12, or 24. In example embodiments, the effector moiety of Formula XI may be represented by Formula XII:



[Formula XII].

e) PEG Moieties

In other aspects, the effector moiety is a moiety comprising poly(ethylene glycol) (PEG, PEO, or POE). PEG is an oligomer or polymer of ethylene oxide and has the chemical structure H-(O-CH2-CH2)n-OH wherein the element in parentheses is repeated. PEGylation (or pegylation) is a process in which PEG polymer chains are attached to another molecule (e.g., a binding polypeptide), which is then described as PEGylated (or pegylated). PEGylation can serve to reduce immunogenicity and antigenicity as well as to increase the hydrodynamic size (size in solution) of the molecule it is attached to, reducing renal clearance and prolonging circulation time. PEGylation can also make molecules more water soluble. In one embodiment of the present invention, the PEG moiety may comprise mono-PEG, bi-PEG, or tri-PEG. In another embodiment, the PEG moiety comprises 3 to 3.5 PEG.

VI. Conjugation of Effector Moieties to Binding Polypeptides

In certain embodiments, effector moieties are conjugated (either directly or through a linker moiety) to an oxidized glycan (e.g., an oxidized N-linked glycan) of an altered binding polypeptide, (e.g., an engineered glycan at N114 of an antibody CH1 domain or a native glycan at N297 of an antibody F domain). The term "oxidized glycan" means that an alcohol substituent on the glycan has been oxidized, providing a carbonyl substituent. The carbonyl

substituent can react with suitable nitrogen nucleophile to form a carbon-nitrogen double bond. For example, reaction of the carbonyl group with an aminooxy group or hydrazine group would form an oxime or hydrazine, respectively. In one embodiment, the carbonyl substituent is an aldehyde. Suitable oxidized glycans include oxidized galactose and oxidized sialic acid.

In one embodiment, the modified polypeptide of Formula (II) may be of Formula (II):

$$Ab(Gal\text{-}C(O)H)_x(Gal\text{-}Sia\text{-}C(O)H)_y$$

Formula (II),

wherein

A) Ab is an antibody or other binding polypeptide as defined herein;

B) Gal is a component derived from galactose;

C) Sia is a component derived from sialic acid;

D) x is 0 to 5; and

E) y is 0 to 5,

wherein at least one of x and y is not 0.

Any art recognized chemistry can be employed to conjugate an effector moiety (e.g., an effector moiety comprising a linker moiety) to a glycan (see e.g., Hermanson, G.T., Bioconjugate Techniques. Academic Press (1996), which is incorporated herein ion its entirety). In certain embodiments, a saccharide residue (e.g., a sialic acid or galactose residue) of the glycan is first oxidized (e.g., using sodium periodate treatment of sialic acid or galactose oxidase treatment of galactose) to generate a reactive aldehyde group. This aldehyde group is reacted with effector moiety an aminooxy group or hydrazine group to form an oxime or hydrazone linker, respectively. Exemplary methods employing this general reaction scheme are set forth in Examples 10 to 15.

In certain embodiments, the native or engineered glycans of a binding polypeptide are first pre-treated with glycosyltransferase enzymes *in vitro* to provide a terminal saccharide residue that is suitably reactive. For example, sialylation may be achieved first using a combination of galactosyltransferase (Gal T) and sialyltransferase (Sial T). In certain embodiments, biantennary glycans that lack galatose (G0F or G0) or that contain only one

galactose (G1F or G1) can be converted to higher-order galactosylated or sialylated structures suitable for conjugation (G1F, G1, G2F, G2, G1S1F, G1S1, G2S1F, G2S1, G2S2F, or G2S2).

An exemplary conjugation scheme for producing sialylated glycoconjugates is shown in **Figure 25C**. In an exemplary embodiment, sialic acid residues are introduced enzymatically and site specifically into the glycan of an antibody (e.g., a native glycan at Asn-297) using a combination of galactosyltransferase (Gal T) and sialyltransferase (Sial T). Introduced sialic acid residues are subsequently oxidized with a low concentration of sodium periodate to yield reactive sialic acid aldehydes suitably reactive with linkers (e.g., aminooxy linkers) to generate antibody–effector moiety conjugates (e.g., oxime-linked antibody-effector moiety conjugates). By controlling the number of glycan and the number of sialic residues with *in vitro* remodeling, the skilled artisan has precise control over the drug-antibody ratio (DAR) of the antibody-effector moiety conjugates. For example, if ~1 sialic acid is added onto a single biantennary glycan (A1F) in each of heavy chain, an antibody or binding polypeptide with a DAR of 2 can be homogeneously obtained.

VII. Modified Binding Polypeptides

In certain embodiments, the invention provides modified polypeptides which are the product of the conjugating effector moieties are conjugated (either directly or through a linker moiety) to one or more oxidized glycans (e.g., an oxidized N-linked glycan) of an altered binding polypeptide (e.g., an engineered glycan at N114 of an antibody CH1 domain or a native glycan at N297 of an antibody F domain).

In one embodiment, the binding polypeptide can be of Formula (III):

$$Ab(Gal\text{-}C(H)=N\text{-}Q\text{-}CON\text{-}X)_x(Gal\text{-}Sia\text{-}C(H)=N\text{-}Q\text{-}CON\text{-}X)_y$$

Formula (III),

wherein:

A) Ab is an antibody as defined herein;

B) Q is NH or O;

C) CON is a connector moiety as defined herein;

D) X is a targeting moiety as defined herein;

E) Gal is a component derived from galactose;

F) Sia is a component derived from sialic acid;

G) x is 0 to 5; and

H) y is 0 to 5,

wherein at least one of x and y is not 0.

In one embodiment, the binding polypeptide can be of Formula (III) can be of Formula (IIIa):

$$Ab(Gal\text{-}C(H)=N\text{-}Q\text{-}CH_2\text{-}C(O)\text{-}Z\text{-}X)_x(Gal\text{-}Sia\text{-}C(H)=N\text{-}Q\text{-}CH_2\text{-}C(O)\text{-}Z\text{-}X)_y$$

Formula (IIIa),

wherein:

A) Ab is an antibody;

B) Q is NH or O;

C) Z is Cys-$(MC)_a$-$(VC)_b$-$(PABC)_c$-$(C_{16}H_{32}O_8\ C_2H_4)_f$ -, wherein

i. Cys is a component derived cysteinamide;

ii. MC is a component derived from maleimide;

iii. VC is a component derived from valine coupled with citruline;

iv. PABC is a component derived from 4-aminobenzyl carbamate;

v. X is an effector moiety (e.g., a targeting moiety as defined herein);

vi. a is 0 or 1;

vii. b is 0 or 1;

viii. c is 0 or 1; and

ix. f is 0 or 1;

D) X is a therapeutic agent as defined herein;

E) Gal is a component derived from galactose;

F) Sia is a component derived from sialic acid;

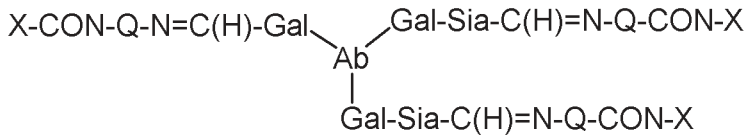
G) x is 0 to 5; and

H) y is 0 to 5,

wherein at least one of x and y is not 0.

It is to be understood that the Formula (III) is not intended to imply that the antibody, the Gal substituent, and the Gal-Sia subsituent are connected in a chain-like manner. Rather, when such substituents are present, the antibody is connected directly to each substituent. For

example, a binding polypeptide of Formula (III) in which x is 1 and y is 2 could have the arrangement shown below:



Formula (III)

The CON substituent in Formula (III) and components therein are as described with regard to Formula (I) for effector moieties.

In one embodiment, Q is NH. In another embodiment, Q is O.

In one embodiment, x is 0.

The antibody Ab of Formula (III) may be any suitable antibody as described herein.

In one embodiment, there is provided a method for preparing the binding polypeptide of Formula (III), the method comprising reacting an effector moiety of Formula (I):

NH_2-Q-CON-X

Formula (I),

wherein:

A) Q is NH or O;

B) CON is a connector moiety; and

C) X is an effector moiety (e.g., a targeting moiety as defined herein),

with a modified antibody of Formula (II)

$Ab(OXG)_r$

Formula (II)

wherein

A) OXG is an oxidized glycan; and

B) r is selected from 0 to 4;

In one embodiment, there is provided a method for preparing the binding polypeptide of Formula (III), the method comprising reacting an effector moiety of Formula (I):

NH_2-Q-CON-X

Formula (I),

wherein:

A) Q is NH or O;

B) CON is a connector moiety; and

C) X is an effector moiety (e.g., a targeting moiety as defined herein),

with a modified antibody of Formula (IIa)

$$Ab(Gal\text{-}C(O)H)_x(Gal\text{-}Sia\text{-}C(O)H)_y$$

Formula (IIa),

wherein

A) Ab is an antibody as described herein;

B) Gal is a component derived from galactose;

C) Sia is a component derived from sialic acid;

D) x is 0 to 5; and

E) y is 0 to 5,

wherein at least one of x and y is not 0.

VII. Methods of Treatment with Modified Antibodies

In one aspect, the invention provides methods of treating or diagnosing a patient in need thereof comprising administering an effective amount of a binding polypeptide disclosed herein. In certain embodiments of the present disclosure provide kits and methods for the diagnosis and/or treatment of disorders, e.g., neoplastic disorders in a mammalian subject in need of such treatment. In certain exemplary embodiments, the subject is a human.

The binding polypeptides of the current disclosure are useful in a number of different applications. For example, in one embodiment, the subject binding polypeptides are useful for reducing or eliminating cells bearing an epitope recognized by the binding domain of the binding polypeptide. In another embodiment, the subject binding polypeptides are effective in reducing the concentration of or eliminating soluble antigen in the circulation. In one embodiment, the binding polypeptides may reduce tumor size, inhibit tumor growth and/or

prolong the survival time of tumor-bearing animals. Accordingly, this disclosure also relates to a method of treating tumors in a human or other animal by administering to such human or animal an effective, non-toxic amount of modified antibody. One skilled in the art would be able, by routine experimentation, to determine what an effective, non-toxic amount of modified binding polypeptide would be for the purpose of treating malignancies. For example, a therapeutically active amount of a modified antibody or one or more fragments thereof may vary according to factors such as the disease stage (e.g., stage I versus stage IV), age, sex, medical complications (e.g., immunosuppressed conditions or diseases) and weight of the subject, and the ability of the modified antibody to elicit a desired response in the subject. The dosage regimen may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

In general, the compositions provided in the current disclosure may be used to prophylactically or therapeutically treat any neoplasm comprising an antigenic marker that allows for the targeting of the cancerous cells by the modified antibody.

VIII. Methods of Administering Modified Antibodies or Fragments Thereof

Methods of preparing and administering binding polypeptides of the current disclosure to a subject are well known to or are readily determined by those skilled in the art. The route of administration of the binding polypeptides of the current disclosure may be oral, parenteral, by inhalation or topical. The term parenteral as used herein includes intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, rectal or vaginal administration. While all these forms of administration are clearly contemplated as being within the scope of the current disclosure, a form for administration would be a solution for injection, in particular for intravenous or intraarterial injection or drip. Usually, a suitable pharmaceutical composition for injection may comprise a buffer (e.g. acetate, phosphate or citrate buffer), a surfactant (e.g. polysorbate), optionally a stabilizer agent (e.g. human albumin), etc. However, in other methods compatible with the teachings herein, the modified antibodies can be delivered directly to the site of the adverse cellular population thereby increasing the exposure of the diseased tissue to the therapeutic agent.

In one embodiment, the binding polypeptide that is administered is a binding polypeptide of Formula (III):

$$Ab(Gal\text{-}C(H)=N\text{-}Q\text{-}CON\text{-}X)_x(Gal\text{-}Sia\text{-}C(H)=N\text{-}Q\text{-}CON\text{-}X)_y$$

Formula (III),

wherein:

A) Ab is an antibody as defined herein;

B) Q is NH or O;

C) CON is a connector moiety as defined herein;

D) X is an effector moiety (e.g., a targeting moiety as defined herein);

E) Gal is a component derived from galactose;

F) Sia is a component derived from sialic acid;

G) x is 0 to 5; and

H) y is 0 to 5,

wherein at least one of x and y is not 0.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. In the compositions and methods of the current disclosure, pharmaceutically acceptable carriers include, but are not limited to, 0.01-0.1 M, e.g.,0.05M phosphate buffer, or 0.8% saline. Other common parenteral vehicles include sodium phosphate solutions, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present such as for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like. More particularly, pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In such cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and will typically be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity

can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal and the like. In many cases, isotonic agents wil be included, for example, sugars, polyalcohols, such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

In any case, sterile injectable solutions can be prepared by incorporating an active compound (e.g., a modified binding polypeptide by itself or in combination with other active agents) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle, which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, exemplary methods of preparation include vacuum drying and freeze-drying, which yields a powder of an active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The preparations for injections are processed, filled into containers such as ampoules, bags, bottles, syringes or vials, and sealed under aseptic conditions according to methods known in the art. Further, the preparations may be packaged and sold in the form of a kit such as those described in co-pending U.S.S.N. 09/259,337 and U.S.S.N. 09/259,338 each of which is incorporated herein by reference. Such articles of manufacture will typically have labels or package inserts indicating that the associated compositions are useful for treating a subject suffering from, or predisposed to autoimmune or neoplastic disorders.

Effective doses of the compositions of the present disclosure, for the treatment of the above described conditions vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic. Usually, the patient is a human but non-human mammals including transgenic

mammals can also be treated. Treatment dosages may be titrated using routine methods known to those of skill in the art to optimize safety and efficacy.

For passive immunization with a binding polypeptide, the dosage can range, e.g., from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg (e.g., 0.02 mg/kg, 0.25 mg/kg, 0.5 mg/kg, 0.75 mg/kg, lmg/kg, 2 mg/kg, etc.), of the host body weight. For example dosages can be 1 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg, e.g., at least 1 mg/kg. Doses intermediate in the above ranges are also intended to be within the scope of the current disclosure. Subjects can be administered such doses daily, on alternative days, weekly or according to any other schedule determined by empirical analysis. An exemplary treatment entails administration in multiple dosages over a prolonged period, for example, of at least six months. Additional exemplary treatment regimens entail administration once per every two weeks or once a month or once every 3 to 6 months. Exemplary dosage schedules include 1-10 mg/kg or 15 mg/kg on consecutive days, 30 mg/kg on alternate days or 60 mg/kg weekly. In some methods, two or more monoclonal antibodies with different binding specificities are administered simultaneously, in which case the dosage of each antibody administered falls within the ranges indicated.

Binding polypeptides of the current disclosure can be administered on multiple occasions. Intervals between single dosages can be weekly, monthly or yearly. Intervals can also be irregular as indicated by measuring blood levels of modified binding polypeptide or antigen in the patient. In some methods, dosage is adjusted to achieve a plasma modified binding polypeptide concentration of 1-1000 μg/ml and in some methods 25-300 μg/ml. Alternatively, binding polypeptides can be administered as a sustained release formulation, in which case less frequent administration is required. For antibodies, dosage and frequency vary depending on the half-life of the antibody in the patient. In general, humanized antibodies show the longest half-life, followed by chimeric antibodies and nonhuman antibodies.

The dosage and frequency of administration can vary depending on whether the treatment is prophylactic or therapeutic. In prophylactic applications, compositions containing the present antibodies or a cocktail thereof are administered to a patient not already in the disease state to enhance the patient's resistance. Such an amount is defined to be a "prophylactic effective dose." In this use, the precise amounts again depend upon the patient's state of health and general immunity, but generally range from 0.1 to 25 mg per dose,

especially 0.5 to 2.5 mg per dose. A relatively low dosage is administered at relatively infrequent intervals over a long period of time. Some patients continue to receive treatment for the rest of their lives. In therapeutic applications, a relatively high dosage (e.g., from about 1 to 400 mg/kg of antibody per dose, with dosages of from 5 to 25 mg being more commonly used for radioimmunoconjugates and higher doses for cytotoxin-drug modified antibodies) at relatively short intervals is sometimes required until progression of the disease is reduced or terminated, oruntil the patient shows partial or complete amelioration of disease symptoms . Thereafter, the patient can be administered a prophylactic regime.

Binding polypeptides of the current disclosure can optionally be administered in combination with other agents that are effective in treating the disorder or condition in need of treatment (e.g., prophylactic or therapeutic). Effective single treatment dosages (i.e., therapeutically effective amounts) of 90Y-labeled modified antibodies of the current disclosure range from between about 5 and about 75 mCi, such as between about 10 and about 40 mCi. Effective single treatment non-marrow ablative dosages of ^{131}I-modified antibodies range from between about 5 and about 70 mCi, orbetween about 5 and about 40 mCi. Effective single treatment ablative dosages (i.e., may require autologous bone marrow transplantation) of ^{131}I-labeled antibodies range from between about 30 and about 600 mCi, such as between about 50 and less than about 500 mCi. In conjunction with a chimeric antibody, owing to the longer circulating half-life vis-a-vis murine antibodies, an effective single treatment non-marrow ablative dosages of iodine-131 labeled chimeric antibodies range from between about 5 and about 40 mCi, such as less than about 30 mCi. Imaging criteria for, e.g., the ^{111}In label, are typically less than about 5 mCi.

While the binding polypeptides may be administered as described immediately above, it must be emphasized that in other embodiments binding may be administered to otherwise healthy patients as a first line therapy. In such embodiments the binding polypeptides may be administered to patients having normal or average red marrow reserves and/or to patients that have not, and are not, undergoing. As used herein, the administration of modified antibodies or fragments thereof in conjunction or combination with an adjunct therapy means the sequential, simultaneous, coextensive, concurrent, concomitant, or contemporaneous administration or application of the therapy and the disclosed antibodies. Those skilled in the art will appreciate that the administration or application of the various components of the combined therapeutic regimen may be timed to enhance the overall effectiveness of the

treatment. For example, chemotherapeutic agents could be administered in standard, well known courses of treatment followed within a few weeks by radioimmunoconjugates of the present disclosure. Conversely, cytotoxin associated binding polypeptides could be administered intravenously followed by tumor localized external beam radiation. In yet other embodiments, the modified binding polypeptide may be administered concurrently with one or more selected chemotherapeutic agents in a single office visit. A skilled artisan (e.g. an experienced oncologist) would be readily be able to discern effective combined therapeutic regimens without undue experimentation based on the selected adjunct therapy and the teachings of the instant specification.

In this regard it will be appreciated that the combination of the binding polypeptides and the chemotherapeutic agent may be administered in any order and within any time frame that provides a therapeutic benefit to the patient. That is, the chemotherapeutic agent and binding polypeptides may be administered in any order or concurrently. In selected embodiments the binding polypeptides of the present disclosure will be administered to patients that have previously undergone chemotherapy. In yet other embodiments, the binding polypeptides and the chemotherapeutic treatment will be administered substantially simultaneously or concurrently. For example, the patient may be given the binding polypeptides while undergoing a course of chemotherapy. In some embodiments the modified antibody will be administered within one year of any chemotherapeutic agent or treatment. In other embodiments the binding polypeptides will be administered within 10, 8, 6, 4, or 2 months of any chemotherapeutic agent or treatment. In still other embodiments the binding polypeptide will be administered within 4, 3, 2, or 1 week(s) of any chemotherapeutic agent or treatment. In yet other embodiments the binding polypeptides will be administered within 5, 4, 3, 2, or 1 day(s) of the selected chemotherapeutic agent or treatment. It will further be appreciated that the two agents or treatments may be administered to the patient within a matter of hours or minutes (i.e. substantially simultaneously).

It will further be appreciated that the binding polypeptides of the current disclosure may be used in conjunction or combination with any chemotherapeutic agent or agents (e.g. to provide a combined therapeutic regimen) that eliminates, reduces, inhibits or controls the growth of neoplastic cells in vivo. Exemplary chemotherapeutic agents that are compatible with the current disclosure include alkylating agents, vinca alkaloids (e.g., vincristine and vinblastine), procarbazine, methotrexate and prednisone. The four-drug combination MOPP

(mechlethamine (nitrogen mustard), vincristine (Oncovin), procarbazine and prednisone) is very effective in treating various types of lymphoma. In MOPP-resistant patients, ABVD (e.g., adriamycin, bleomycin, vinblastine and dacarbazine), ChIVPP (CH1orambucil, vinblastine, procarbazine and prednisone), CABS (lomustine, doxorubicin, bleomycin and streptozotocin), MOPP plus ABVD, MOPP plus ABV (doxorubicin, bleomycin and vinblastine) or BCVPP (carmustine, cyclophosphamide, vinblastine, procarbazine and prednisone) combinations can be used. Arnold S. Freedman and Lee M. Nadler, Malignant Lymphomas, in HARRISON'S PRINCIPLES OF INTERNAL MEDICINE 1774-1788 (Kurt J. Isselbacher et al, eds., 13th ed. 1994) and V. T. DeVita et al, (1997) and the references cited therein for standard dosing and scheduling. These therapies can be used unchanged, or altered as needed for a particular patient, in combination with one or more binding polypeptides of the current disclosure as described herein.

Additional regimens that are useful in the context of the present disclosure include use of single alkylating agents such as cyclophosphamide or chlorambucil, or combinations such as CVP (cyclophosphamide, vincristine and prednisone), CHOP (CVP and doxorubicin), C-MOPP (cyclophosphamide, vincristine, prednisone and procarbazine), CAP-BOP (CHOP plus procarbazine and bleomycin), m-BACOD (CHOP plus methotrexate, bleomycin and leucovorin), ProMACE-MOPP (prednisone, methotrexate, doxorubicin, cyclophosphamide, etoposide and leucovorin plus standard MOPP), ProMACE-CytaBOM (prednisone, doxorubicin, cyclophosphamide, etoposide, cytarabine, bleomycin, vincristine, methotrexate and leucovorin) and MACOP-B (methotrexate, doxorubicin, cyclophosphamide, vincristine, fixed dose prednisone, bleomycin and leucovorin). Those skilled in the art will readily be able to determine standard dosages and scheduling for each of these regimens. CHOP has also been combined with bleomycin, methotrexate, procarbazine, nitrogen mustard, cytosine arabinoside and etoposide. Other compatible chemotherapeutic agents include, but are not limited to, 2-Chlorodeoxyadenosine (2-CDA), 2'-deoxycoformycin and fludarabine.

For patients with intermediate-and high-grade NHL (non-Hodgkin's lymphoma), who fail to achieve remission or relapse, salvage therapy is used. Salvage therapies employ drugs such as cytosine arabinoside, carboplatin, cisplatin, etoposide and ifosfamide given alone or in combination. In relapsed or aggressive forms of certain neoplastic disorders the following protocols are often used: IMVP-16 (ifosfamide, methotrexate and etoposide), MIME (methyl-gag, ifosfamide, methotrexate and etoposide), DHAP (dexamethasone, high dose cytarabine

and cisplatin), ESHAP (etoposide, methylpredisolone, HD cytarabine, cisplatin), CEPP(B) (cyclophosphamide, etoposide, procarbazine, prednisone and bleomycin) and CAMP (lomustine, mitoxantrone, cytarabine and prednisone) each with well-known dosing rates and schedules.

The amount of chemotherapeutic agent to be used in combination with the modified antibodies of the current disclosure may vary by subject or may be administered according to what is known in the art. See for example, Bruce A Chabner et al, Antineoplastic Agents, in GOODMAN & GILMAN'S THE PHARMACOLOGICAL BASIS OF THERAPEUTICS 1233-1287 ((Joel G. Hardman et al, eds., 9th ed. 1996).

As previously discussed, the binding polypeptides of the present disclosure, immunoreactive fragments or recombinants thereof may be administered in a pharmaceutically effective amount for the in vivo treatment of mammalian disorders. In this regard, it will be appreciated that the disclosed binding polypeptides will be formulated to facilitate administration and promote stability of the active agent.

A pharmaceutical compositions in accordance with the present disclosure can comprise a pharmaceutically acceptable, non-toxic, sterile carrier such as physiological saline, nontoxic buffers, preservatives and the like. For the purposes of the instant application, a pharmaceutically effective amount of the modified binding polypeptide, immunoreactive fragment or recombinant thereof, conjugated or unconjugated to a therapeutic agent, shall be held to mean an amount sufficient to achieve effective binding to an antigen and to achieve a benefit, e.g., to ameliorate symptoms of a disease or disorder or to detect a substance or a cell. In the case of tumor cells, the modified binding polypeptide can interact with selected immunoreactive antigens on neoplastic or immunoreactive cells and provide for an increase in the death of those cells. Of course, the pharmaceutical compositions of the present disclosure may be administered in single or multiple doses to provide for a pharmaceutically effective amount of the modified binding polypeptide.

In keeping with the scope of the present disclosure, the binding polypeptides of the disclosure may be administered to a human or other animal in accordance with the aforementioned methods of treatment in an amount sufficient to produce a therapeutic or prophylactic effect. The binding polypeptides of the disclosure can be administered to such human or other animal in a conventional dosage form prepared by combining the antibody of the disclosure with a conventional pharmaceutically acceptable carrier or diluent according to

known techniques. It will be recognized by one of skill in the art that the form and character of the pharmaceutically acceptable carrier or diluent is dictated by the amount of active ingredient with which it is to be combined, the route of administration and other well-known variables. Those skilled in the art will further appreciate that a cocktail comprising one or more species of binding polypeptides described in the current disclosure may prove to be particularly effective.

IX. Expression of Binding polypeptides

In one aspect, the invention provides polynucleotides encoding the binding polypeptides disclosed herein. A method of making a binding polypeptide comprising expressing these polynucleotides are also provided.

Polynucleotides encoding the binding polypeptides disclosed herein are typically inserted in an expression vector for introduction into host cells that may be used to produce the desired quantity of the claimed antibodies, or fragments thereof. Accordingly, in certain aspects, the invention provides expression vectors comprising polynucleotides disclosed herein and host cells comprising these vectors and polynucleotides.

The term "vector" or "expression vector" is used herein for the purposes of the specification and claims, to mean vectors used in accordance with the present invention as a vehicle for introducing into and expressing a desired gene in a cell. As known to those skilled in the art, such vectors may easily be selected from the group consisting of plasmids, phages, viruses and retroviruses. In general, vectors compatible with the instant invention will comprise a selection marker, appropriate restriction sites to facilitate cloning of the desired gene and the ability to enter and/or replicate in eukaryotic or prokaryotic cells.

Numerous expression vector systems may be employed for the purposes of this invention. For example, one class of vector utilizes DNA elements which are derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MOMLV), or SV40 virus. Others involve the use of polycistronic systems with internal ribosome binding sites. Additionally, cells which have integrated the DNA into their chromosomes may be selected by introducing one or more markers which allow selection of transfected host cells. The marker may provide for prototrophy to an auxotrophic host, biocide resistance (e.g., antibiotics) or resistance to heavy

metals such as copper. The selectable marker gene can either be directly linked to the DNA sequences to be expressed, or introduced into the same cell by cotransformation. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include signal sequences, splice signals, as well as transcriptional promoters, enhancers, and termination signals. In some embodiments the cloned variable region genes are inserted into an expression vector along with the heavy and light chain constant region genes (such as human genes) synthesized as discussed above.

In other embodiments the binding polypeptides featured in the invention may be expressed using polycistronic constructs. In such expression systems, multiple gene products of interest such as heavy and light chains of antibodies may be produced from a single polycistronic construct. These systems advantageously use an internal ribosome entry site (IRES) to provide relatively high levels of polypeptides in eukaryotic host cells. Compatible IRES sequences are disclosed in U.S. Pat. No. 6,193,980 which is incorporated by reference herein. Those skilled in the art will appreciate that such expression systems may be used to effectively produce the full range of polypeptides disclosed in the instant application.

More generally, once a vector or DNA sequence encoding an antibody, or fragment thereof, has been prepared, the expression vector may be introduced into an appropriate host cell. That is, the host cells may be transformed. Introduction of the plasmid into the host cell can be accomplished by various techniques well known to those of skill in the art. These include, but are not limited to, transfection (including electrophoresis and electroporation), protoplast fusion, calcium phosphate precipitation, cell fusion with enveloped DNA, microinjection, and infection with intact virus. See, Ridgway, A. A. G. "Mammalian Expression Vectors" Chapter 24.2, pp. 470-472 Vectors, Rodriguez and Denhardt, Eds. (Butterworths, Boston, Mass. 1988). The transformed cells are grown under conditions appropriate to the production of the light chains and heavy chains, and assayed for heavy and/or light chain protein synthesis. Exemplary assay techniques include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), or flourescence-activated cell sorter analysis (FACS), immunohistochemistry and the like.

As used herein, the term "transformation" shall be used in a broad sense to refer to the introduction of DNA into a recipient host cell that changes the genotype and consequently results in a change in the recipient cell.

Along those same lines, "host cells" refers to cells that have been transformed with vectors constructed using recombinant DNA techniques and encoding at least one heterologous gene. In descriptions of processes for isolation of polypeptides from recombinant hosts, the terms "cell" and "cell culture" are used interchangeably to denote the source of antibody unless it is clearly specified otherwise. In other words, recovery of polypeptide from the "cells" may mean either from spun down whole cells, or from the cell culture containing both the medium and the suspended cells.

In one embodiment, the host cell line used for antibody expression is of mammalian origin; those skilled in the art can determine particular host cell lines which are best suited for the desired gene product to be expressed therein. Exemplary host cell lines include, but are not limited to, DG44 and DUXB11 (Chinese Hamster Ovary lines, DHFR minus), HELA (human cervical carcinoma), CVI (monkey kidney line), COS (a derivative of CVI with SV40 T antigen), R1610 (Chinese hamster fibroblast) BALBC/3T3 (mouse fibroblast), HAK (hamster kidney line), SP2/O (mouse myeloma), BFA-1c1BPT (bovine endothelial cells), RAJI (human lymphocyte), 293 (human kidney). In one embodiment, the cell line provides for altered glycosylation, e.g., afucosylation, of the antibodyexpressed therefrom (e.g., PER.C6.RTM. (Crucell) or FUT8-knock-out CHO cell lines (Potelligent.RTM. Cells) (Biowa, Princeton, N.J.)). In one embodiment NS0 cells may be used. Host cell lines are typically available from commercial services, the American Tissue Culture Collection or from published literature.

In vitro production allows scale-up to give large amounts of the desired polypeptides. Techniques for mammalian cell cultivation under tissue culture conditions are known in the art and include homogeneous suspension culture, e.g. in an airlift reactor or in a continuous stirrer reactor, or immobilized or entrapped cell culture, e.g. in hollow fibers, microcapsules, on agarose microbeads or ceramic cartridges. If necessary and/or desired, the solutions of polypeptides can be purified by the customary chromatography methods, for example gel filtration, ion-exchange chromatography, chromatography over DEAE-cellulose and/or (immuno-) affinity chromatography.

One or more genes encoding binding polypeptides can also be expressed non-mammalian cells such as bacteria or yeast or plant cells. In this regard it will be appreciated that various unicellular non-mammalian microorganisms such as bacteria can also be transformed; i.e. those capable of being grown in cultures or fermentation. Bacteria, which

are susceptible to transformation, include members of the enterobacteriaceae, such as strains of Escherichia coli or Salmonella; Bacillaceae, such as Bacillus subtilis; Pneumococcus; Streptococcus, and Haemophilus influenzae. It will further be appreciated that, when expressed in bacteria, the polypeptides can become part of inclusion bodies. The polypeptides must be isolated, purified and then assembled into functional molecules.

In addition to prokaryotes, eukaryotic microbes may also be used. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among eukaryotic microorganisms although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, (Stinchcomb *et al.*, Nature, 282:39 (1979); Kingsman *et al.*, Gene, 7:141 (1979); Tschemper *et al.*, Gene, 10:157 (1980)) is commonly used. This plasmid already contains the TRP1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, Genetics, 85:12 (1977)). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

EXAMPLES

The present invention is further illustrated by the following examples which should not be construed as further limiting. The contents of the Sequence Listing, figures and all references, patents, and published patent applications cited throughout this application are expressly incorporated herein by reference.

Example 1. Design, preparation, and characterization of 2C3 anti-CD-52 hyperglycosylation antibody mutants

Multiple hyperglycosylation mutations were designed in the heavy chain of the anti-CD-52 antibody, 2C3, for the purpose of adding a bulky group to an interaction interface (e.g., the FcRn binding site to modulate antibody pharmacokinetics), for modulating antibody effector function by changing its interaction with FcγRs, or to introduce a novel cross-linking site subsequence chemical modification for effector moiety conjugation, including but not limited to, drugs, toxins, cytotoxic agents, radionucleotides and the like. The hyperglycosylated 2C3 mutants are set forth in Table 3.

Table 3. Hyperglycosylated 2C3 anti-CD-52 mutants

Mutation	Desired Benefit	Applications
A114N	Glycosylation at Asn-Ser-Thr	1) Control 2) Effector moiety conjugation
Y436T	Glycosylation at Asn434 Inhibition of FcRn binding	1) Transplant and other indications which need short half-life
Y436S	Glycosylation at Asn434 Inhibition of FcRn binding	1) Transplant and other indications which need short half-life
S440N	Glycosylation at Asn-Leu-Ser	1) Control 2) Effector moiety conjugation
S442N	Glycosylation at Asn-Leu-Ser	1) Control 2) Effector moiety conjugation
Add NGT to C-terminal	Glycosylation	1) Control 2) Effector moiety conjugation

S298N/Y300S	Glycosylation at Asn298 Reduced effector function	1) Reduce effector function 2) Effector moiety conjugation

1A. Creation of 2C3 anti-CD-52 antibody hyperglycosylation mutants

The A114N mutation, designated based upon the Kabat numbering system (equivalent to EU position 118), was introduced into the CH1 domain of 2C3 by mutagenic PCR. To create the full-length antibody, the VH domain plus the mutated A114N residue was inserted by ligation independent cloning (LIC) into the pENTR-LIC-IgG1 vector encoding antibody CH domains 1-3. All other mutations were introduced on pENTR-LIC-IgG1 by site-directed mutagenesis with a QuikChange site-directed mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, USA). The WT 2C3 VH was cloned into mutated vectors by LIC. Full-length mutants were cloned into the pCEP4(-E+I)Dest expression vector by Gateway cloning. Fc mutations were designated based on the EU numbering system. Mutations were confirmed by DNA sequencing. Amino acid sequences of the WT 2C3 heavy and light chains and the mutated 2C3 heavy chains are set forth in Table 4. Mutated amino acids are shaded and the consensus glycosylation target sites created by the mutation are underlined.

Table 4. Amino acid sequences of 2C3 anti-CD-52 antibodies

SEQ ID NO	Name	Amino Acid Sequence
1	Anti-CD-52 WT light chain	DIVMTQTPLSLSVTPGQPASISCKSSQSLLYSNGKTY LNWLLQKPGQSPQRLIYLVSKLDSGVPDRFSGSGSG TDFTLKISRVEAEDVGVYYCVQGTHLHTFGQGTRL EIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYP REAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLS STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNR GEC
2	Anti-CD-52 WT heavy chain	VQLVESGGGLVQPGGSLRLSCAASGFTFNTYWMN WVRQAPGKGLEWVGQIRLKSNNYATHYAESVKGR FTISRDDSKNSLYLQMNSLKTEDTAVYYCTPVDFW GQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALG CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGK

SEQ ID NO	Name	Amino Acid Sequence
3	Anti-CD-52 A114N heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYWMN WVRQAPGKGLEWVGQIRLKSNNYATHYAESVKGR FTISRDDSKNSLYLQMNSLKTEDTAVYYCTPVDFW GQGTTVTVSSNSTKGPSVFPLAPSSKSTSGGTAALG CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGK
4	Anti-CD-52 Y436S heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYWMN WVRQAPGKGLEWVGQIRLKSNNYATHYAESVKGR FTISRDDSKNSLYLQMNSLKTEDTAVYYCTPVDFW GQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALG CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHSTQKSLSLSPGK
5	Anti-CD-52 S440N heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYWMN WVRQAPGKGLEWVGQIRLKSNNYATHYAESVKGR FTISRDDSKNSLYLQMNSLKTEDTAVYYCTPVDFW GQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALG CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKNLSLSPGK
6	Anti-CD-52 S442N heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYWMN WVRQAPGKGLEWVGQIRLKSNNYATHYAESVKGR FTISRDDSKNSLYLQMNSLKTEDTAVYYCTPVDFW GQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALG CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG

SEQ ID NO	Name	Amino Acid Sequence
		LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLNLSPGK
7	Anti-CD-52 NGT heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYWMN WVRQAPGKGLEWVGQIRLKSNNYATHYAESVKGR FTISRDDSKNSLYLQMNSLKTEDTAVYYCTPVDFW GQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALG CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGKNGT
8	Anti-CD-52 S298N / Y300S heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYWMN WVRQAPGKGLEWVGQIRLKSNNYATHYAESVKGR FTISRDDSKNSLYLQMNSLKTEDTAVYYCTPVDFW GQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALG CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYNNTSRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGK

The mutants and WT control were transfected into HEK293-EBNA cells in a 6-well plate format. As shown in **Figure 9A**, the expression level was found to be ~0.1 µg/ml, as analyzed by SDS-PAGE and Western blot. Expression of mutants in conditioned media was also measured by protein A capture on Biacore. Concentration was determined using the dissociation response 6 minutes after injection into immobilized Protein A. CHO-produced WT 2C3 serially diluted in media from 90µg/mL to 1.5ng/mL was used as a standard curve. Concentrations were calculated down to ~0.2µg/mL by a calibration curve using a 4-

parameter fit. As shown in **Figure 9B**, relative expressions levels were low and generally corresponded with the Western blot results.

1B. Verification of hyperglycosylation

To determine whether additional glycosylation sites were introduced by mutation, 2C3 mutant and wild-type proteins were treated with the universal deglycosylating enzyme PNGase F and protein samples were analyzed by SDS-PAGE and Western blot. As shown in **Figure 10**, only the A114N mutant had an increased apparent molecular weight, indicating the presence of an additional N-linked carbohydrate.

Small scale antibody preparations were produced to purify the 2C3 mutants for further verification of glycosylation site introduction. As shown in **Figure 11**, it was confirmed by SDS-PAGE that only the A114N mutant had additional glycosylation sites introduced.

1C. Binding properties of 2C3 anti-CD-52 mutants

Biacore was used to compare the binding properties of the purified proteins. Mouse and SEC-purified human FcRn-HPC4 were immobilized on a CM5 chip via amine coupling. Each antibody was diluted to 200, 50, and 10nM and injected over the immobilized Fc receptors. Campath, CHO-produced WT 2C3, and DEPC-treated Campath were included as positive and negative controls. As shown in **Figure 13**, the Y436S mutant displayed about a 2-fold decrease in binding to human FcRn. Interestingly, binding of this mutant to mouse FcRn was not affected. None of the other 2C3 mutations had any considerable effect on human or mouse FcRn binding.

Biacore was used to compare the antigen binding properties of the purified proteins using the CD-52 peptide 741 Biacore binding assay. CD-52 peptide 741 and control peptide 777 were immobilized to a CM5 chip. Antibodies were serially diluted 2-fold from 60 to 0.2nM in HBS-EP and injected in duplicate for 3 min followed by a 5 min dissociation in buffer at a50µL/min flow-rate. GLD52 lot 17200-084 was included as a control. The surface was regenerated with 1 pulse of 40mM HCl. A 1:1 binding model was used to fit the 7.5 to 0.2nM curves. As shown in **Figure 16**, the A114N mutant had a slightly lower CD-52 binding affinity while the NGT mutant had a slightly higher affinity than the rest of the mutants in this assay. The CD-52 peptide 741 Biacore binding assay was repeated with

protein purified from larger scale prep. As shown in **Figure 17**, the A114N mutant exhibited CD-52 peptide binding that was comparable to WT 2C3.

1D. Charge characterization of the A114N mutant

Isoelectric focusing (IEF) was performed to characterize the charge of the 2C3 mutants. Purified protein was run on immobilized pH gradient (pH3-10) acrylamide (IPG) gels. As shown in **Figure 18A**, A114N was found to have more negative charges, likely due to sialic acid residues. Intact MS data confirmed the complex structure with sialic acids on the A114N mutant. In contrast, the WT 2C3 was shown to have G0F and G1F as the dominant glycosylation species (**Figures 18C and 18D**, respectively).

Example 2. Preparation of hyperglycosylation mutants in several antibody backbones

In addition to the 2C3 anti-CD-52 antibody, the A114N mutation was engineered in several other antibody backbones to confirm that the unique hyperglycosylation site could be introduced into unrelated heavy chain variable domain sequences. The hyperglycosylated anti-TEM1, anti-FAP, and anti-Her2 mutants are set forth in Table 5.

Table 5. A114N and/or S298N mutants designed in several unrelated antibody backbones

Mutation	Antibody	Desired benefits	Applications
A114N	anti-TEM1 anti-FAP anti-Her2	Additional glycosylation site at the elbow hinge of heavy chain for site-specific carbohydrate-mediated conjugation	1) Control 2) Aminooxy toxin conjugation via exposed sialic acid or galactose group (SAM or GAM)

Mutation	Antibody	Desired benefits	Applications
S298N / T299A / Y300S (NNAS)	anti-Her2	Switch the glycosylation from Asn297 to an engineered Asn298. Expect solvent exposed and complex carbohydrates at S298N, offering conjugation site and means to remove effector function	1) Aminooxy toxin conjugation via exposed sialic acid or galactose group (SAM or GAM) 2) Reduced effector function
A114N / NNAS	anti-Her2	Potential for increased conjugation yield with two conjugation sites	1) Control 2) Aminooxy toxin conjugation via exposed sialic acid or galactose group (SAM or GAM)

2A. Creation of anti-TEM1 and anti-FAP antibody hyperglycosylation mutants

The A114N mutation, designated based upon the Kabat numbering system, was introduced into the CH1 domain of anti-TEM1 and anti-FAP by mutagenic PCR. To create the full-length antibody, the mutated VH plus residue 114 was inserted by ligation independent cloning (LIC) into the pENTR-LIC-IgG1 vector encoding antibody CH domains 1-3. Full-length mutants were then cloned into the pCEP4(-E+I)Dest expression vector by Gateway cloning. Mutations were confirmed by DNA sequencing. Amino acid sequences of the anti-TEM1 wild-type and mutated heavy and light chains are set forth in Table 6. Mutated amino acids are shaded and the consensus glycosylation target sites created by the mutation are underlined.

Table 6. Amino acid sequences of anti-TEM1 and anti-FAP antibodies

SEQ ID NO	Name	Amino Acid Sequence
9	Anti-TEM1 WT light chain	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWY QQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTL TISRLEPEDFAVYYCQQYGSSPWTFGQGTKVEIKRT

SEQ ID NO	Name	Amino Acid Sequence
	(clone #187)	VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
10	Anti-TEM1 WT heavy chain (clone #187)	QVQLQESAPGLVKPSETLSLTCTVSGGSIRSYYWSW IRQPPGKGLEYIGYIYYTGSAIYNPSLQSRVTISVDTS KNQFSLKLNSVTAADTAVYYCAREGVRGASGYYY YGMDVWGQGTTVTVSSASTKGPSVFPLAPSSKSTS GGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLF PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ VYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ QGNVFSCSVMHEALHNHYTQKSLSLSPGK
11	Anti-TEM1 A114N	QVQLQESAPGLVKPSETLSLTCTVSGGSIRSYYWSW IRQPPGKGLEYIGYIYYTGSAIYNPSLQSRVTISVDTS KNQFSLKLNSVTAADTAVYYCAREGVRGASGYYY YGMDVWGQGTTVTVSSNSTKGPSVFPLAPSSKSTS GGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLF PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ VYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ QGNVFSCSVMHEALHNHYTQKSLSLSPGK*

The mutants and wild-type control were transfected into HEK293-EBNA cells in a triple flask format and purified on HiTrap protein A columns (GE Healthcare Biosciences, Pittsburgh, PA, USA). As analyzed by A280 on a NanoDrop spectrophotometer, the expression of anti-FAP A114N and anti-FAP A114C was about 3µg/ml and about1µg/ml, respectively. The expression of anti-TEM1 A114N was about 0.04µg/ml.

2B. Verification of hyperglycosylation

To confirm that the additional glycosylation site was introduced into the A114N mutants, purified protein from the A114N mutants was analyzed on reducing SDS-PAGE along with wild-type control protein. One additional glycosylation site would add 2000-3000

Daltons to the molecular weight of the heavy chain. As shown in **Figure 20**, SDS-PAGE indicated that the anti-FAP and anti-TEM1 A114N mutants heavy chain bands had increased apparent molecular weight, consistent with successful introduction of an additional glycosylation site to both antibodies.

2C. Creation of anti-Her2 antibody hyperglycosylation mutants

The Her-2 A114N, Her-2 A114N/NNAS, and WT Her-2 antibodies were created by ligation independent cloning. The VH domain of Herceptin was synthesized and PCR-amplified with two LIC-compatible sets of primers, either WT or bearing the A114N mutation. To obtain a full-length antibody, amplified VH inserts (WT or A114N) were cloned into two pENTR vectors encoding CH 1-3 domains, pENTR-LIC-IgG1 WT and pENTR-LIC-IgG1 NNAS, resulting in three full-length mutants (A114N, NNAS, A114N/NNAS) and WT control as entry clones on pENTR. These mutants were cloned into the pCEP4(-E+I)Dest expression vector, by Gateway cloning. Mutations were confirmed by DNA sequencing. Amino acid sequences of the anti-Her-2 wild-type and mutated heavy and light chains are set forth in **Table 7**. Mutated amino acids are shaded and the consensus glycosylation target sites created by the mutation are underlined.

Table 7. Amino acid sequences of anti-Her-2 antibodies

SEQ ID NO	Name	Amino Acid Sequence
12	Anti-Her-2 WT light chain	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAW YQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFT LTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRT VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
13	Anti-Her-2 WT heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHW VRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTIS ADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFY AMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNT KVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYT LPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO	Name	Amino Acid Sequence
14	Anti-Her-2 A114N heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHW VRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTIS ADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFY AMDYWGQGTLVTVSSNSTKGPSVFPLAPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNT KVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYT LPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGK
15	Anti-Her2 NNAS heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHW VRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTIS ADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFY AMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNT KVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNNASRVVSVLTVLHQDWL NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYT LPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGK
16	Anti-Her2 A114N / NNAS heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHW VRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTIS ADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFY AMDYWGQGTLVTVSSNSTKGPSVFPLAPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNT KVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNNASRVVSVLTVLHQDWL NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYT LPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGK

2D. Expression of the A114N anti-Her2 antibody hyperglycosylation mutant

The A114N anti-Her2 and wild-type constructs were transfected with Lipofectamine-2000 (2.5:1 ratio of reagent to DNA) and XtremeGene HP (3:1 ratior of reagent to DNA) into

HEK293-EBNA cells in 12 triple flasks. Octet measurement of aliquots from day 3 conditioned media (CM) showed that protein expression was consistent across 6 flasks for both Lipofectamine-2000 and XtremeGene HP. As shown in Table 8, the overall transfection efficiency was about 30% higher with XtremeGene HP. Conditioned media collected on day 3 was pooled together for both transfection conditions and purified by protein A column. Octet measurement showed 1.8 ug/ml antibody in the serum-containing mock media versus 0 ug/ml in no serum mock media.

Table 8. A114N anti-Her2 hyperglycosylation mutant expression

		Lipofectamine-2000	**XtremeGene HP**
Purified protein from protein A column	Concentration (mg/ml)	1.72	3.18
	Volume (ml)	3.5	3.5
	Total protein (mg)	6.02	11.13
Buffer-exchanged protein	Concentration (mg/ml)	15.59	16.86
	Volume (ml)	0.2	0.36
	Total protein (mg)	3.1	6.07
	%Recovery	51.8	54.5

Conditioned media from day 6 was collected and purified separately for each transfection condition. Both eluates were buffer-exchanged separately into PBS, pH 7.2, and concentrated ~15-fold using Amicon-4 (50 kD cut-off) columns. Day 6 CM showed higher expression level compared to Day 3 CM. As shown in Table 8, a total of 3 mg of Herceptin A114N 15.59 mg/ml (from Lipofectamine transfection) and 6 mg of Herceptin A114N 16.86 mg/ml (from XtremeGene HP transfection) was produced from day 6 conditioned media for additional downstream applications, such as antibody-drug conjugation.

2E. SDS-PAGE and HIC analyis of the A114N anti-Her2 mutant

Prior to conjugation, purified A114N Herceptin was characterized by SDS-PAGE and HIC (hydrophobic interaction chromatography). As shown in **Figure 21**, the quality of purified A114N Herceptin was determined to be suitable for further downstream applications.

2F. Conjugation to engineered glycosylation

It was demonstrated that: a) a glycosylation site was introduced at Kabat position 114 (EU position 118) site on anti-TEM1; b) the A114N mutant had hyperglycosylation on the heavy chain by reducing SDS-PAGE; and c) the A114N hyperglycosylated mutant had complex carbohydrate structure by intact LC/MS, including terminal sialic acids and galactose, which are ideal for SAM and GAM conjugation. To confirm that the engineered glycosylation site was suitable for conjugation, anti-TEM1 A114N was conjugated with a 5kDa PEG via aminooxy chemistry. As shown in **Figure 22**, PEG was successfully conjugated to anti-TEM1 A114N through an aminooxy linkage. This mutant was also successfully prepared on the anti-FAP and anti-CD-52 2C3 backbones (not shown). These data demonstrate that the glycosylation site at N114 is useful for conjugation of effector moieties.

Example 3: **Generation of S298N/Y300S Fc mutants**

Engineered Fc variants were designed and generated in which a new glycosylation site was introduced at EU position Ser 298, next to the naturally-occurring Asn297 site. The glycosylation at Asn297 was either maintained or ablated by mutation. Mutations and desired glycosylation results are set forth in Table 9.

Table 9: Glycosylation states of various antibody variants

#	Mutation	Desired Glycosylation State	Applications
17	N297Q	No glycosylation (agly)	Agly Control
18	T299A	No glycosylation (agly)	Agly Control, unknown effector function
19	N297Q/S298N/Y300S (NSY)	No glycosylation at 297 but engineered glycosylation site at 298	Reduced effector function; Conjugation via exposed sialic acid or galactose groups.
20	S298N/T299A/Y300S (STY)	No glycosylation at 297 but engineered glycosylation site at 298	Reduced effector function; Conjugation via exposed sialic acid or galactose groups.
21	S298N/Y300S (SY)	Two potential glycosylation sites at 297 & 298; Alterations in glycosylation pattern.	Reduced effector function; Conjugation via exposed sialic acid or galactose groups.
22	Wild-type	297	control

3A. Creation of H66 αβ-TCR antibody altered glycosylation variants

Mutations were made on the heavy chain of αβ T-cell receptor antibody clone #66 by Quikchange using a pENTR_LIC_IgG1 template. The VH domain of HEBE1 Δab IgG1 #66 was amplified with LIC primers before being cloned into mutated or wild-type pENTR_LIC_IgG1 by LIC to create full-length mutant or wild-type antibodies. The subcloning was verified with DraIII/XhoI double digest, producing an approximately 1250 bp-sized insert in the successful clones. Those full-length mutants were then cloned into an expression vector, pCEP4(-E+I)Dest, via Gateway cloning. The mutations were confirmed by DNA sequencing.Amino acid sequences of the WT H66 anti-αβTCR heavy and light

chains and the mutated H66 heavy chains are set forth in Table 10. Mutated amino acids are shaded and the consensus glycosylation target sites created by the mutation are underlined.

Table 10: Amino acid sequences of H66 anti-αβTCR antibodies

SEQ ID NO	Name	Amino Acid Sequence
23	Anti-αβTCR clone H66 light chain	EIVLTQSPATLSLSPGERATLSCSATSSVSYMHWYQQ KPGQAPRRLIYDTSKLASGVPARFSGSGSGTSYTLTIS SLEPEDFAVYYCQQWSSNPLTFGGGTKVEIKRTVAAP SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYE KHKVYACEVTHQGLSSPVTKSFNRGEC*
24	Anti-αβTCR clone H66 heavy chain	EVQLLQSGGGLVQPGGSLRLSCAASGYKFTSYVMHW VRQAPGKGLEWVGYINPYNDVTKYNEKFKGRFTLSR DNSKNTLYLQMNSLRAEDTAVYYCARGSYYDYDGF VYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMIIEALIIN HYTQKSLSLSPGK*
25	Anti-αβTCR clone H66 S298N/Y300S heavy chain	EVQLLQSGGGLVQPGGSLRLSCAASGYKFTSYVMHW VRQAPGKGLEWVGYINPYNDVTKYNEKFKGRFTLSR DNSKNTLYLQMNSLRAEDTAVYYCARGSYYDYDGF VYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNNTSRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK*
26	Anti-αβTCR clone H66 S298N/ T299A/ Y300S heavy chain	EVQLLQSGGGLVQPGGSLRLSCAASGYKFTSYVMHW VRQAPGKGLEWVGYINPYNDVTKYNEKFKGRFTLSR DNSKNTLYLQMNSLRAEDTAVYYCARGSYYDYDGF VYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK

SEQ ID NO	Name	Amino Acid Sequence
		TKPREEQYNNASRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK*
27	Anti-αβTCR clone H66 N297Q/ S298N/ Y300S heavy chain	EVQLLQSGGGLVQPGGSLRLSCAASGYKFTSYVMHW VRQAPGKGLEWVGYINPYNDVTKYNEKFKGRFTLSR DNSKNTLYLQMNSLRAEDTAVYYCARGSYYDYDGF VYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYQNTSRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK*

The mutant, wild-type, and two aglycosylated control (HEBE1 Agly IgG4 and HEBE1 Δab IgG1 in pCEP4) constructs were transfected into HEK293-EBNA cells in triple-flasks for expression. Proteins were purified from 160 ml of conditioned media (CM) with 1 ml HiTrap protein A columns (GE) using a multi-channel peristaltic pump. Five micrograms of each resulting supernatant were analyzed on 4-20% Tris-Glycine reducing and non-reducing SDS-PAGE gels (see **Figure 2**). The heavy chains of the aglycosylated mutants (N297Q, T299A, and Agly controls), have migrated further (arrowhead), consistent with the loss of the glycans in these antibodies. The heavy chains of the engineered glycosylated antibodies (NSY, STY, SY, Δab, and wt control, arrows), however, migrate similarly to the wild-type control. This result is consistent with the existence of an engineered glycosylation site at EU position 298. SEC-HPLC analysis indicated that all mutants are expressed as monomers.

3B. Glycosylation analysis by LC-MS

The engineered H66 IgG1 Fc variants were partially reduced with 20mM DTT at 37°C for 30 min. The samples were then analyzed by capillary LC/MS on an Agilent 1100

capillary HPLC system coupled with a QSTAR qq TOF hybrid system (Applied Biosystems). A Bayesian protein reconstruction with baseline correction and computer modeling in Analyst QS 1.1 (Applied Bisoystem) was used for data analysis. In the S298N/T299A/Y300S H66 antibody mutant, one glycosylation site was observed at amino acid 298 with bi-antennary and tri- antennary complex-type glycans detected as the major species alongside G0F, G1F and G2F (see **Figure 34**). This altered glycosylation profile is consistent which shifted glycosylation at N298 instead of the wild-type glycosylation site at N297.

3C.Binding properties of αβTCR antibody mutants to human FcγRIIIa and FcγRI using Biacore

Biacore was used to assess binding to recombinant human FcγRIIIa (V158 & F158) and FcγRI. All four flowcells of a CM5 chip were immobilized with anti-HPC4 antibody via the standard amine coupling procedure provided by Biacore. The anti-HPC4 antibody was diluted to 50µg/mL in 10mM sodium acetate pH 5.0 for the coupling reaction and injected for 25 min at 5µL/min. Approximately 12,000 RU of antibody was immobilized to the chip surface. Recombinant human FcγRIIIa-V158 and FcγRIIIa-F158 were diluted to 0.6µg/mL in binding buffer (HBS-P with 1mM $CaCl_2$) and injected to flowcells 2 and 4, respectively, for 3 min at 5µL/min to capture 300 – 400 RU receptor on the anti-HPC4 chip. In order to distinguish between the low binders, three times more rhFcγRIIIa was captured on the anti-HPC4 surface than usually used in this assay. Flowcells 1 and 3 were used as reference controls. Each antibody was diluted to 200nM in binding buffer and injected over all four flowcells for 4 min, followed by 5 min dissociation in buffer. The surfaces were regenerated with 10mM EDTA in HBS-EP buffer for 3 min at 20µL/min. The results of these experiments are shown in **Figure 3**.

Biacore was also used to compare the FcγRI binding. Anti-tetra His antibody was buffer exchanged into 10mM sodium acetate pH 4.0 using a Zeba Desalting column and diluted to 25µg/mL in the acetate buffer for amine coupling. Two flowcells of a CM5 chip were immobilized with ~9000 RU of the anti-Tetra-His antibody after 20 min injection at 5µL/min. As in the previous experiment, ten times more FcγRI was captured to the anti-tetra-His surface in order to compare samples with weak binding. Recombinant human FcγRI was diluted 10µg/mL in HBS-EP binding buffer and injected to flowcell 2 for 1 min at

5μL/min to capture ~1000 RU receptor to the anti-tetra-His chip. A single concentration of antibody, 100nM, was injected for 3 min at 30μL/min over the captured receptor and control surface. Subsequently, dissociation was monitored for three minutes. The surface was then regenerated with two 30 second injections of 10mM glycine pH 2.5 at 20μL/min. The results of these experiments are shown in **Figure 4**.

These results demonstrate a striking decrease in binding of the glycoengineered mutants to FcγRIIIa or FcγRI. H66 S298N/T299A/Y300S in particular has almost completely abolished binding to both receptors. This mutant was chosen for more detailed analysis.

3D. Stability characterization using Circular Dichroism (CD)

The stability of the S298N/T299A/Y300S antibody mutant was monitored by a Far-UV CD thermo melting experiment in which the CD signal at 216nm and 222nm was monitored as increasing temperature lead to the unfolding of the antibody (denaturation).

Temperature was controlled by a thermoelectric peltier (Jasco model AWC100) and was increased at a rate of 1°C/min from 25-89 °C. The CD spectra were collected on a Jasco 815 spectrophotometer at a protein concentration of approximately 0.5 mg/mL in PBS buffer in a quartz cuvette (Hellma, Inc) with a path length of 10 mm. The scanning speed was 50 nm/min and a data pitch of 0.5 nm. A bandwidth of 2.5 nm was used with a sensitivity setting of medium. The CD signal and HT voltage were collected from 210-260 nm with data intervals of 0.5 nm and at temperature intervals of 1 °C and four replicate scans were performed for each sample. The results demonstrate that both delta AB H66 and the S298N/T299A/Y300S H66 mutant exhbit similar thermal behaviors and have approximately the same onset temperature for degradation (around 63℃) (**Figure 35**), further suggesting that they have comparable stability.

Example 4: Functional analysis of Fc-engineered mutants

Fc-engineered mutants were assessed through a PBMC proliferation assay and a cytokine release assay. In the PBMC proliferation assay, human PBMC were cultured with increasing concentrations of therapeutic antibody for 72 hours, ^{3}H-thymidine was added and

cells were harvested 18 hours later. For the T cell depletion/Cytokine Release assay, human PBMC were cultured with increasing concentrations of therapeutic antibody and were analyzed daily for cell counts and viability (Vi-Cell, Beckman Coulter) out to day 7. Cell supernatants were also harvested, stored at -20°C and analyzed on an 8-plex cytokine panel (Bio-Rad).

Normal donor PBMC were thawed and treated under the following conditions (all in media containing complement): Untreated; BMA031, moIgG2b 10ug/ml; OKT3, moIgG2a 10ug/ml; H66, huIgG1 deltaAB 10ug/ml, 1ug/ml and 0.1ug/ml; H66, huIgG1 S298N/T299A/Y300S 10ug/ml, 1ug/ml and 0.1ug/ml.

Cytokines were harvested at day 2 (D2) and day 4 (D4) for Bioplex Analysis (IL2, IL4, IL6, IL8, IL10, GM-CSF, IFNg, TNFa). Cells were stained at D4 for CD4, CD8, CD25 and abTCR expression.

The results, shown in **Figures 5-8**, demonstrate that H66 S298N/T299A/Y300S behaved similarly to the H66 deltaAB in all the cell based assays performed, showing minimal T-cell activation by CD25 expression, binding to abTCR (with slightly different kinetics to deltaAB), and minimal cytokine release at both D2 and D4 time points. The S298N/T299A/Y300S mutant thus eliminated effector function as effectively as the deltaAB mutation.

Example 5: Preparation and characterization of an engineered Fc variant in the anti-CD52 antibody backbone.

In addition to the H66 anti-αβTCR antibody, the S298N/Y300S mutation was also engineered in an anti-CD52 antibody backbone (clone 2C3). This mutant was then examined in order to determine whether the observed effector function modulation seen in the S298N/Y300S H66 anti-αTCR antibody was consistent in another antibody backbone.

5A. Creation of 2C3 anti-CD52 antibody altered glycosylation variants

First, S298N/Y300S 2C3 variant DNA was prepared by quick change mutagenesis using pENTR_LIC_IgG1, and WT 2C3 VH was cloned into the mutated vector by LIC. Full-length mutants were cloned into the pCEP4 (-E+I)Dest expression vector using Gateway

technology. Mutations were subsequently confirmed by DNA sequencing and the sequences are set forth in Table 11. Mutated amino acids are shaded and the consensus glycosylation target sites created by the mutation are underlined. The mutants were then transfected into HEK293-EBNA cells in a 6-well plate format and the protein was purified from conditioned media. Anti-CD52 2C3 wild-type antibody was produced in parallel as a control. The expression level was found to be 0.1µg/mL using SD-PAGE and Western blot analyses (**Figure 9A**). Expression of mutants in neat conditioned media was also measured by protein A capture on Biacore. Concentration was determined using the dissociation response after a six-minute injection to immobilized protein A. CHO-produced WT 2C3 serially diluted in media from 90 µg/mL to 1.5ng/mL was used as a standard curve. Concentrations were calculated within approximately 0.2 µg/mL by a calibration curve using a 4-parameter fit. Relative expression levels were low and generally agree with the Western blot data (**Figure 9B**).

Table 11: Anti-CD52 clone 2C3 antibody sequences

SEQ ID NO	Name	Amino Acid Sequence
28	Anti-CD-52 2C3 WT light chain	DIVMTQTPLSLSVTPGQPASISCKSSQSLLYSNGKTYLNWL LQKPGQSPQRLIYLVSKLDSGVPDRFSGSGSGTDFTLKISR VEAEDVGVYYCVQGTHLHTFGQGTRLEIKRTVAAPSVFIF PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVT HQGLSSPVTKSFNRGEC*
29	Anti-CD-52 2C3 WT heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYWMNWVR QAPGKGLEWVGQIRLKSNNYATHYAESVKGRFTISRDDS KNSLYLQMNSLKTEDTAVYYCTPVDFWGQGTTVTVSSAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSV FLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPGK*
30	Anti-CD-52 2C3 S298N/Y300S heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYWMNWVR QAPGKGLEWVGQIRLKSNNYATHYAESVKGRFTISRDDS KNSLYLQMNSLKTEDTAVYYCTPVDFWGQGTTVTVSSAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN

SEQ ID NO	Name	Amino Acid Sequence
		SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSV FLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNNTSRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPGK*

5B. Glycosylation analysis using PNGaseF

To evaluate the additional glycosylation sites introduced by the mutation, the enriched S298N/Y300S mutant was de-glycosylated with PNGase F. Deglycosylation did not demonstrate any apparent change in molecular weight, which indicates that no additional carbohydrate was present (**Figure 10**). Small scale preparations were performed in order to purify these mutants for further characterization and the results reconfirmed that there was not an additional carbohydrate present on the S298N/Y300S mutant (**Figure 11**).

5C.Binding properties of 2C3 anti-CD52 antibody mutants to human FcγRIIIa using Biacore

Biacore was also used to characterize the antigen-binding, FcγRIII, and binding properties of the purified antibodies (see **Figures 12, 13, and 14**). The S298N/Y300S 2C3 variant bound to the CD52 peptide tightly and the binding sensorgram was undistinguishable from the wild-type control, demonstrating that this mutation does not affect its antigen binding (**Figure 12A**).

To assay for Fc effector function, FcγRIII receptor (Val158) was used in binding studies. The mutant and wild-type control antibody were diluted to 200nM and injected to HPC4-tag captured FcγRIIIa. FcγRIII binding was almost undetectable for the S298N/Y300S mutant, which indicated a loss of effector function by this variant (**Figure 12B** and **Figure 14A**). To further assay for Fc effector function, the FcγRIII receptor (Phe158) was also used in binding studies. The mutant and wild-type control antibodies were diluted to 200nM and injected to HPC4-tag captured FcγRIIIa. FcγRIII binding was almost undetectable for the S298N/Y300S mutant, which indicates a loss of effector function with

the Phe158 variant (**Figure 14B**). Finally, Biacore was used to compare the FcRn binding properties of the purified proteins. Mouse and SEC-purified human FcRn-HPC4 were immobilized to a CM5 chip via amine coupling. Each antibody was diluted to 200, 50, and 10 nM and injected over the receptors. Campath, CHO-produced WT 2C3, and DEPC-treated Campath were included as positive and negative controls. These data show that the mutant binds to both human and murine FcRn receptor with the same affinity as the wild-type antibody control and that it likely has no alterations in its circulation half-life or other pharmacokinetic properties. (see **Figure 12C**, **Figure 13A and B**). Accordingly, the S298N/Y300S mutation is applicable to antibodies in general, to reduce or eliminate undesired Fc effector function, for example through engagement of human Fcγ receptors.

Example 6: Circulating Immune Complex Detection in the S298N/Y300S mutant.

Circulating immune complex detection was also investigated using a C1q binding assay for the S298N/Y300S mutant and WT control. High binding Costar 96-well plates were coated overnight at 4°C with 100µl of 2-fold serially diluted 2C3 Abs at concentrations ranging from 10 - 0.001 µg/ml in coating buffer (0.1M $NaCHO_3$ pH 9.2). ELISA analysis showed that C1q binding is reduced for the S298N/Y300S mutant compared to WT (**Figure 15A**). The binding of anti-Fab Ab to the coated 2C3 Abs confirmed equivalent coating of the wells (**Figure 15B**).

Example 7: Separation and analysis of S298N/Y300S mutant using Isoelectric Focusing.

A pH 3-10 Isoelectric Focusing (IEF) gel was run to characterize the S298N/Y300S mutants. S298/Y300S was found to have more negative charges, and therefore, likely more sialic acid molecules (**Figure 18A**). Both the S298N/Y300S mutant and WT 2C3 were shown by intact MS to have G0F and G1F as the dominant glycosylation species (**Figure 18 B and D**, respectively).

Example 8: Antigen binding affinity of S298N/Y300S.

Biacore was used to compare the antigen binding affinity of WT anti-CD52 2C3 Ab and the S298N/Y300S mutant that had been prepared and purified from both smaller (**Figure 16**) and larger (**Figure 17**) scale expressions. CM5 chips immobilized with CD52 peptide

741 and control peptide 777 were obtained. Antibodies were serially diluted 2-fold from 60 to 0.2nM in HBS-EP and were then injected over the chip surface for 3 min followed by a 5 min dissociation in buffer at a flow rate of 50µl/min. The surface was then regenerated with a pulse of 40mM HCl. These analyses were performed in duplicate and demonstrate that the S298N/Y300S mutant and WT 2C3 antibodies show comparable CD52 peptide binding.

A media screening platform was designed to test functional binding properties prior to purification in order to screen antibodies created during small scale transfections. These tests were performed using Octet (**Figure 19A**) to determine concentration and used Protein A biosensors and a GLD52 standard curve. Samples were diluted to 7.5 and 2nM in HBS-Ep for a CD52 binding comparison using Biacore (**Figure 19B**). The results of the peptide binding assay showed that both the S298N/Y300S mutant and the WT 2C3 antibodies have comparable CD52 peptide binding. Furthermore, these analyses demonstrate that Octet and Biacore work well to predict antigen binding by antibodies from small scale transfections.

Example 9: Preparation of S298N/Y300S, S298N/T299A/Y300S, and N297Q/S298N/Y300S altered glycosylation mutants in additional antibody backbones.

In addition to the anti-αβ-TCR antibody and 2C3 anti-CD-52 antibody, the S298/Y300S, S298N/T299A/Y300S, and N297Q/S298N/Y300S mutations were engineered in other antibody backbones to confirm that the additional tandem glycosylation site could be introduced into unrelated heavy chain variable domain sequences. The alternatively glycosylated anti-CD-52 12G6 and anti-Her2 mutants are set forth in Tables 12 and 13. Mutated amino acids are shaded and the consensus glycosylation target sites created by the mutation are underlined.

Table 12: Anti-CD52 clone 12G6 antibody sequences

SEQ ID NO	Name	Amino Acid Sequence
31	Anti-CD-52 12G6 WT light chain	DIVMTQTPLSLSVTPGQPASISCKSSQSLLYSNGKTYLNWV LQKPGQSPQRLIYLVSKLDSGVPDRFSGSGSGTDFTLKISRV EAEDVGVYYCVQGSHFHTFGQGTKLEIKRTVAAPSVFIFPP

SEQ ID NO	Name	Amino Acid Sequence
		SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNS QESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQ GLSSPVTKSFNRGEC
32	Anti-CD-52 12G6 WT heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFPFSNYWMNWVRQ APGKGLEWVGQIRLKSNNYATHYAESVKGRFTISRDDSKN SLYLQMNSLKTEDTAVYYCTPIDYWGQGTTVTVSSASTKG PSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK*
33	Anti-CD-52 12G6 S298N/Y300S heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFPFSNYWMNWVRQ APGKGLEWVGQIRLKSNNYATHYAESVKGRFTISRDDSKN SLYLQMNSLKTEDTAVYYCTPIDYWGQGTTVTVSSASTKG PSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNNTSRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK*
34	Anti-CD-52 12G6 S298N/ T299A/ Y300S heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFPFSNYWMNWVRQ APGKGLEWVGQIRLKSNNYATHYAESVKGRFTISRDDSKN SLYLQMNSLKTEDTAVYYCTPIDYWGQGTTVTVSSASTKG PSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNNASRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK*
35	Anti-CD-52 12G6 N297Q/ S298N/ Y300S heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFPFSNYWMNWVRQ APGKGLEWVGQIRLKSNNYATHYAESVKGRFTISRDDSKN SLYLQMNSLKTEDTAVYYCTPIDYWGQGTTVTVSSASTKG PSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP

SEQ ID NO	Name	Amino Acid Sequence
		KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYQNTSRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK*

Table 13: Anti-Her2 antibody sequences

SEQ ID NO	Name	Amino Acid Sequence
36	Anti-Her2 WT light chain	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKP GKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDF ATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVT EQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPV TKSFNRGEC*
37	Anti-Her2 WT heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQA PGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAY LQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTV SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTY ICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPS VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPGK*
38	Anti-Her2 S298N/T299A/ Y300S heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQA PGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAY LQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTV SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTY ICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPS VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV DGVEVHNAKTKPREEQYNNASRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPGK*

Example 10. Generation of altered antibodies containing reactive glycan moieties

In order to generate antibodies containing glycan moieties capable of reacting with derivatized effector moieties, an anti-HER antibody was first glycosylated in vitro using glycosyltransferase and relevant sugar nucleotide donors. For example, to introduce the sialic acid residues, donor antibodies were first galactosylated with β-galactosyltransferase, followed with sialylation with α2,6-sialyltransferase according to the methods of Kaneko et al. (Kaneko, Y., Nimmerjahn, F., and Ravetch, J. V. (2006) Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science 313*, 670-3) . The reaction was performed in a one-pot synthesis step using β-galactosyltransferase (50mU/mg, Sigma) and α2,6-sialyltranafrease (5ug/mg, R&D system) with donor sugar nucleotide substrates, UDP-galactose (10mM) and CMP-sialic acid (10mM) in 50mM MES buffer (pH 6.5) containing 5mM $MnCl_2$. The reaction mixture containing 5mg/ml anti-HER2 antibody was incubated for 48 hours at 37 °C. The sialylation was verified using MALDI-TOF MS analysis of permethylated glycans released from the antibody with PNGase F, sialic acid content analysis using Dionex HPLC and lectin blotting with SNA, a lectin specific for α2,6-sialic acid.

MALDI-TOF analysis of glycans released by PNGase F treatment of the sialylated anti-HER2 antibody indicated that native glycans had been completely remodeled with a mainly monosialylated biantennary structure, A1F (**Figure 27A**) together with small amount of disialylated species. Treatment of the antibody with higher amounts of α2,6-sialyltransferase produced more homogenous populations of the A1F glycoform, suggesting that either the enzyme activity or glycan localization may prevent full sialylation. Sialic acid content was determined to be ~2 mol per mol of antibody, which is consistent with A1F glycan as the major glycoform species (**Figure 27B**). Lectin blotting with a SAN lectin, *Sambucus nigra agglutinin* specific for α2,6-linked sialic acid, confirmed that the sialic acid was present in an α2,6-linkage configuration (**Figure 27C**).

In conclusion, although the native protein glycans are somewhat heterogeneous, remodeling through galactosyl and sialyltransferases yields a nearly homogeneous antibody with monosialylated but fully galactosylated biantennary glycans (A1F). The introduction of only ~1 sialic acid on the two galactose acceptors on each branched glycan may be due to limited accessibility of one of the galactoses from glycans which are often buried in the antibody or non-covalent interactions of the glycans with the protein surface.

Example 11. Oxidation of altered antibodies containing reactive glycan moieties

Once the sialylation was verified, the in-process oxidation of sialylated anti-HER2 antibody with various concentrations of periodate (0.25 to 2mM) was investigated. The sialylated antibody was first buffer-exchanged into 25mM Tris-HCl (pH 7.5) containing 5mM EDTA followed by buffer exchange with PBS buffer. The buffered antibody mixture was then applied to protein A Sepharose column pre-equilibrated with PBS buffer. After the column was washed with 15 column volumes of PBS, 15 column volumes of PBS containing 5mM EDTA, and 30 column volumes of PBS, it was then eluted with 25mM citrate phosphate buffer (pH 2.9). The eluates were immediately neutralized with dibasic phosphate buffer and the antibody concentrated using Amicon ultra from Millipore. Following purification, the sialylated anti-HER2 antibody then was oxidized with sodium periodate (Sigma) in 100mM sodium acetate buffer (pH 5.6) on ice in the dark for 30 minutes, and the reaction quenched with 3% glycerol on ice for 15minutes. The product was desalted and exchanged into 100mM sodium acetate (pH 5.6) by 5 rounds of ultrafiltration over 50kDa Amicons. **Figure 28A** shows sialic acid content analysis of sialylated antibody titrated with various amounts of periodate. Complete oxidation of the sialic acid residues was achieved at a periodate concentration above 0.5mM. Indeed, a periodate concentration as low as 0.5mM was enough to fully oxidize the introduced sialic acid. Accordingly, a 1mM concentration of periodate was chosen for oxidation of sialylated antibody for drug conjugation.

Oxidation can have adverse effects on the integrity of an antibody. For example, the oxidation of methionine residues, including Met-252 and Met-428, located in Fc CH3 region, close to FcRn binding site is known to affect FcRn binding which is critical for prolonging antibody serum half-life (Wang, W., et al. (2011) Impact of methionine oxidation in human IgG1 Fc on serum half-life of monoclonal antibodies. *Mol Immunol 48*, 860-6). Accordingly, to examine the potential side effects of periodate oxidation on methionine residues (e.g., Met-252) critical for FcRn interaction, the oxidation state of the sialylated antibody was determined by LC/MS analysis of a trypsin peptide digest. This analysis revealed ~30% oxidation of Met-252 and < 10% oxidation of Met-428 after treatment of the sialylated trastuzumab with 1mM periodate. To determine the impact of this degree of methionine oxidation on FcRn binding, the FcRn binding kinetics for each antibody was evaluated using surface plasmon resonance (BIACORE). This analysis revealed that oxidation state correlated

with a minor loss in FcRn binding (12% and 26% reduction to mouse and human FcRn, see **Figures 28B and 28C** respectively). Notably, a ~25% reduction in the Ka for human FcRn has been reported to have no effect on the serum half-life in a human FcRn transgenic mouse, since a single intact FcRn site on each antibody is sufficient to provide functionality and the PK advantage (Wang et al., *Id).*

In summary, these data indicate that the introduction of periodate-sensitive sialic acid residues by sialyltransferase treatment permits the use of much lower concentrations of periodate, resulting in minimal side effects on antibody-FcRn interactions and antibody integrity as assessed by aggregation (≤1%). Thus, the use of sialylated antibodies provides a wider window of oxidation conditions to be employed, allowing the reproducible generation of active glycoconjugates without an effect on serum half-life.

The galactose in a hyperglycosylated antibody mutant can also be oxidized specifically using galactose oxidase to generate an aldehyde group for conjugation. To confirm this approach, an A114N anti-TEM1 antibody was concentrated to 13-20 mg/ml and then treated with 20mU/mg sialidase in PBS for 6 hours at 37°C. The desialated product was then oxidized with galactose oxidase ("GAO"), first with 5 ug GAO/mg protein overnight at 37°C followed by addition of 2 ug GAO/mg protein and incubation for an additional 5 hours. Sodium acetate was added to adjust the pH to 5.6 (0.1 v/v, pH5.6), and DMSO was added to achieve a final reaction concentration of 16%, were added prior to conjugation. The hyperglycosylation mutant A114N anti-HER antibody (15mg/ml) was similarly desialylated with sialidase (20mU/mg) and oxidized with 5ug GAO per mg protein in a single reaction overnight at 37 °C.

Example 12. Synthesis of Reactive Effector Moieties

In order to facilitate conjugation with aldehyde-derivatized antibody glycoforms, candidate drug effector moieties (e.g., Momomethyl Auristatin E (MMAE) and Dolastatin 10 (Dol10)) were derivatized with aminooxy-cystamide to contain functional groups (e.g., aminooxy-cys) specifically reactive with the aldehyde.

Briefly, to generate aminooxy-cystamide as a starting material, S-Trityl-L-cysteinamide (362 mg, 1 mmol) was added to a 3 mL of a DMF solution of t-*BOC-aminooxyacetic acid N-hydroxysuccinimide* ester (289 mg, 1 mmol). The reaction was

complete after 3 h as evident from HPLC analysis. The reaction mixture was subsequently diluted with 30 ml of dichloromethane and was washed with 0.1 M sodium bicarbonate solution (2 x 20 mL), water (2 x 20 mL), and brine (2 x 20 mL). The solution was dried over anhydrous sodium sulfate, filtered and concentrated to dryness. To this dried residue was added 3 mL of TFA followed by150 µL of triethylsilane. The resulting solution was precipitated from t-butyl methyl ether and the process repeated three times. After filtration, the residue was dried under reduced pressure yielding 205 mg of an off white solid (67% yield). The compound was used for next step without further purification.

To generate aminooxy-derivatized MMAE (Aminooxy-Cys-MC-VC-PABC-MMAE), 30.1 mg of aminooxy-cystamide (0.098 mmol, 2 eq.) was combined with 64.6 mg of MC-VC-PABC-MMAE (0.049 mmol), and 100 µL of triethylamine in 3 mL of DMF. The resulting reaction mixture was stirred at room temperature for 15 minutes, by which time reaction was complete according to HPLC analysis. The compound was purified by preparative HPLC yielding 45 mg (62%) of the desired product as an off-white solid. Reversed-phase HPLC analysis suggested the purity of the compound to be >96%. ESI calcd for C73H116N14O18S $(MH)^+$ 1509.8501; found, m/z 1509.8469.

To generate aminooxy-derivatized Dol10 (Aminooxy-Cys-MC-VC-PABC-PEG8-Dol10), 7.4 mg (0.024 mmol, 3 eq.) of aminooxy-cystamide, 12 mg (0.008 mmol) of MC-VC-PABC-PEG8-Dol10 and 30 µL triethylamine were combined in in 3 mL of DMF. The reaction was complete within 15 minutes according to HPLC analysis. Preparative HPLC purification resulted in 6.2 mg (46%) of the desired product as an offwhite solid. Reversed-phase HPLC analysis suggests the purity of the compound to be >96%. ESI calcd for C80H124N16O19S2 $(MH)^+$ 1678.0664; found, *m/z* 1678.0613.

Example 13. Sialic acid-mediated (SAM) conjugation of Reactive Effector Moieties

Following desalting, drug-linkers of Example 11 were combined with the oxidized, sialylated antibodies of Example 10 with 75% DMSO (0.167 v/v) at a concentration of 25mM to achieve a 24:1 molar ratio of drug-linker to antibody and a final antibody concentration at 5 mg/ml. The mixture was incubated overnight at room temperature. The unincorporated drug-linkers and any free drugs were scavenged using BioBeads. The product was buffer-exchanged into Histidine-Tween buffer using PD-10 columns and sterile

filtered. The endotoxin levels were determined and less than 0.1EU/mg ADC was achieved for in vivo study.

Figure 29A-C shows a hydrophobic interaction chromatograph (HIC) of different sialylated antibodies (anti FAP B11 and G11 and the anti-HER2 antibody of Example 11) glycoconjugated to AO-MMAE. Sialylated HER2 antibody was also conjugated with the drug-linker, AO-Cys-MC-VC-PABC-PEG8-Dol10 (**Figure 29D**). This analysis reveals that there are mainly one or two drug conjugates per antibody with a drug-to-antibody ratio (DAR) ranging from 1.3-1.9. The increased retention time of the Dol10 glycoconjugate (**Figure 29D**) as compared to the MMAE glycoconjugate (**Figure 29C**) is likely due to the greater hydrophobicity of Dol10.

LC-MS analysis was also conducted with an anti-HER antibody conjugated with two different drug-linkers (AO-MMAE or AO-PEG8-Dol10) at 30mg scale. This analysis showed similar DAR values of 1.7 and 1.5 following conjugation, which is comparable to HIC analysis. Size-exclusion chromatograpy (SEC) showed very low levels (1%) of aggregates in these conjugates.

Example 14. Galactose-mediated (GAM) conjugation of Reactive Effector Moieties

The galactose aldehyde generated with galactose oxidase on the A114N antiTEM1 hyperglycosylation mutant antibody as described in Example 11 was conjugated with 24 molar excess of aminooxy-MC-VC-PABC-MMAE drug-linker over antibody by overnight incubation at 25°C, yielding a ADC conjugate with a DAR of 1.72.

To the galactose oxidase-treated antiHER antibody prepared as described in Example 11, one tenth reaction volume of 1M sodium acetate, pH5.6, was added to adjust the pH to 5.6 and DMSO was added to make the final concentration of 14% before adding 24eq. aminooxy MC-VC-PABC-MMAE drug linker. The reactions were incubated for overnight at room temperature. Free drug and drug-linker were scavenged with Biobeads and the product buffer exchanged by SEC (65% yield). The product conjugate was analyzed by HIC. As shown in **Figure 30**, AO-MMAE had been conjugated to ~60 % of the molecules.

Example 15. In vitro ADC Cell Proliferation Assays

The *in vitro* activity of the anti-HER and anti-FAP glycoconjugate molecules were also compared with corresponding thiol conjugates containing the same drug moiety linked via

thiol linkages to hinge region cysteines of the same donor antibody. The thiol conjugates contained approximately twice the number of drugs per antibody (DAR) than the glycoconjugates. Thiol-based conjugation was performed as described by Stefano et al (Methods in Molecular Biology 2013, in press). Her2+ SK-BR-3 and Her2- MDA-MB-231 cell lines were then employed to evaluate the relative efficacy of each ADC. The results of this analysis are presented in Table 15 below

Table 15. EC_{50} comparison of glycoconjugates and thiol conjugates

	DAR	EC_{50} (ng/ml)
Anti-HER-MC-VC-PABC-MMAE (Thiol MMAE)	3.8*	2.3
AntiHER-AO-Cys-MC-VC-PABC-MMAE (Glyco MMAE)	1.7*	4.7
Anti-HER-MC-VC-PABC-PEG8-Dol10 (Thiol Dol10)	3.9*	0.45
Anti-HER-AO-Cys-MC-VC-PABC-PEG8-Dol10 (Glyco Dol10)	1.5*	0.97
Anti FAP B11-MC-VC-PABC-MMAE (Thiol MMAE), CHO+FAP	3.3**	382.4
Anti FAP B11-AO-Cys-MC-VC-PABC-MMAE (Glyco MMAE), CHO+FAP	1.5**	682.4

Note: * DAR determined by LC-MS; ** DAR determined by HIC

Figures 31(A-D) show a comparison of in vitro potency of anti-HER glycoconjugate and its counterpart thiol conjugate. Cell viability was determined following 72 hr exposure of the conjugates to Her2 antigen expressing (SK-BR-3) cells (**Figures 31A and C**) or non-expressing (MDA-MB-231) cells (**Figures 31B and D**). The ADCs contained either MMAE or PEG8-Dol10 linked to the glycans ("glyco") or by conventional chemistry to hinge region cysteines ("thiol"). As shown in **Figures 31A and C**, ~2-fold lower EC_{50} was observed for the thiol conjugates compared to the glycoconjugates, which is consistent with 2-fold higher DAR in the former than the latter. No toxicity was observed with the Her2- cell line with any antibody up to 100ug/ml.

Similar trends were also observed in the cell proliferation for ADC prepared with antibodies against a tumor antigen (FAP) which is highly expressed by reactive stromal

fibroblasts in epithelial cancers including colon, pancreatic and breast cancer (Teicher, B. A. (2009) Antibody-drug conjugate targets. *Curr Cancer Drug Targets 9*, 982-1004). These conjugates were again prepared by conjugating either aminooxy MMAE drug-linker or maleimido MMAE drug-linker to glycans or a thiol group. Cell proliferation assays of these conjugates showed that EC_{50} of the thiol conjugate had ~100-fold higher potency on the CHO cells transfected with human FAP than the same cells lacking FAP expression as depicted in **Figure 32**, which shows a comparison of in vitro potency of anti FAP B11 glycoconjugate and thiol conjugate. Cell viability was determined following exposure of the conjugates to CHO cells transfected with or without FAP antigen. The ADCs contained MMAE linked to the glycans ("glyco") or by conventional chemistry to hinge region cysteines ("thiol"). Note that the ~2-fold lower EC50 for the thiol compared to the glycoconjugates is consistent with the relative amounts of drug delivered per antibody assuming similar efficiencies for target binding and internalization in antigen expressing CHO cells. In parallel, a glycoconjugate of anti FAP (B11) ADC with a DAR of 1.5 as described previously was assayed and showed an ~2-fold higher EC_{50} than comparator thiol conjugate (DAR 3.3).

As shown in **Figure 36**, similar trends were observed in the cell proliferation assay for ADC prepared with the anti-HER antibody bearing the A114N hyperglycosylation mutation and AO-MMAE as described in Example 14, when assayed on SK-BR-3 expressing cells or MDA-MB-231 cells. The A114N glycoconjugate clearly shows enhanced cell toxicity against the Her2 expressing cell line over the non-expressing line. The relative toxicity compared to the SialT glycoconjugate prepared with the same antibody is consistent with the lower drug loading of this preparation.

A cell proliferation assay was also performed for ADC prepared with the anti-TEM1 antibody bearing the A114N hyperglycosylation mutation and AO-MMAE prepared as described in Example 14. Higher toxicity was observed with the TEM1-expressing cells lines SJSA-1 and A673 compared to the non-expressing MDA-MB-231 line. The level of toxicity compared with a conventional thiol conjugate with the same antibody was in keeping with the drug loading (DAR) of this preparation.

	SJSA-1	A673-RPMI	A673-DMEM-RPMI	MDA-MB-231
	IC50	IC50	IC50	IC50
antiTEM1 A114N-AO-MC-VC-PABC-MMAE	3 µg/ml	3.2 µg/ml	2.2 µg/ml	40 µg/ml
antiTEM1-MC-VC-PABC-MMAE	4 µg/ml	1 µg/ml	0.9 µg/ml	20 µg/ml

In summary, the site-specific conjugation of the drugs through the glycans with cleavable linkers produces ADCs with toxicities and in vitro efficacy that are equivalent to conventional thiol-based conjugates, as demonstrated using different antibodies and different drug-linkers. Moreover, below 2mM periodate, the level of drug conjugation correlates with the reduction of sialic acid. Increasing periodate concentration above 2mM produces little benefit, as expected from the complete conversion of sialic acid to the oxidized form. However, under all conditions, the number of drugs per antibody was slightly lower than the sialic acid content, indicating that some of the oxidized sialic acids may similarly not be available for coupling, either because of being buried or otherwise due to steric hindrance arising from the bulk of the drug-linker.

Example 16. In vivo Characterization of Antibody Drug Conjugates

Efficacy of anti-HER glycoconjugates were also evaluated in a Her2+ tumor cell xenograft mode and compared with thiol conjugate comparators having ~2-fold higher DAR. Beige/SCID mice were implanted with SK-OV-3 Her2+ tumor cells which were allowed to establish tumors of ~150 mm^3 prior to initiation of treatment. ADCs at 3 or 10mg/kg doses were injected through tail vein on days 38, 45, 52 and 59. There were ~10 mice per group. The tumor volume of mice in different group was measured and their survival was recorded. The survival curve was plotted based on Kaplan-Meier method.

Figures 33(A-D) show a comparison of in vivo efficacy of the anti-HER glycoconjugates and thiol conjugates in a Her2+ tumor cell xenograft model. Beige/SCID mice implanted with SK-OV-3 Her2+ tumor cells were dosed with MMAE (**Figures 33 A and B**) and PEG8-Dol10 (**Figures 33 C and D**) containing glycoconjugates or a thiol conjugate comparators with ~2-fold higher DAR. The tumor growth kinetics of the MMAE conjugates is shown in **Figure 33A**. In this case, the glycoconjugate showed a significantly higher efficacy than the naked antibody alone (black) but less than a thiol conjugate comparator having a ~2-fold higher DAR (green). The MMAE glycoconjugate showed

significant tumor regression and a ~20 day delay in tumor growth (**Figure 33A**) and ~2-fold increase in survival time from first dose (**Figure 33B**). The thiol MMAE conjugate showed near-complete tumor suppression at the same dose of ADC (10 mg/kg).

The in vivo efficacy of a PEG8-Dol10 glycoconjugate ("Glyco Dol10') and a thiol conjugate comparator with ~2-fold higher DAR ("Thiol Dol10") was also determined in the same Her2+ tumor cell xenograft model. Both conjugates showed lower efficacy than MMAE conjugates as described previously. However, the aminooxy-PEG8-Dol10 glycoconjugate ("Glyco Dol10") at 10 mg/kg showed a 15-day delay in tumor growth (**Figure 33C**) and ~20 day (1.7-fold) increase in survival time following first administration (**Figure 33D**). The thiol conjugate was more efficacious at the same dose, showing a 2-fold increase in survival. At a lower dose (3 mg/kg), the thiol conjugate showed a lesser efficacy than the glycoconjugate at 10 mg/kg. This dose corresponds to 80 umol PEG8-Dol10 drug per kg dose, compared to 110 umol PEG8-Dol10 drug per kg dose for the glycoconjugate.

These data demonstrate that site-specific conjugation of drugs onto sialic acid of antibody glycans yields molecules with comparable potency as ADCs generated via thiol-based chemistry. The somewhat lower in vivo efficacy likely stems from the fewer number of drugs which are carried by each antibody into the tumor cells by the internalization of each antibody-bound antigen. Although we have not compared these glycoconjugates with thiol conjugates of the same DAR, the efficacy observed at different doses of the two ADCs representing comparable levels of administered drug shows that the glycoconjugates have comparable intrinsic efficacy as their thiol counterparts, indicating no deleterious effect of conjugation at this site. Moreover, a 10mg/kg dose of the Dol10 glycoconjugate which introduced only 28% more drug provided a 2-fold increase in survival over the thiol conjugate (at 3mg/kg), suggesting these conjugates may even provide superior efficacies at the same DAR. Given the apparent limitation in sialic acid incorporation at native glycans, higher drug loading could be achieved by a number of different strategies including the use of branched drug linkers or the introduction of additional glycosylation sites and using the same method.

Example 17. Conjugation of Targeting Moieties

Figure 37 demonstrates the overall scheme for the conjugation of targeting moieties to existing carbohydrates or engineered glycosylation sites. This conjugation can be

performed through the attachment of neoglycans, glycopeptides, or other targeting moieties to oxidized sialylated antibodies (**Figures 38 and 39**). Moieties suitable for conjugation may include those containing aminooxy linkers (**Figure 40 and 41**).

Example 18. Conjugation through sialic acid in native Fc glycans

Man-6-P hexamannose aminooxy was conjugated to either a polyclonal antibody or monoclonal antibody specifically targeting a Man-6-P receptor. The SDS-PAGE and MALDI-TOF MS analyses of the conjugation of the rabbit polyclonal antibody with Man-6-P hexamannose aminooxy is shown in **Figure 42**. **Figure 43** depicts the results of surface plasmon resonance experiments used to assess the binding of control and Man-6-P hexamannose conjugated rabbit polyclonal IgG antibodies to cation-independent Man-6-P receptor (CI-MPR). *In vitro* analyses of this conjugated antibody demonstrates increased uptake into both HepG2 (*Homo sapiens* liver hepatocellular carcinoma) and RAW (*Mus musculus* murine leukemia) cell lines (**Figure 44**). Cultures were stained with anti-rabbit-Alexa 488 antibody counterstained with DAPI.

Antibodies conjugated with Man-6-P or lactose aminooxy moieties were further tested through SDS-PAGE and lectin blotting and compared with unconjugated antibodies (**Figure 45**). MALDI-TOF intact protein analyses of the control and conjugated antibodies demonstrate that the conjugates have approximately two glycan moieties per antibody, while control antibodies have none (**Figure 46**).

Example 19. Conjugation through sialic acid to hinge cysteine residues in antibody

Man-6-P hexamannose maleimide (**Figure 41**) was conjugated to either a polyclonal antibody or monoclonal antibody.

The conjugation of a polyclonal antibody with Man-6-P hexamannose maleimide through hinge cysteines was examined through SDS-PAGE, lectin blotting, and Man-6-P quantitation (to determine the number of glycans conjugated per antibody) (**Figure 47**). Conjugation of a polyclonal antibody with lactose maleimide was also examined through the use of SDS-PAGE and galacose quantitation of the control antibody, conjugated antibody, and filtrate are shown in **Figure 48**. Little increased aggregation was observed in hinge cysteine-conjugated polyclonal antibodies by size exclusion chromatography (SEC) (**Figure 50**).

The conjugation of a monoclonal antibody with Man-6-P hexamannose maleimide through hinge cysteines was also examined through SDS-PAGE and glycan quantitation (to determine the number of glycans conjugated per antibody) (**Figure 49**). Little increased aggregation was observed in hinge cysteine-conjugated polyclonal antibodies by size exclusion chromatography (SEC) (**Figure 51**).

The binding of Man-6-P receptor (CI-MPR) to bis Man-6-P hexamannose conjugated polyclonal and monoclonal antibodies through native Fc glycans or hinge disulfides was also demonstrated through gel shift on a native PAGE (**Figure 55**).

Example 20. Preparation of fully galactosylated monoclonal antibody and conjugation of a trigalactosylated glycopeptide to the sialylated antibody

A mouse monoclonal antibody mutant with an STY mutation (NNAS) was modified with sialidase and galactosyltransferase for making mainly native trigalactosylated glycans (2 glycans per antibody). The same mutant was also sialylated with sialyltransferase and conjugated with a trivalent galactose containing glycopeptide (**Figure 68**) using SAM approach. The sialic acid content of the enzyme modified antibodies was examined (**Figure 52**). Further, MALDI-TOF analysis of the glycans released from control and desialylated/galactosylated (**Figure 53**) NNAS as well as the glycans released from control and sialylated (**Figure 54**) NNAS were examined. SDS-PAGE (4-12%NuPAGE) and lectin blotting of enzyme modified and conjugated NNAS are shown in (**Figure 56**). Terminal galactose quantitation was also measured for the control NNAS antibody, desialylated/galactosylated NNAS antibody, and glycopeptide conjugated NNAS antibody (**Figure 57**).

Example 21. Preparation of α2,3 sialylated lactose maleimide using a chemoenzyme approach and subsequent conjugation to non-immune rabbit IgG through hinge disulfides.

Carbohydrate-binding proteins (including Siglec proteins) are able to bind more efficiently to areas with greater sialic acid density. Thus, the monosialylated glycans on a given antibody may not provide enough sialic acid density to facilitate binding to other Siglec proteins. Therefore, a hinge disulfide conjugation approach for introducing multiple copies of sialylated glycans was investigated. To produce sialylated glycans for conjugation, lactose

maleimide (5mg) was sialylated in vitro with α2,3 sialyltransferase from *Photobacterium damsela* in Tris buffer (pH 7.5) for 2 hrs at 37℃. A control glycan was incubated without sialyltransferase and compared with the original glycans. MALDI-TOF MS analysis showed that the incubation of lactose maleimide without enzyme in Tris buffer (pH 7.5) for 2 hrs at 37℃ did not change the expected molecular weight of the molecule, suggesting that the examined condition did not result in maleimide hydrolysis. The MALDI-TOF and Dionex HPLC analysis of glycans modified with α2,3 sialyltransferase indicate the presence of sialyllactose, although not as major peak (data not shown). Therefore, the sialyllactose maleimide was additionally purified using QAE-sepharose columns and each fraction was subsequently analyzed using MALDI-TOF and Dionex HPLC. These analyses indicated that sialyllactose maleimide existed as major species in the 20mM NaCl eluate from QAE column (**Figure 58**). The amount of sialylated glycans purified was estimated using sialic acid quantitation analysis of the samples, indicating a recovery of ~1.8mg sialyllactose maleimide.

Subsequent conjugation of a rabbit polyclonal antibody with this sialyllactose maleimide was tested using thiol-maleimide chemistry. A rabbit IgG antibody (1mg) was reduced with TCEP at a 4 molar excess (over the antibody) for 2 hrs at 37℃ before being conjugated to 24 molar excess of sialyllactose for 1hr at room temperature. The conjugate was then buffer-exchanged into PBS for analysis on SDS-PAGE (**Figure 59A**). Sialic acid quantitation was also performed using Dionex HPLC (**Figure 59B**). Aliquots of control and thiol conjugate were treated with or without sialidase (1U per mg) overnight at 37℃ before supernatants were recovered through filtration (10 kDa MWCO). The sialic acid content of the supernatants was measured and compared to samples treated without sialidase. There are approximately 4 α2,3 sialyllactose moieties coupled per antibody.

Example 22. Preparation of α2,6 sialyllactose maleimide by sialylating lactose maleimide and conjugation to hinge disulfides of a rabbit polyclonal antibody through α2,3- or α2,6- linkages resulting in high sialylation

The conjugation of multiple copies of α2,6- sialylated glycans to the hinge disulfides of a rabbit polyclonal antibody was also investigated. Since the α2,3 sialyllactose maleimide was successfully produced using a chemoenzyme approach (see above, Example 21), a similar method was used to produce α2,6 sialyllactose maleimide (minor modifications of the protocol included the use of a different sialyltransferase). To produce α2,6 sialylated glycan for conjugation, lactose maleimide (~5mg) was sialylated in vitro with 0.5U of a bacterial

α2,6 sialyltransferase from *Photobacterium damsela* in Tris buffer (pH 8) for 1 hr at 37℃. After enzymatic reaction, the product was applied to a QAE-sepharose column. The column was washed with 10 fractions of 1ml 2mM Tris (pH 8), 5 fractions of 1ml of Tris buffer containing 20mM NaCl, and 5 fractions of 1ml Tris buffer containing 70mM NaCl. The aliquots from each fraction were analyzed using Dionex HPLC alongside lactose and α2,6 sialyllactose standards. The oligosaccharide profiles of the standards and one of the eluted fractions are shown in **Figures 60 (A-D)**. The fractions containing α2,6 sialyllactose maleimide were also analyzed and confirmed by MALDI-TOF. The glycan in one of the fractions can be seen in **Figure 61**.

The amount of α2,6 sialyllactose maleimide purified was then estimated using sialic acid quantitation analysis which indicated a recovery of ~1.5mg sialyllactose maleimide. Once the glycan was prepared, the conjugation of antibody with either α2,6 sialyllactose maleimide or α2,3 sialyllactose maleimide was tested using thiol chemistry. A rabbit polyclonal IgG antibody (1mg) was buffer-exchanged and reduced with TCEP at a 4 molar excess (over antibody) for 2 hrs at 37℃. The reduced antibody was then split in half: one portion was conjugated to 24 molar excess of α2,6 sialyllactose maleimide, and the other to α2,3 sialyllactose maleimide for 1hr at room temperature. The two conjugates were then buffer-exchanged into PBS before SDS-PAGE analysis (**Figure 62A**) and sialic acid quantitation using Dionex HPLC (**Figure 62B**). Sialic acid quantitation was was used to estimate the number of glycan conjugated. Aliquots of control antibody and thiol-conjugated antibody were treated with or without sialidase (1U per mg) overnight at 37℃ before supernatants were recovered through filtration (10 kDa MWCO). The sialic acid content of the supernatants was measured and compared to samples treated without sialidase (control). The analysis demonstrated that approximately 7 glycans (either α2,3- or α2,6-sialyllactose glycans) were conjugated to the polyclonal antibody by this method.

Example 23. PEGylation of NNAS using GAM chemistry

A mouse NNAS (S298N/T299A/Y300S) mutant monoclonal antibody was galactosylated and desialylated, generating a Gal NNAS monoclonal antibody without any protease degradation. This antibody was modified with galactose oxidase (GAO) to generate galactose aldehyde. The galactose aldehyde was then conjugated with 2 or 5kDa of aminooxy polyethylene glycol (PEG). **Figure 63** depicts the characterization of control and enzyme

modified (desalylated/galactosylated) NNAS mutant antibodies using SDS-PAGE and lectin blotting. **Figure 64** depicts the characterization through reducing SDS-PAGE of the PEGylation of a control antibody and Gal NNAS with various amounts of galactose oxidase. These results demonstrate that Gal NNAS can be PEGylated efficiently with significant amounts of mono-, bi-, and tri-PEG conjugated per heavy chain. **Figure 66** depicts the characterization through reducing SDS-PAGE of the PEGylation of a control antibody and Gal NNAS with various molar excess of PEG over antibody. Protein Simple scans characterizing the PEGylation of the antibodies demonstrate that approximately 1.5-1.7 PEG moieties are conjugated per heavy chain (or about 3-3.4 PEG per antibody) (**Figures 65** and **67**).

Example 24. PEGylation of NNAS using GAM chemistry

An NNAS antibody was galactosylated with 50mU/mg galactosyltransferase and subsequently desialylated with 1U/mg sialidase in 50mM MES buffer (pH 6.5). Desialylated fetuin and NNAS as well as galactosylated NNAS were then treated with galactose oxidase (57mU/mg)/catalase in the presence or absence of 0.5mM copper acetate before conjugation with 25 molar excess of 5kDa aminoxy PEG (**Figure 69A**). In another experiment, galactosylated NNAS was treated with galactose oxidase (57mU/mg)/catalase in the presence of 0, 0.02, 0.1 and 0.5mM copper acetate before conjugation with 25 molar excess of 5kDa aminoxy PEG (**Figure 69B**). Antibody oxidized with galactose oxidase in the presence of copper acetate showed a higher degree of PEGylation than the same antibody reacted with galactose oxidase in the absence of copper acetate. Significantly higher levels of PEGylation were observed when the conjugation was performed in a reaction containing copper sulfate in concentrations above 0.1mM.

Example 25. Modification of wild-type and mutant Herceptin using sialidase/galactosyltransferase

Wild-type and mutant (A114N, NNAS, and A114N/NNAS) Herceptin antibodies were enzymaticically modified with 50mU/mg galactosyltransferase and subsequently desialylated with 1U/mg sialidase in 50mM MES buffer (pH 6.5). The modified antibodies were analyzed using SDS-PAGE (reducing and nonreducing), lectin blotting with ECL (a plant lectin specific for terminal galactose), and terminal galactose quantitation using Dionex

HPLC analysis of released galactose by galactosidase (**Figure 70**). Enzyme modified antibodies containing approximately three to nine terminal galactose were obtained with the NNAS and NNAS/A114N double mutants demonstrating a higher level of terminal galactose than the wild-type and A114N mutant.

Example 26. PEGylation of wild-type and mutant antibodies using the SAM conjugation method

Wild-type and (A114N, NNAS, and A114N/NNAS) Herceptin antibodies were PEGylated using sialic acid-mediated (SAM) conjugation. The antibodies were subsequently oxidized with 2mM periodate. After buffer exchange, the oxidized antibodies were PEGylated with 25 molar excess of 5kDa aminoxy PEG. The sialic acid content of the wild-type and mutant antibodies was measured using Dionex HPLC (**Figure 71**). The PEGylated antibodies were then analyzed using reducing and non-reducing SDS-PAGE (**Figure 72**). Further, the PEGylation (PAR, number of PEG per antibody) was estimated by analyzing the scanned gels using ProteinSimple (**Figure 73**). The NNAS, A114N, and A114N/NNAS mutants all showed higher PAR (2.7-4.6) than wild-type Herceptin antibodies (1.4).

Example 27. Investigation of uptake of glycoengineered antibodies with galactose containing glycan ligands

A polyclonal antibody was either enzymatic modified with galactosyltransferase (Gal Transferase), conjugated to lactose aminoxy (Gal-Glc to 297: conjugated to sialic acid in glycans from Asn-297 of sialylated antibody), or conjugated to lactose maleimide (Gal-Glc to Hinge: conjugated to cysteines in hinge disulfides). The control, modified, or conjugated antibodies were then incubated with HepG2 cells (a hepatocyte cell line expressing ASGPR) for 1-2 hrs at 37°C before the uptaken antibodies were measured using Immunofluorescence staining (**Figure 74**). The results showed increased HepG2 cell uptake of enzymatic modified or lactose conjugated antibodies.

Example 28. Conjugation of a trivalent GalNAc glycan to Herceptin

Herceptin (anti-Her2) was sialylated and conjugated with a trivalent GalNAc glycan (**Figure 75**) for targeting ASGPR using the SAM conjugation method. Subsequently, surface

plasmon resonance experiments (Biacore) were used to asses the binding of these trivalent GalNAc glycan-conjugated antibodies to ASGPR subunit H1 (**Figure 76**).

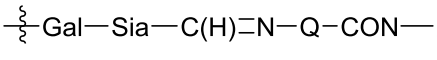
Example 29. Conjugation of trivalent GalNAc glycan and trivalent galactose containing glycopeptide to a recombinant lysosomal enzyme

A recombinant lysosomal enzyme was conjugated with either trivalent GalNAc glycan or trivalent galactose containing glycopeptides (**Figure 77**) for targeting ASGPR using the SAM conjugation method. Subsequently, surface plasmon resonance experiments (Biacore) were used to asses the binding of these trivalent GalNAc glycan-conjugated and trivalent galactose containing glycopeptide-conjugated enzymes to ASGPR subunit H1 (**Figure 78**). The results showed strong ASGPR binding of trivalent GalNAc glycan conjugated recombinant lysosomal enzyme. **Figures 79(A-D)** show a number of exemplary trivalent GalNAc moieties. Examples are shown that have no spacer, a 12Å spacer, a 1 kDa (approximately 80 Å) spacer, and both a ~20 Å spacer and a 1 kDa (approximately 80 Å) spacer. A second recombinant lysosomal enzyme (rhGAA) was also conjugated with trivalent GalNAc glycan C12. **Figure 80** shows a graph depicting the results of conjugation of periodate oxidized recombinant lysosomal enzyme rhGAA with an excess of trivalent GalNAc glycan C12. The resulting glycan per GAA conjugated is depicted with 20, 40, 80, and 200-fold molar excess glycan over rhGAA. **Figure 81** depicts ASGPR binding of a recombinant lysosomal enzymes conjugated with trivalent GalNAc glycan C12 on Biacore. Enzymes conjugated with 20 (conjugate 1), 40, 80, and 200-fold (conjugate 4) excess of glycan all show strong binding to ASGPR subunit 1. There is no significant difference in binding among the conjugates (conjugates 1 to 4).

CLAIMS

We claim:

1. A binding polypeptide comprising at least one modified glycan, wherein the glycan comprises at least one moiety of Formula (IV):



Formula (IV),

wherein:

A) Q is NH or O;

B) CON is a connector moiety;

C) X is a targeting moiety;

D) Gal is a component derived from galactose; and

E) Sia is a component derived from sialic acid, wherein Sia is present or absent, and wherein the targeting moiety binds to a cell.

2. The binding polypeptide of claim 1, wherein one or more of the following conditions are met:

a) the cell is a mammalian cell;

b) the binding polypeptide is internalized by the cell;

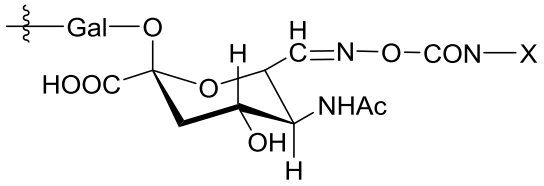
c) the targeting moiety binds to a mannose 6 phosphate receptor on the cell;

d) the targeting moiety binds to a sialic acid-binding immunoglobulin-type lectin (Siglec) on the cell;

e) the targeting moiety binds to a C-type lectin receptor, a galectin, or a L-type lectin receptor;

f) Q is O;

g) the glycan comprises at least one moiety of the following structural formula:



;

h) the targeting moiety is a glycopeptide capable of binding an asialoglycoprotein receptor (ASGPR) on a cell;

i) the binding protein comprises an N-glycan glycoform selected from the group consisting of: a G0 glycoform, a G1 glycoform, and a G2 glycoform;

j) the N-glycan glycoform is selected from the group consisting of: a G1S1 glycoform, a G2S1 glycoform, and a G2S2 glycoform; and

k) the N-glycan glycoform is selected from the group consisting of: a G1F glycoform, a G2F glycoform, a G1S1F glycoform, a G2S1F glycoform, and a G2S2F glycoform.

3. The binding polypeptide of claim 2, wherein one or more of the following conditions are met:

a) the mammalian cell is selected from an immune cell, a liver cell, a tumor cell, a vascular cell, an epithelial cell, or a mesenchymal cell;

b) the amount of the binding polypeptide internalized by the cell is greater than the amount of a reference binding polypeptide lacking a targeting moiety internalized by the cell;

c) the targeting moiety comprises a mannose 6 phosphate moiety;

d) the Siglec is sialoadhesin (Siglec-1), CD22 (Siglec-2), CD33 (Siglec-3), myelin-associated glycoprotein (MAG (Siglec-4)), Siglec-5, Siglec-6, Siglec-7, Siglec-8, Siglec-9, Siglec-10, Siglec-11, Siglec-12, Siglec-14, or Siglec-15;

e) the targeting moiety comprises an α2,3-, α2,6-, or α2,8-linked sialic acid residue;

f) the targeting moiety binds to DEC-205 (CD205; lymphocyte antigen 75), macrophage mannose receptor (MMR; CD206), Dectin-1, Dectin-2, macrophage-inducible C-type lectin (Mincle), dendritic cell-specific ICAM3-grabbing nonintegrin (DC-SIGN, CD209), DC NK lectin group receptor-1 (DNGR-1), Langerin (CD207), a lectican, an asialoglycoprotein receptor (ASGPR), C-lectin receptor dendritic cell immunoreceptor (CLEC4A; CLECSF6; DCIR), macrophage galactose-type lectin (MGL), a DC receptor, a collectin, a selectin, an NK-cell receptor, a multi- C-type lectin domain (CTLD) endocytic receptor, a Reg group (type VII) lectin, chondrolectin, tetranectin, polycystin, attractin (ATRN), eosinophil major basic protein (EMBP), DiGeorge Syndrome Critical

Region Gene 2 (DGCR2), Thrombomodulin, Bimlec, a group XVI lectin (SEEC), or a group XVII lectin (CBCP/Frem1/QBRICK); and

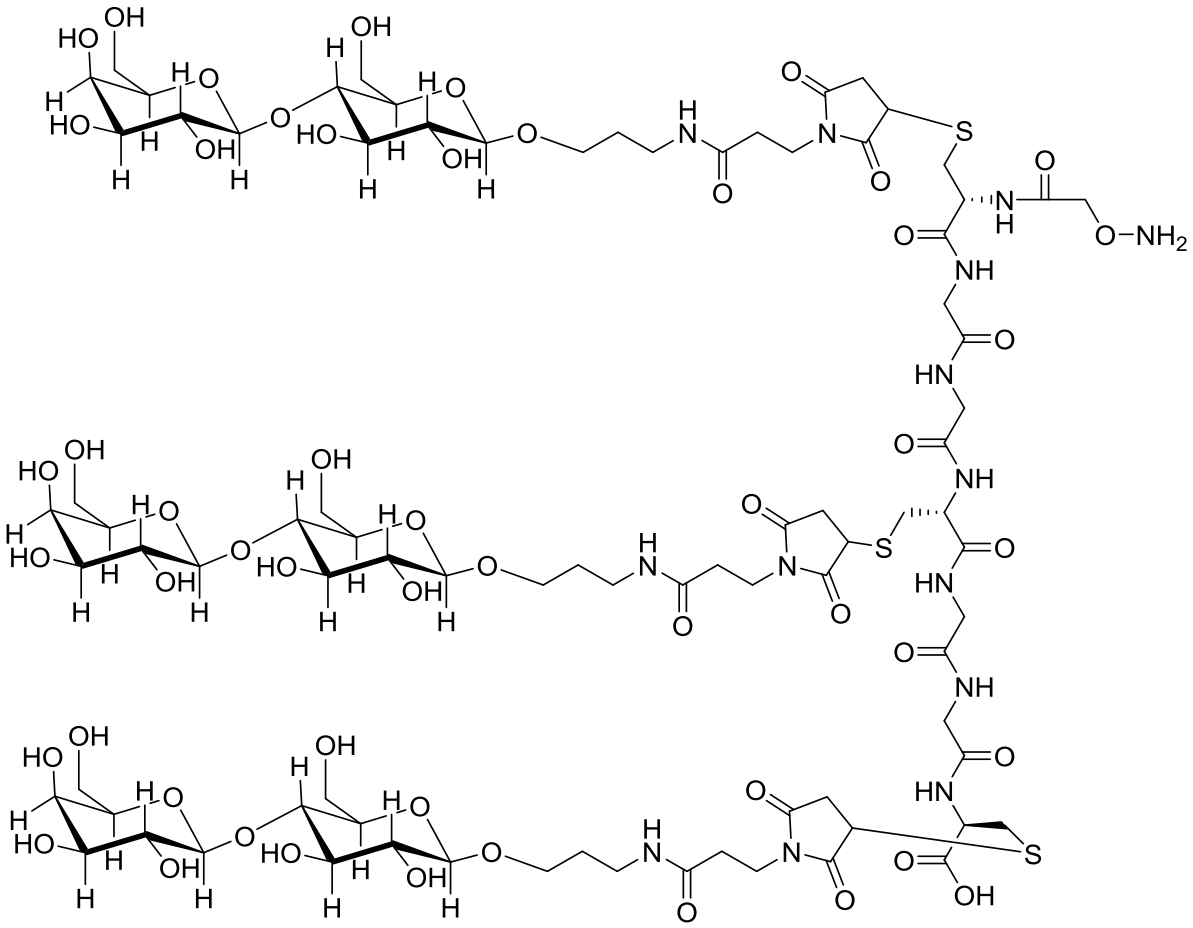
g) the targeting moiety is a trivalent GalNAc glycan moiety.

4. The binding polypeptide of claim 2, wherein the mammalian cell is selected from a B cell, a T cell, a dendritic cell, a natural killer (NK) cell, a macrophage, a neutrophil, a hepatocyte, a liver sinusoidal endothelial cell, or a hepatoma cell.

5. The binding polypeptide of claim 3,

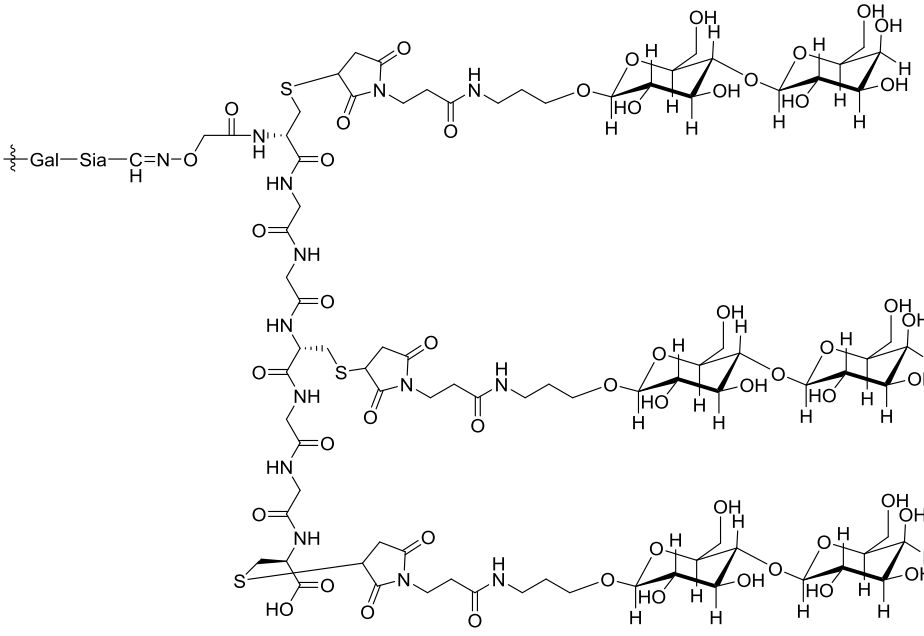
wherein the targeting moiety comprises an α2,3-siallylactose moiety or an α2,6-siallylactose moiety, or

wherein the targeting moiety is a tri-galactosylated glycopeptide or lactose3-Cys3Gly4, optionally wherein Sia is present and the lactose$_3$-Cys$_3$Gly$_4$ moiety is represented by Formula V:

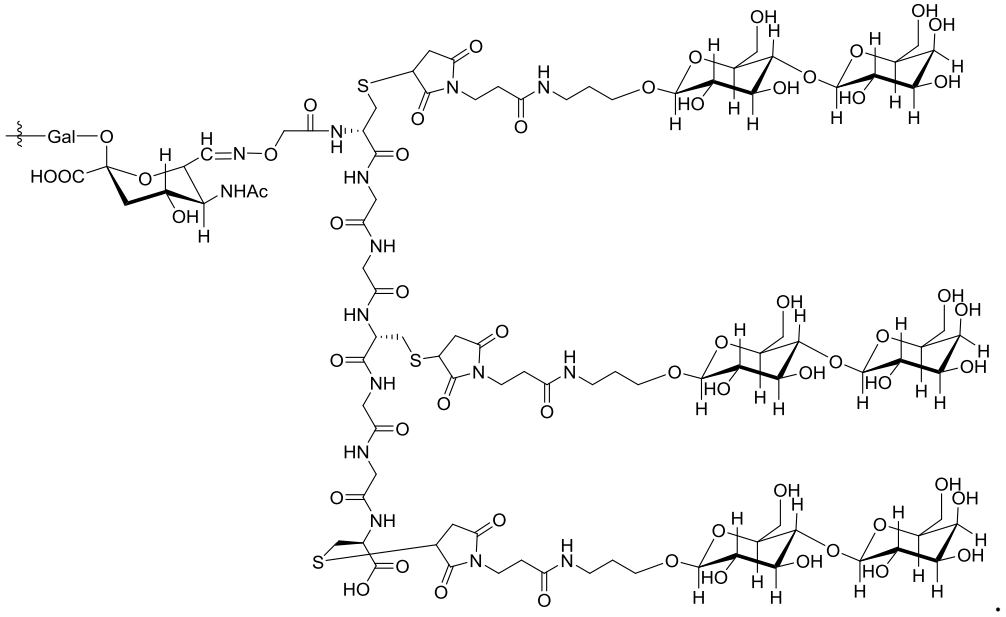


Formula V

6. The binding polypeptide of claim 5, wherein the glycan comprises at least one moiety selected from the following structural formulae:



; or

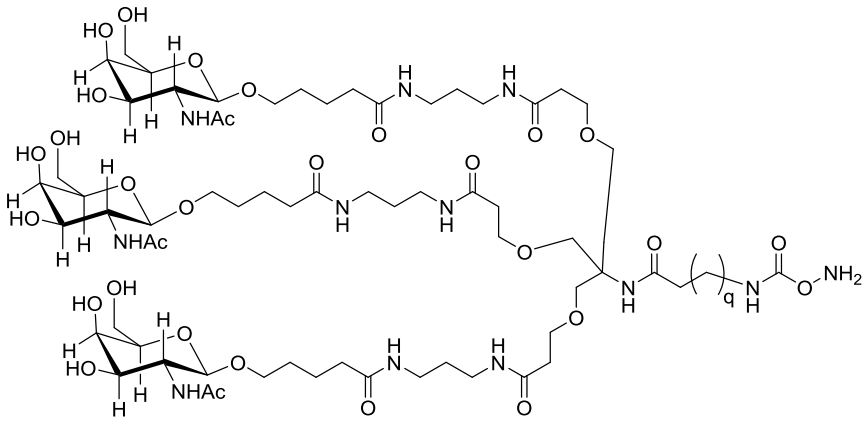


.

7. The binding polypeptide of claim 2, wherein the targeting moiety is a trivalent GalNAc glycan moiety.

8. The binding polypeptide of claim 7, wherein one or more of the following conditions are met:

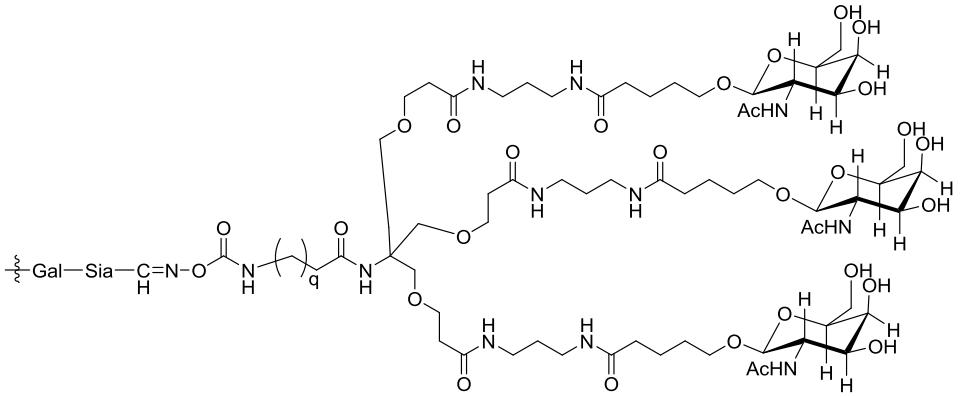
a) Sia is present and the trivalent GalNAc glycan moiety is represented by Formula VIII:



Formula VIII

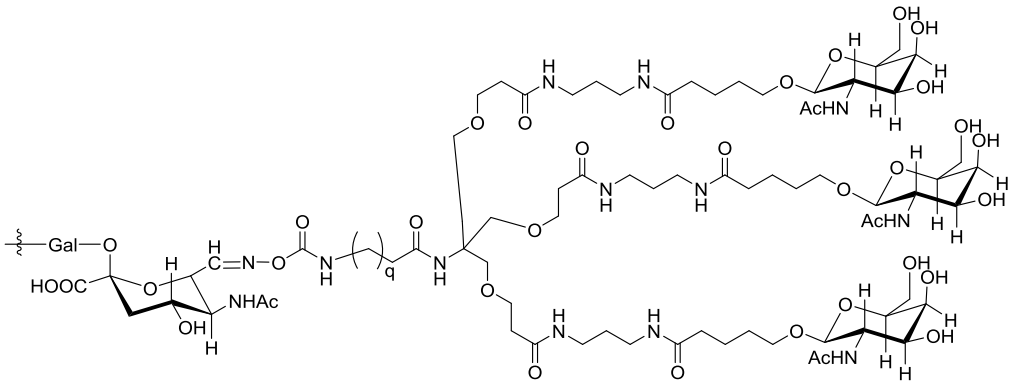
wherein q is an integer between 1 and 29 inclusive;

b) the glycan comprises at least one moiety having the following structural formula:



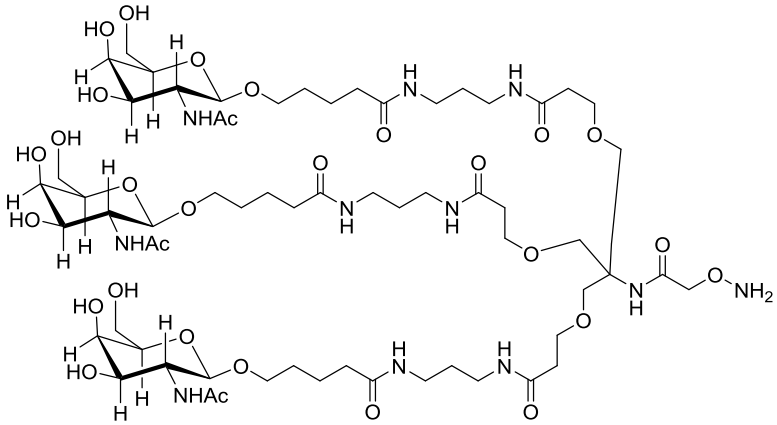
wherein q is an integer between 1 and 29 inclusive;

c) the glycan comprises at least one moiety having the following structural formula:

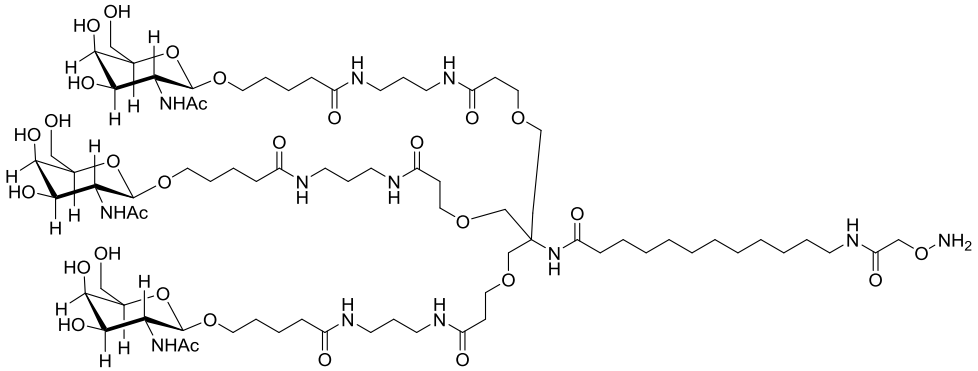


wherein q is an integer between 1 and 29 inclusive;

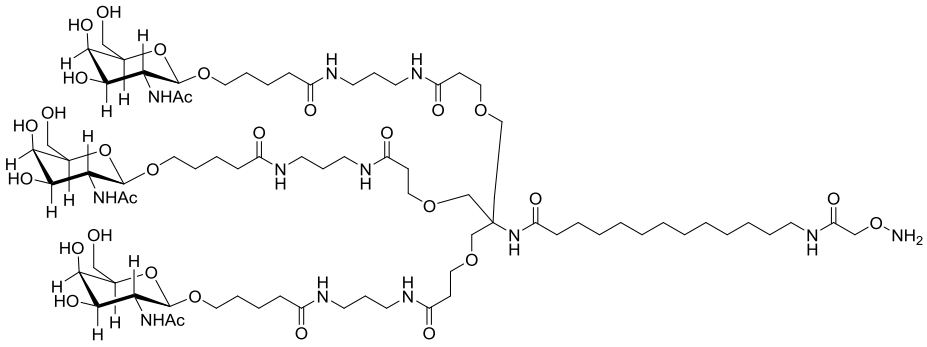
d) the trivalent GalNAc glycan moiety is represented by Formula VII, Formula XIII, or Formula XIV:



Formula VII

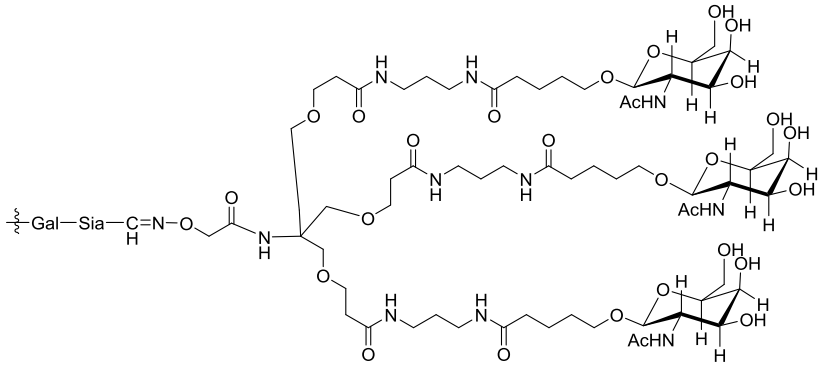


Formula XIII or



Formula XIV; and

e) the glycan comprises at least one moiety selected from the following structural formulae:



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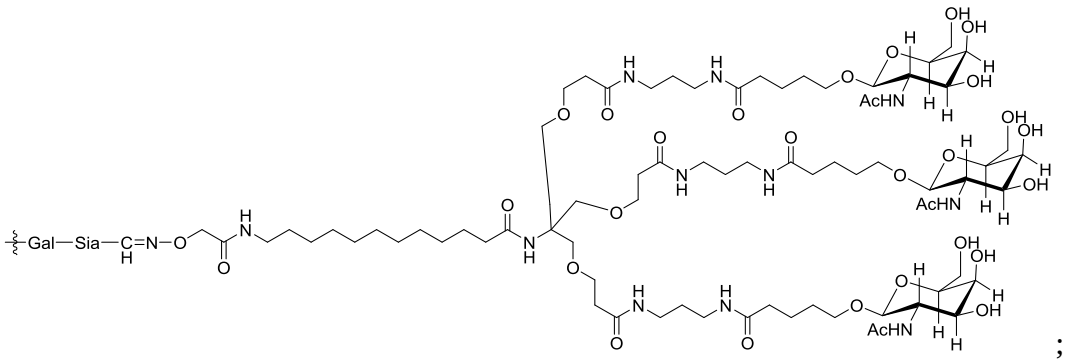

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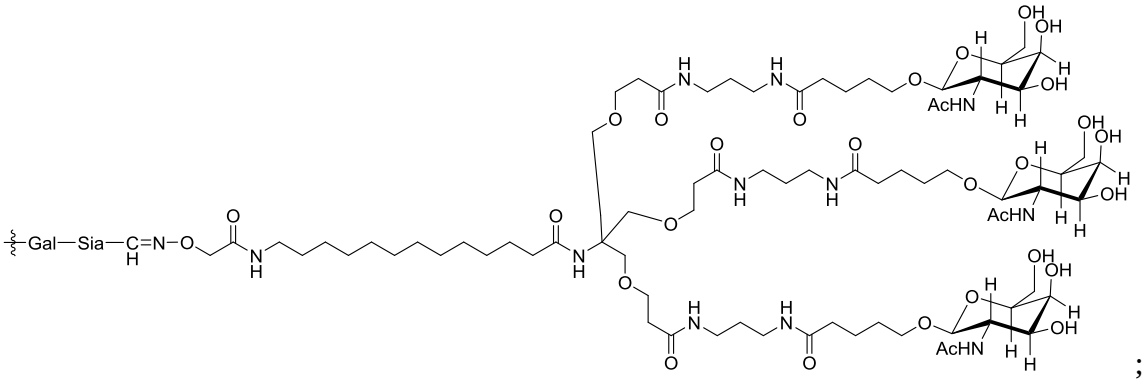

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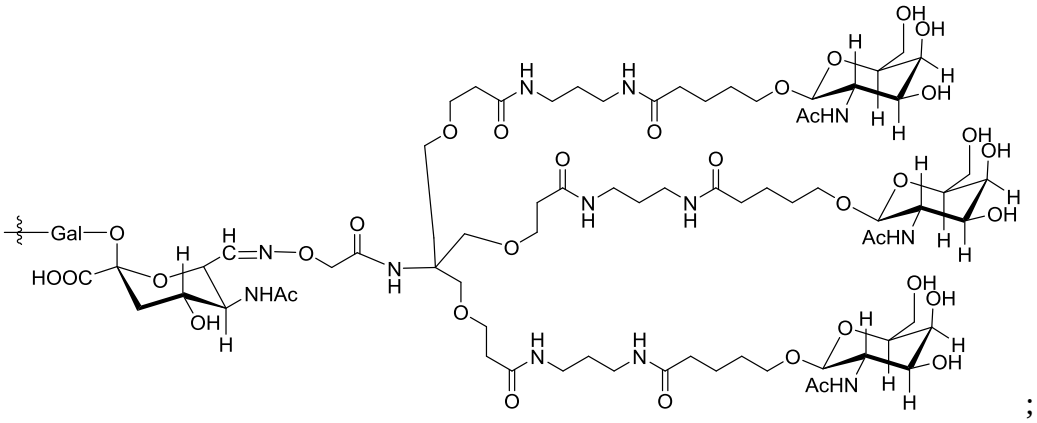

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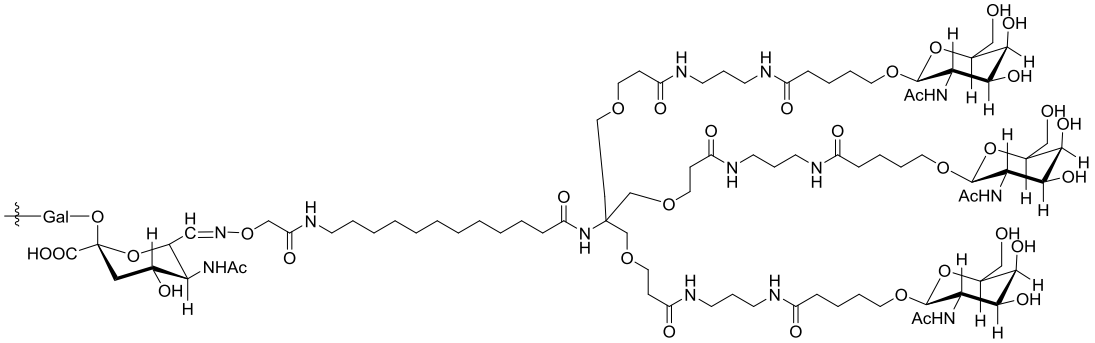
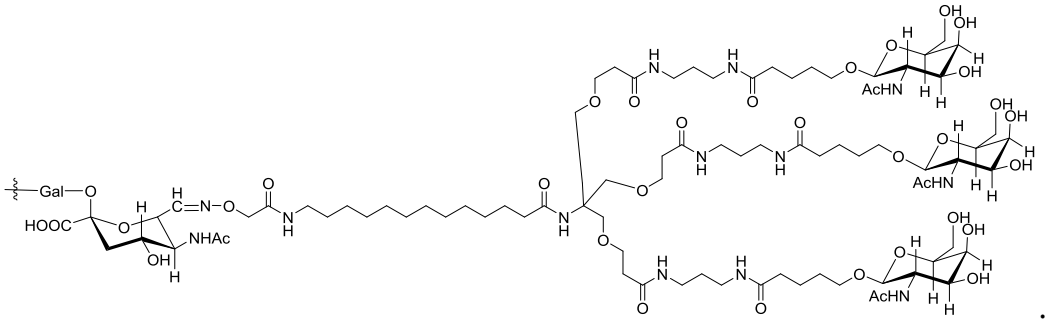


; or



.

9. The binding polypeptide of any one of the preceding claims, wherein one or more of the

following conditions are met:

a) the binding polypeptide comprises an Fc domain, wherein the Fc domain is optionally human;

b) the binding polypeptide comprises a CH1 domain;

c) the connector moiety comprises a pH-sensitive linker, disulfide linker, enzyme-sensitive linker or other cleavable linker moiety;

d) the connector moiety comprises a linker moiety selected from the group consisting of linker moieties depicted in Table 2 and 14;

e) the binding polypeptide is an antibody or immunoadhesin;

f) the modified glycan is N-linked to the binding polypeptide via an asparagine residue at amino acid position 297 of the Fc domain, according to EU numbering;

g) the modified glycan is N-linked to the binding polypeptide via an asparagine residue at amino acid position 298 of the Fc domain, according to EU numbering; and

h) the modified glycan is N-linked to the binding polypeptide via an asparagine residue at amino acid position 114 of the CH1 domain, according to Kabat numbering.

10. A method of making the binding polypeptide of any one of the preceding claims, the method comprising reacting an effector moiety of Formula (I):

NH_2-Q-CON-X

Formula (I),

wherein:

A) Q is NH or O;

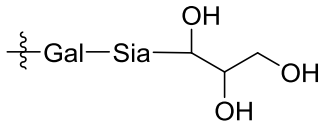
B) CON is a connector moiety; and

C) X is a targeting moiety,

with a precursor binding polypeptide comprising an oxidized glycan.

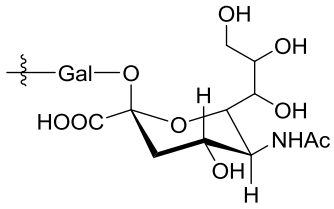
11. The method of claim 10, wherein one or more of the following conditions are met:

a) the initial binding polypeptide comprises at least one moiety of the following structural formula:



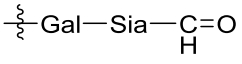
;

b) the initial binding polypeptide comprises at least one moiety of the following structural formula:



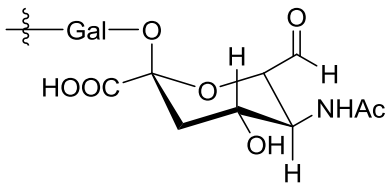
;

c) the precursor binding polypeptide comprises an oxidized glycan comprises at least one moiety of the following structural formula:



;

d) the precursor binding polypeptide comprises an oxidized glycan comprises at least one moiety of the following structural formula: and



;

e) the precursor binding polypeptide comprising an oxidized glycan is generated by reacting an initial binding polypeptide comprising a glycan with a mildly oxidizing agent,

optionally wherein the mildly oxidizing agent is sodium periodate,

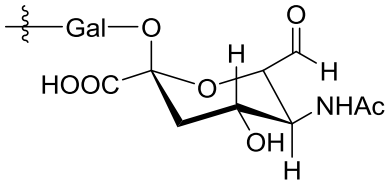
wherein less than 1mM sodium periodate is employed, or

wherein the mildly oxidizing agent is periodate or galactose oxidase.

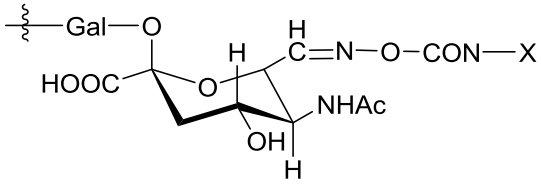
12. The method of claim 10 comprising reacting an effector moiety of the following structural formula:

NH_2-O-CON-X

with the precursor binding polypeptide comprising an oxidized glycan comprising at least one moiety of the following structural formula:



to form a binding polypeptide of the following structural formula:



,

wherein the targeting moiety is optionally a trivalent GalNAc glycan moiety.

13. The method of claim 11, wherein the reacting step is conducted in the presence of a salt comprising a metal ion, optionally wherein

the metal ion is a copper ion,

the salt is copper acetate, or

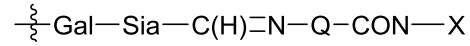
the salt comprises a metal ion present at a concentration of at least 0.1 mM.

14. The method of any of any one of the preceding claims wherein the binding polypeptide comprising the glycan comprises one or two terminal sialic acid residues, optionally wherein the one or two terminal sialic acid residues are introduced by treatment of the binding polypeptide with a sialyltransferase or combination of sialyltransferase and galactosyltransferase.

15. A composition comprising a binding polypeptide of any one of the preceding claims and a pharmaceutically acceptable carrier or excipient, optionally wherein the ratio of targeting moiety to binding polypeptide is at least about 2 or equal to or more than about 4.

16. Use of an effective amount of the composition of claim 15 in the manufacture of a medicament for the treatment of neoplastic disorders in a patient in need thereof.

17. A binding polypeptide comprising at least one modified glycan comprising at least one moiety of Formula (IV):



Formula (IV)

wherein:

A) Q is NH or O;

B) CON is a connector moiety;

C) X is a moiety comprising PEG;

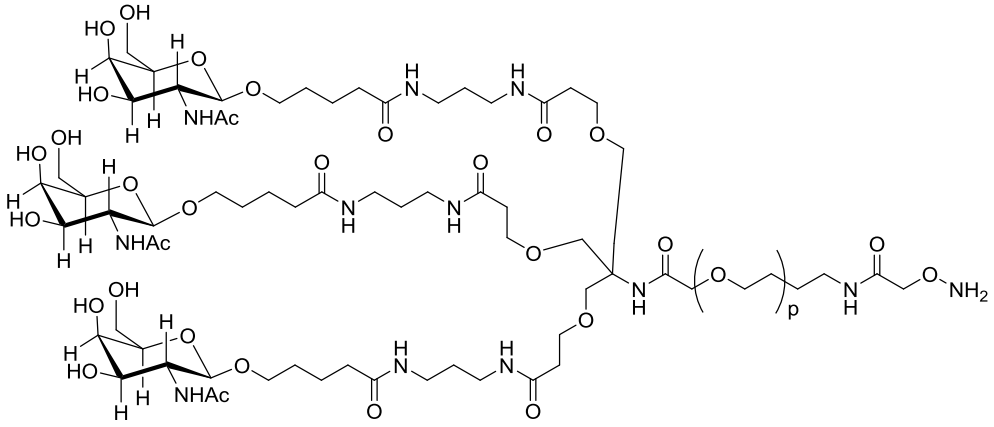
D) Gal is a component derived from galactose; and

E) Sia is a component derived from sialic acid,

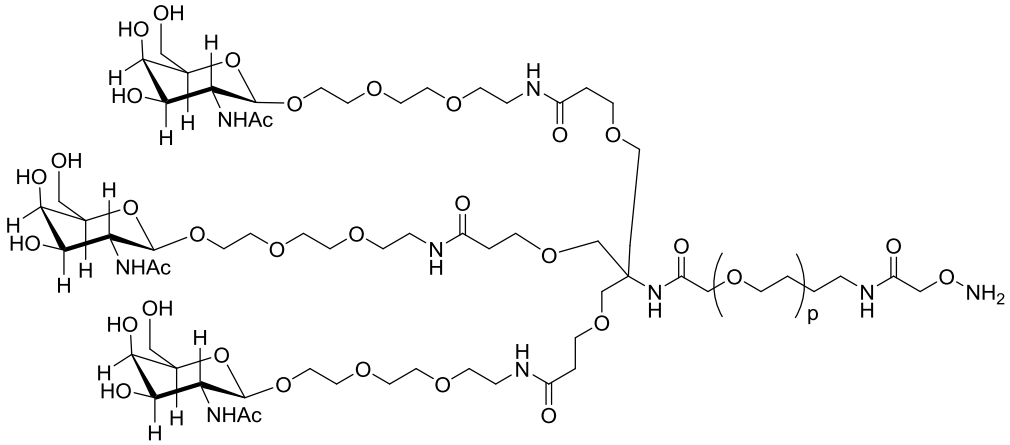
wherein Sia is present or absent.

18. The binding polypeptide of claim 17, wherein one or more of the following conditions are met:

a) the glycan comprises at least one moiety represented by Formula IX or Formula XI:

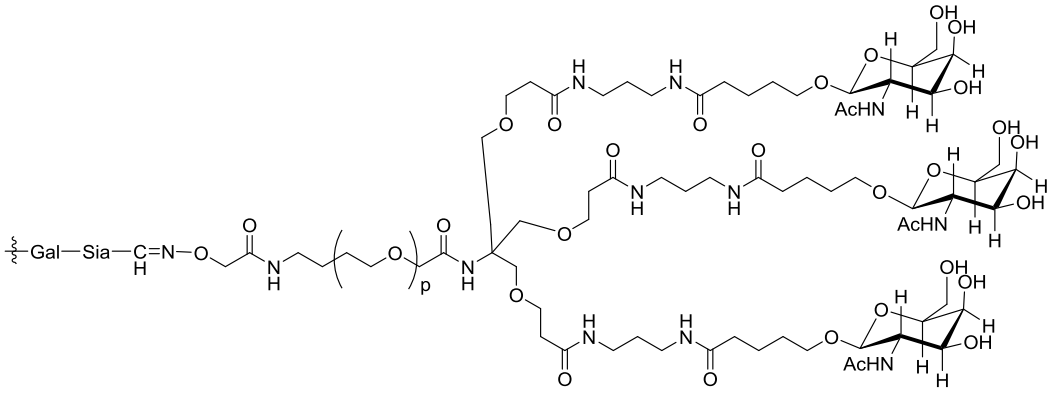


[Formula IX], wherein p has a value of 1 to 32; or

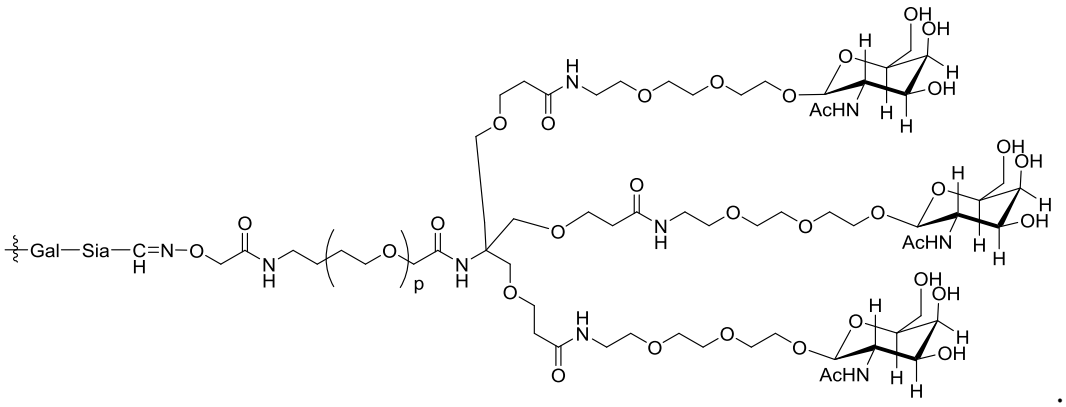


[Formula XI], wherein p has a value of 1 to 32;

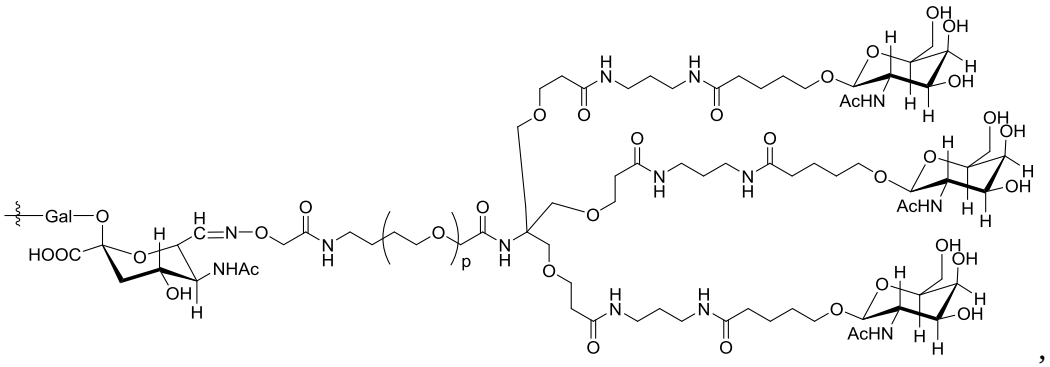
b) the glycan comprises at least one moiety selected from the following structural formulae:



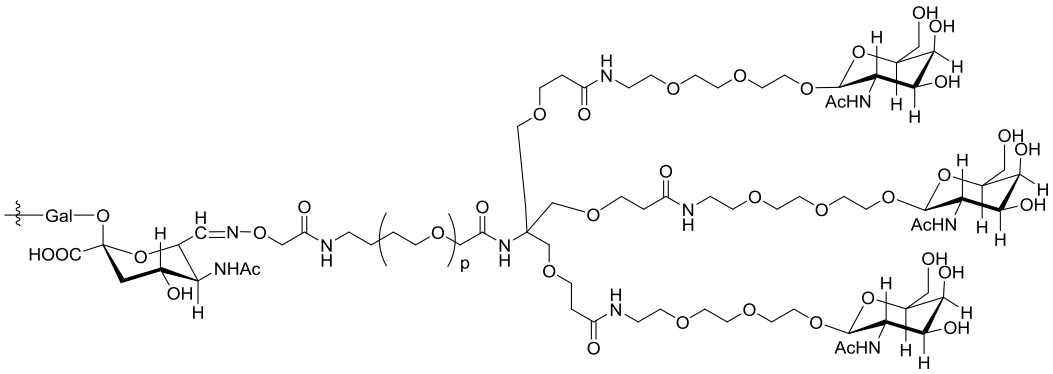
wherein p has a value of 1 to 32;



wherein p has a value of 1 to 32;

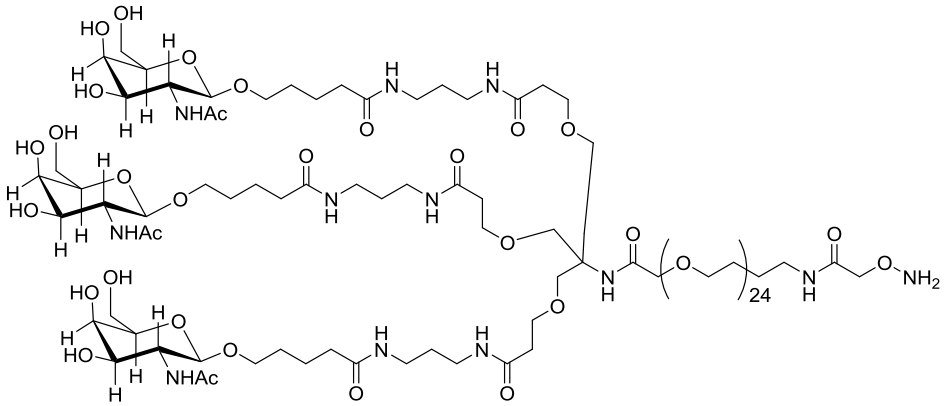


wherein p has a value of 1 to 32; or

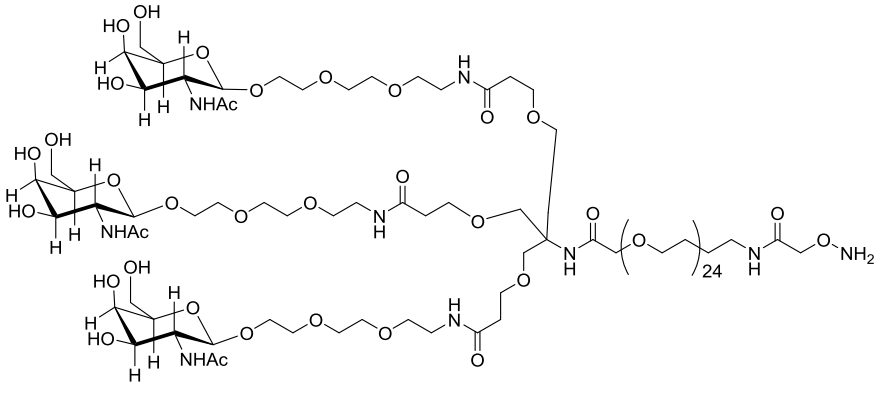


wherein p has a value of 1 to 32;

c) the targeting moiety comprises PEG and is represented by Formula X or Formula XII:

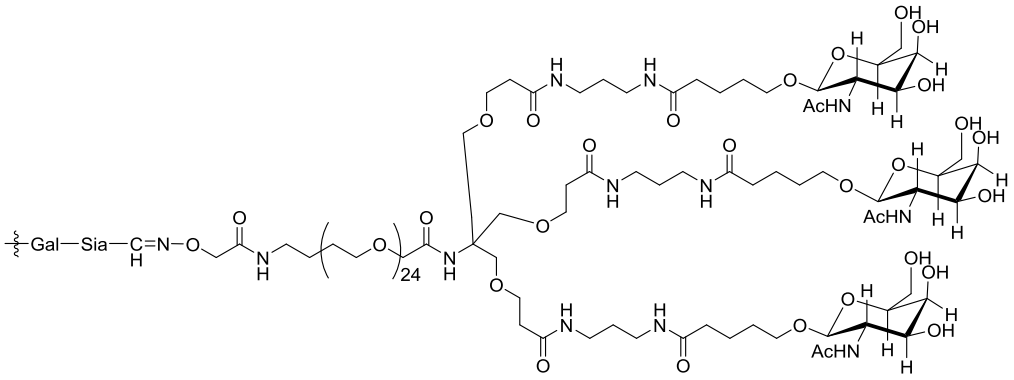


[Formula X]; or



[Formula XII];

d) the glycan comprises at least one moiety selected from the following structural formulae:



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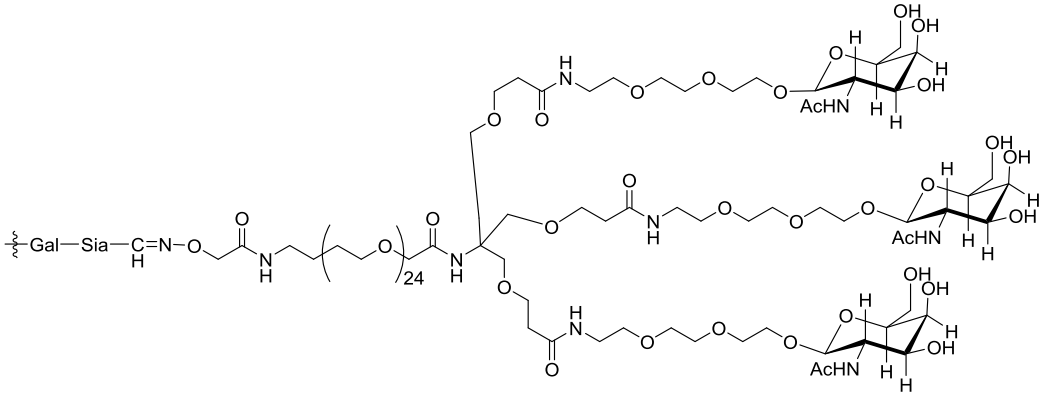

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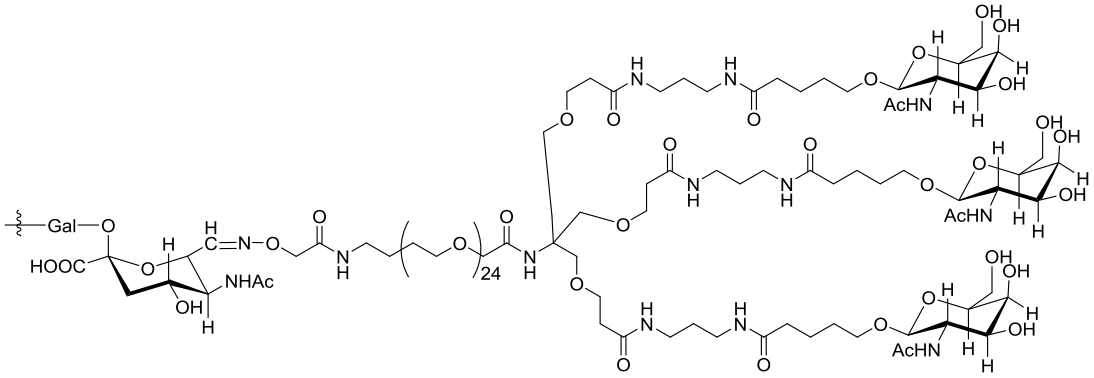
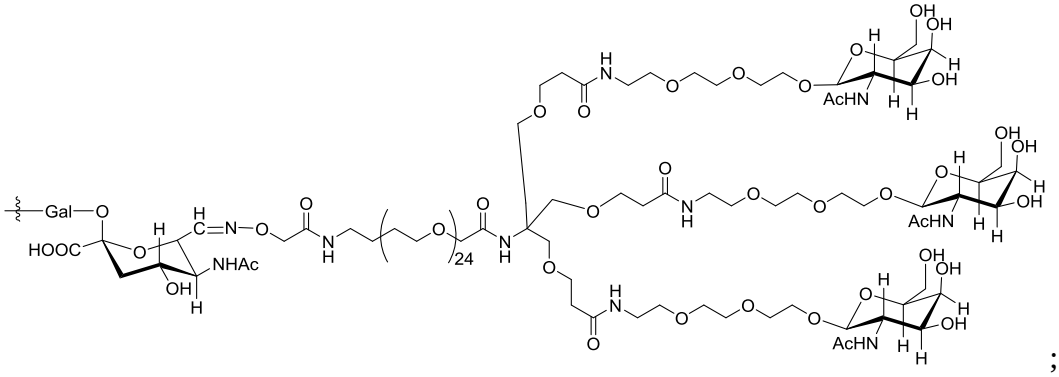


; or



;

e) the PEG comprises mono-PEG, bi-PEG, or tri-PEG, wherein

the PEG optionally comprises 3 to 3.5 PEG; and

f) the binding polypeptide is an antibody or immunoadhesin.

18. A method of making a PEGylated binding polypeptide, wherein the method comprises:

(a) reacting a binding polypeptide comprising at least one glycan with a mildly oxidizing agent in the presence of a salt comprising a metal ion to generate an oxidized binding polypeptide comprising at least one oxidized glycan, and

(b) conjugating the oxidized binding polypeptide with at least one moiety comprising PEG, thereby generating the PEGylated binding polypeptide.

19. The method of claim 18, wherein one or more of the following conditions are met:

a) the metal ion is a copper ion;

b) the salt is copper acetate;

c) the salt comprising a metal ion is present at a concentration of at least 0.1 mM;

d) the mildly oxidizing agent is periodate oxidase or galactose oxidase; and

e) the at least one glycan is a modified glycan.