



- (51) **International Patent Classification:**  
*A61K 47/48* (2006.01) *A61P 35/00* (2006.01)
- (21) **International Application Number:**  
PCT/US2012/031253
- (22) **International Filing Date:**  
29 March 2012 (29.03.2012)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**  
61/468,981 29 March 2011 (29.03.2011) US  
61/468,997 29 March 2011 (29.03.2011) US
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- (81) **Designated States (unless otherwise indicated, for every kind of national protection available):** AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
- (84) **Designated States (unless otherwise indicated, for every kind of regional protection available):** ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

- without international search report and to be republished upon receipt of that report (Rule 48.2(g))



WO 2012/135522 A2

(54) **Title:** PROCESS FOR MANUFACTURING CONJUGATES OF IMPROVED HOMOGENEITY

(57) **Abstract:** The invention provides processes for manufacturing cell-binding agent-cytotoxic agent conjugates of improved homogeneity comprising performing the modification reaction at a lower temperature. The inventive processes comprise contacting a cell-binding agent with a bifunctional crosslinking reagent at a temperature of about 15° C or less to covalently attach a linker to the cell-binding agent and thereby prepare a mixture comprising cell-binding agents having linkers bound thereto.

## PROCESS FOR MANUFACTURING CONJUGATES OF IMPROVED HOMOGENEITY

## CROSS-REFERENCE TO RELATED APPLICATIONS

**[0001]** This patent application claims the benefit of U.S. Provisional Patent Application No. 61/468,997, filed March 29, 2011, and U.S. Provisional Patent Application No. 61/468,981, filed March 29, 2011, which are incorporated by reference in their entireties herein.

## BACKGROUND OF THE INVENTION

**[0002]** Antibody-Drug-Conjugates (ADC's) which are useful for the treatment of cancer and other diseases are commonly composed of three distinct elements: a cell-binding agent; a linker; and a cytotoxic agent. Commonly used manufacturing processes comprise a modification step, in which the cell-binding agent is reacted with a bifunctional linker at room temperature (about 20° C) or above to form a cell-binding agent covalently attached to a linker having a reactive group, and a conjugation step, in which the modified cell-binding agent is reacted with a cytotoxic agent to form a covalent chemical bond from the linker (using the reactive group) to the cytotoxic agent.

**[0003]** Optimizing the modification step (reaction of the cell-binding agent with the linker) requires maximizing the reaction of the linker with the cell-binding agent and minimizing side reactions of the reactive group on the linker, with, for example, water and reactive groups on the cell-binding agent. These side reactions are especially problematic where the reactive group on the linker is a very reactive functional group, such as a maleimide. The side reactions can lead to undesirable reaction products, such as cell-binding agents crosslinked to themselves, as well as cell-binding agents having linkers that are unable to react with the cytotoxic agent.

**[0004]** In view of the foregoing, there is a need in the art to develop an improved process for preparing cell-binding agents having a linker bound thereto that results in a high yield of the desired species of cell-binding agents having a linker bound thereto and that is compatible with large scale manufacturing processes. The invention provides such a process. These and other advantages of the invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

## BRIEF SUMMARY OF THE INVENTION

**[0005]** The invention provides a process for preparing a cell-binding agent having a linker bound thereto, which process comprises contacting a cell-binding agent with a bifunctional crosslinking reagent at a temperature of about 15° C or less to covalently attach a linker to the cell-binding agent and thereby prepare a mixture comprising the cell-binding agents having linkers bound thereto.

**[0006]** In one embodiment, the invention provides a process for preparing a conjugate comprising a cell-binding agent chemically coupled to a cytotoxic agent, which process comprises (a) contacting a cell-binding agent with a bifunctional crosslinking reagent at a temperature of about 15° C or less to covalently attach a linker to the cell-binding agent and thereby prepare a first mixture comprising the cell-binding agents having linkers bound thereto, (b) subjecting the first mixture to tangential flow filtration, selective precipitation, non-adsorptive chromatography, adsorptive filtration, adsorptive chromatography, or a combination thereof and thereby prepare a purified first mixture of cell-binding agents having linkers bound thereto, (c) conjugating a cytotoxic agent to the cell-binding agents having linkers bound thereto in the purified first mixture by reacting the cell-binding agents having linkers bound thereto with a cytotoxic agent in a solution having a pH of about 4 to about 9 to prepare a second mixture comprising (i) cell-binding agent chemically coupled through the linker to the cytotoxic agent, (ii) free cytotoxic agent, and (iii) reaction by-products, and (d) subjecting the second mixture to tangential flow filtration, selective precipitation, non-adsorptive chromatography, adsorptive filtration, adsorptive chromatography, or a combination thereof to purify the cell-binding agents chemically coupled through the linkers to the cytotoxic agent from the other components of the second mixture and thereby prepare a purified second mixture of cell-binding agents chemically coupled through the linkers to the cytotoxic agent.

**[0007]** Another embodiment of the invention provides a process for preparing a conjugate comprising a cell-binding agent chemically coupled to a cytotoxic agent, which process comprises (a) contacting a cell-binding agent with a bifunctional crosslinking reagent at a temperature of about 15° C or less to covalently attach a linker to the cell-binding agent and thereby prepare a first mixture comprising cell-binding agents having linkers bound thereto, (b) conjugating a cytotoxic agent to the cell-binding agents having linkers bound thereto in the first mixture by reacting the cell-binding agents having linkers bound thereto with a cytotoxic agent in a solution having a pH of about 4 to about 9 to prepare a second mixture

comprising (i) cell-binding agent chemically coupled through the linker to the cytotoxic agent, (ii) free cytotoxic agent, and (iii) reaction by-products, and (c) subjecting the second mixture to tangential flow filtration, selective precipitation, non-adsorptive chromatography, adsorptive filtration, adsorptive chromatography, or a combination thereof, to purify the cell binding agents chemically coupled through the linkers to the cytotoxic agent from the other components of the second mixture and thereby prepare a purified second mixture of cell binding agents chemically coupled through the linkers to the cytotoxic agent.

**[0008]** The present invention also includes a conjugate comprising a cell-binding agent chemically coupled to a cytotoxic agent prepared according to the processes described herein.

#### DESCRIPTION OF THE INVENTION

**[0009]** One of ordinary skill in the art will appreciate that conjugates comprising a cell-binding agent, such as an antibody, chemically coupled to a cytotoxic agent (“antibody-cytotoxic agent conjugates”) typically are prepared by modifying an antibody with a bifunctional crosslinking reagent at room temperature (i.e., about 20° C or above), purifying the antibody having linkers bound thereto, conjugating a cytotoxic agent to the antibody having linkers bound thereto, and purifying the antibody- cytotoxic agent conjugate. The invention improves upon such methods by optimizing the modification step in order to maximize reaction of the linker with the cell-binding agent and minimize undesirable side reactions. In particular, it was surprisingly discovered that performing the modification reaction (reaction of the cell-binding agent with the linker) at a lower temperature (e.g., about 15° C or less), extends the interval during which the level of desirable species of cell-binding agents having a linker bound thereto is maximized and before significant levels of undesirable reaction products are formed, thereby making the process suitable for large scale manufacturing. Accordingly, the invention provides processes for manufacturing cell-binding agent-cytotoxic agent conjugates of improved homogeneity comprising performing the modification reaction at a lower temperature.

**[0010]** The invention provides a process for preparing a cell-binding agent having a linker bound thereto, which process comprises contacting a cell-binding agent with a bifunctional crosslinking reagent at a temperature of about 15° C or less to covalently attach a linker to the cell-binding agent and thereby prepare a mixture comprising cell-binding agents having linkers bound thereto. For example, the inventive process comprises contacting a cell-binding agent with a bifunctional crosslinking reagent at a temperature of about 15° C,

about 14° C, about 13° C, about 12° C, about 11° C, about 10° C, about 9° C, about 8° C, about 7° C, about 6° C, about 5° C, about 4° C, about 3° C, about 2° C, about 1° C, or about 0° C, about -1° C, about -2° C, about -3° C, about -4° C, about -5° C, about -6° C, about -7° C, about -8° C, about -9° C, or about -10° C, provided that the solution is prevented from freezing, e.g., by the presence of organic solvent(s) used to dissolve the bifunctional crosslinking reagent. In one embodiment, the inventive process comprises contacting a cell-binding agent with a bifunctional crosslinking reagent at a temperature of about -10° C to about 15° C, about 0° C to about 15° C, about 0° C to about 10° C, about 0° C to about 5° C, about 5° C to about 15° C, about 10° C to about 15° C, or about 5° C to about 10° C. In another embodiment, the inventive process comprises contacting a cell-binding agent with a bifunctional crosslinking reagent at a temperature of about 10° C (e.g., a temperature of 8° C to 12° C or a temperature of 9° C to 11° C).

**[0011]** In one embodiment, the inventive process comprises contacting a cell-binding agent with a bifunctional crosslinking reagent in a solution having a pH of about 7.5 or greater. For example, the inventive process comprises contacting a cell-binding agent with a bifunctional crosslinking reagent in a solution having a pH of about 7.5, about 7.6, about 7.7, about 7.8, about 7.9, about 8.0, about 8.1, about 8.2, about 8.3, about 8.4, about 8.5, about 8.6, about 8.7, about 8.8, about 8.9, or about 9.0. In one embodiment, the inventive process comprises contacting a cell-binding agent with a bifunctional crosslinking reagent in a solution having a pH of about 7.5 to about 9.0, about 7.5 to about 8.5, about 7.5 to about 8.0, about 8.0 to about 9.0, or about 8.5 to about 9.0. In another embodiment, the inventive process comprises contacting a cell-binding agent with a bifunctional crosslinking reagent in a solution having a pH of about 7.8 (e.g., a pH of 7.6 to 8.0 or a pH of 7.7 to 7.9). Any suitable buffering agent can be used. Suitable buffering agents include, for example, a citrate buffer, an acetate buffer, a succinate buffer, and a phosphate buffer. In a preferred embodiment, the buffering agent is selected from the group consisting of HEPPSO (N-(2-Hydroxyethyl)piperazine-N'-(2-hydroxypropanesulfonic acid)), POPSO (Piperazine-1,4-bis-(2-hydroxy-propane-sulfonic acid) dehydrate), HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), HEPPS (EPPS) (4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid), TES (N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid), and a combination thereof.

**[0012]** In one embodiment, the inventive process comprises contacting a cell-binding agent with a bifunctional crosslinking reagent in a solution having a high pH (e.g., about 7.5

or greater) at a low temperature (e.g., about 15° C or less). In a preferred embodiment, the inventive process comprises contacting a cell-binding agent with a bifunctional crosslinking reagent in a solution having a pH about 7.8 at a temperature of about 10° C. In another preferred embodiment, the inventive process comprises contacting a cell-binding agent with a bifunctional crosslinking reagent in a solution having a pH about 8.5 at a temperature of about 0° C.

**[0013]** In accordance with the inventive method, contacting a cell-binding agent with a bifunctional crosslinking reagent produces a first mixture comprising the cell-binding agent having linkers bound thereto, as well as reactants and other by-products. In some embodiments of the invention, the first mixture comprises the cell-binding agent having linkers stably and unstably bound thereto, as well as reactants and other by-products. A linker is “stably” bound to the cell-binding agent when the covalent bond between the linker and the cell-binding agent is not substantially weakened or severed under normal storage conditions over a period of time, which could range from a few months to a few years. In contrast, a linker is “unstably” bound to the cell-binding agent when the covalent bond between the linker and the cell-binding agent is substantially weakened or severed under normal storage conditions over a period of time, which could range from a few months to a few years.

**[0014]** In one embodiment of the invention, purification of the modified cell-binding agent from reactants and by-products is carried out by subjecting the first mixture to a purification process. In this regard, the first mixture can be purified using tangential flow filtration (TFF), e.g., a membrane-based tangential flow filtration process, non-adsorptive chromatography, adsorptive chromatography, adsorptive filtration, or selective precipitation, or any other suitable purification process, as well as combinations thereof. This first purification step provides a purified first mixture, i.e., an increased concentration of the cell-binding agents having linkers bound thereto and a decreased amount of unbound bifunctional crosslinking reagent, as compared to the first mixture prior to purification in accordance with the invention. Preferably, the first mixture is purified using tangential flow filtration.

**[0015]** After purification of the first mixture to obtain a purified first mixture of cell-binding agents having linkers bound thereto, a cytotoxic agent is conjugated to the cell-binding agents having linkers bound thereto in the first purified mixture by reacting the cell-binding agents having linkers bound thereto with a cytotoxic agent in a solution having a pH from about 4 to about 9, wherein a second mixture comprising (i) the cell-binding agent

chemically coupled through the linker to the cytotoxic agent, (ii) free cytotoxic agent, and (iii) reaction by-products is produced.

**[0016]** Optionally, purification of the modified cell-binding agent may be omitted. Thus, in one embodiment of the invention, the first mixture comprising the cell-binding agent having linkers bound thereto, as well as reactants and other by-products, is not subjected to a purification process. In such a situation, the cytotoxic agent may be added simultaneously with the crosslinking reagent or at some later point, e.g., 1, 2, 3, or more hours after addition of the crosslinking reagent to the cell-binding agent. The modified cell-binding agent is conjugated to a cytotoxic agent (e.g., a maytansinoid) by reacting the modified cell-binding agent with the cytotoxic agent in a solution having a pH from about 4 to about 9, wherein the conjugation step results in formation of a mixture of stable cell-binding agent-cytotoxic agent conjugates, non-stable cell-binding agent-cytotoxic agent conjugates, non-conjugated cytotoxic agent (i.e., "free" cytotoxic agent), reactants, and by-products.

**[0017]** The conjugation reaction preferably is performed at a pH of about 4 to about pH 9 (e.g., a pH of about 4.5 to about 8.5, about 5 to about 8, about 5.5 to about 7.5, or about 6.0 to about 7). In some embodiments, the conjugation reaction is performed at a pH of about 6 to about 6.5 (e.g., a pH of 5.5 to 7, a pH of 5.7 to 6.8, a pH of 5.8 to 6.7, a pH of 5.9 to 6.6, or a pH of 6 to 6.5), a pH of about 6 or below (e.g., a pH of about 4 to 6, about 4 to about 5.5, about 5 to 6) or at a pH of about 6.5 or greater (e.g., a pH of 6.5 to about 9, about 7 to about 9, about 7.5 to about 9, or 6.5 to about 8). In one embodiment, the conjugation reaction is performed at a pH of about 4 to a pH less than 6 or at a pH of greater than 6.5 to 9. When the conjugation step is performed at a pH of about 6.5 or greater, some sulfhydryl-containing cytotoxic agents may be prone to dimerize by disulfide-bond formation. In one embodiment, removal of trace metals and/or oxygen from the reaction mixture, as well as optional addition of antioxidants or the use of linkers with more reactive leaving groups, or addition of cytotoxic agent in more than one aliquot, may be required to allow for efficient reaction in such a situation.

**[0018]** The inventive process may optionally include the addition of sucrose to the conjugation step used in the inventive process to increase solubility and recovery of the cell-binding agent-cytotoxic agent conjugates. Desirably, sucrose is added at a concentration of about 0.1% (w/v) to about 20% (w/v) (e.g., about 0.1% (w/v), 1% (w/v), 5% (w/v), 10% (w/v), 15% (w/v), or 20% (w/v)). Preferably, sucrose is added at a concentration of about 1% (w/v) to about 10% (w/v) (e.g., about 0.5% (w/v), about 1% (w/v), about 1.5% (w/v), about

2% (w/v), about 3% (w/v), about 4% (w/v), about 5% (w/v), about 6% (w/v), about 7% (w/v), about 8% (w/v), about 9% (w/v), about 10% (w/v), or about 11% (w/v)). In addition, the conjugation reaction also can comprise the addition of a buffering agent. Any suitable buffering agent known in the art can be used. Suitable buffering agents include, for example, a citrate buffer, an acetate buffer, a succinate buffer, and a phosphate buffer. In a preferred embodiment, the buffering agent is selected from the group consisting of HEPPSO (N-(2-Hydroxyethyl)piperazine-N'-(2-hydroxypropanesulfonic acid)), POPSO (Piperazine-1,4-bis-(2-hydroxy-propane-sulfonic acid) dehydrate), HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), HEPPS (EPPS) (4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid), TES (N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid), and a combination thereof.

**[0019]** Following the conjugation step, the conjugate is subjected to a purification step. In this regard, the conjugation mixture can be purified using tangential flow filtration (TFF), e.g., a membrane-based tangential flow filtration process, non-adsorptive chromatography, adsorptive chromatography, adsorptive filtration, or selective precipitation, or any other suitable purification process, as well as combinations thereof. One of ordinary skill in the art will appreciate that purification after the conjugation step enables the isolation of a stable conjugate comprising the cell-binding agent chemically coupled to the cytotoxic agent.

**[0020]** In one embodiment, the invention provides a process for preparing a conjugate comprising a cell-binding agent chemically coupled to a cytotoxic agent, which process comprises a first purification step after the modification step and a second purification step after the conjugation step. For example, the invention provides a process for preparing a conjugate comprising a cell-binding agent chemically coupled to a cytotoxic agent, which process comprises (a) contacting a cell-binding agent with a bifunctional crosslinking reagent at a temperature of about 15° C or less to covalently attach a linker to the cell-binding agent and thereby prepare a first mixture comprising cell-binding agents having linkers bound thereto, (b) subjecting the first mixture to tangential flow filtration, selective precipitation, non-adsorptive chromatography, adsorptive filtration, adsorptive chromatography, or a combination thereof and thereby prepare a purified first mixture of cell-binding agents having linkers bound thereto, (c) conjugating a cytotoxic agent to the cell-binding agents having linkers bound thereto in the purified first mixture by reacting the cell-binding agents having linkers bound thereto with a cytotoxic agent in a solution having a pH of about 4 to about 9 to prepare a second mixture comprising (i) cell-binding agent chemically coupled through the



linker to the cytotoxic agent, (ii) free cytotoxic agent, and (iii) reaction by-products, and (d) subjecting the second mixture to tangential flow filtration, selective precipitation, non-adsorptive chromatography, adsorptive filtration, adsorptive chromatography, or a combination thereof to purify the cell-binding agents chemically coupled through the linkers to the cytotoxic agent from the other components of the second mixture and thereby prepare a purified second mixture of cell-binding agents chemically coupled through the linkers to the cytotoxic agent.

**[0021]** In one embodiment of the invention, tangential flow filtration (TFF, also known as cross flow filtration, ultrafiltration and diafiltration) and/or adsorptive chromatography resins are utilized in the purification steps. For example, the inventive process can comprise a first purification step using TFF after the modification step and a second purification step using TFF after the conjugation step. Alternatively, the inventive process can comprise a first purification step using adsorptive chromatography after the modification step and a second purification step using adsorptive chromatography after the conjugation step. The inventive process also can comprise a first purification step using adsorptive chromatography after the modification step and a second purification step using TFF after the conjugation step or a first purification step using TFF after the modification step and a second purification step using adsorptive chromatography after the conjugation step.

**[0022]** In one embodiment of the invention, non-adsorptive chromatography is utilized as the purification step. For example, the inventive process can comprise a first purification step using non-adsorptive chromatography after the modification step and a second purification step using non-adsorptive chromatography after the conjugation step.

**[0023]** In another embodiment, the invention provides a process for preparing a conjugate comprising a cell-binding agent chemically coupled to a cytotoxic agent, which process comprises a single purification step after the conjugation step. For example, the inventive process can comprise a process for preparing a conjugate wherein the mixture is not subjected to purification following the modification step. In this respect, the invention provides a process for preparing a conjugate comprising a cell-binding agent chemically coupled to a cytotoxic agent, which process comprises (a) contacting a cell-binding agent with a bifunctional crosslinking reagent at a temperature of about 15° C or less to covalently attach a linker to the cell-binding agent and thereby prepare a first mixture comprising cell-binding agents having linkers bound thereto, (b) conjugating a cytotoxic agent to the cell-binding agents having linkers bound thereto in the first mixture by reacting the cell-binding agents

having linkers bound thereto with a cytotoxic agent in a solution having a pH of about 4 to about 9 to prepare a second mixture comprising (i) cell-binding agent chemically coupled through the linker to the cytotoxic agent, (ii) free cytotoxic agent, and (iii) reaction by-products, and (c) subjecting the second mixture to tangential flow filtration, selective precipitation, non-adsorptive chromatography, adsorptive filtration, adsorptive chromatography, or a combination thereof, to purify the cell binding agents chemically coupled through the linkers to the cytotoxic agent from the other components of the second mixture and thereby prepare a purified second mixture of cell binding agents chemically coupled through the linkers to the cytotoxic agent.

**[0024]** In one embodiment of the invention, the inventive process comprises two separate purification steps following the conjugation step.

**[0025]** Any suitable TFF systems may be utilized for purification, including a Pellicon type system (Millipore, Billerica, MA), a Sartocoon Cassette system (Sartorius AG, Edgewood, NY), and a Centrasette type system (Pall Corp., East Hills, NY).

**[0026]** Any suitable adsorptive chromatography resin may be utilized for purification. Preferred adsorptive chromatography resins include hydroxyapatite chromatography, hydrophobic charge induction chromatography (HCIC), hydrophobic interaction chromatography (HIC), ion exchange chromatography, mixed mode ion exchange chromatography, immobilized metal affinity chromatography (IMAC), dye ligand chromatography, affinity chromatography, reversed phase chromatography, and combinations thereof. Examples of suitable hydroxyapatite resins include ceramic hydroxyapatite (CHT Type I and Type II, Bio-Rad Laboratories, Hercules, CA), HA Ultrogel hydroxyapatite (Pall Corp., East Hills, NY), and ceramic fluoroapatite (CFT Type I and Type II, Bio-Rad Laboratories, Hercules, CA). An example of a suitable HCIC resin is MEP Hypercel resin (Pall Corp., East Hills, NY). Examples of suitable HIC resins include Butyl-Sepharose, Hexyl-Sepharose, Phenyl-Sepharose, and Octyl Sepharose resins (all from GE Healthcare, Piscataway, NJ), as well as Macro-prep Methyl and Macro-Prep t-Butyl resins (Biorad Laboratories, Hercules, CA). Examples of suitable ion exchange resins include SP-Sepharose, CM-Sepharose, and Q-Sepharose resins (all from GE Healthcare, Piscataway, NJ), and Unosphere S resin (Bio-Rad Laboratories, Hercules, CA). Examples of suitable mixed mode ion exchangers include Bakerbond ABx resin (JT Baker, Phillipsburg NJ). Examples of suitable IMAC resins include Chelating Sepharose resin (GE Healthcare, Piscataway, NJ) and Profinity IMAC resin (Bio-Rad Laboratories, Hercules, CA). Examples

of suitable dye ligand resins include Blue Sepharose resin (GE Healthcare, Piscataway, NJ) and Affi-gel Blue resin (Bio-Rad Laboratories, Hercules, CA). Examples of suitable affinity resins include Protein A Sepharose resin (e.g., MabSelect, GE Healthcare, Piscataway, NJ), where the cell-binding agent is an antibody, and lectin affinity resins, e.g. Lentil Lectin Sepharose resin (GE Healthcare, Piscataway, NJ), where the cell-binding agent bears appropriate lectin binding sites. Alternatively an antibody specific to the cell-binding agent may be used. Such an antibody can be immobilized to, for instance, Sepharose 4 Fast Flow resin (GE Healthcare, Piscataway, NJ). Examples of suitable reversed phase resins include C4, C8, and C18 resins (Grace Vydac, Hesperia, CA).

**[0027]** Any suitable non-adsorptive chromatography resin may be utilized for purification. Examples of suitable non-adsorptive chromatography resins include, but are not limited to, SEPHADEX™ G-25, G-50, G-100, SEPHACRYL™ resins (e.g., S-200 and S-300), SUPERDEX™ resins (e.g., SUPERDEX™ 75 and SUPERDEX™ 200), BIO-GEL® resins (e.g., P-6, P-10, P-30, P-60, and P-100), and others known to those of ordinary skill in the art.

**[0028]** In one embodiment, the inventive process further comprises a holding step to release the unstably bound linkers from the cell-binding agent. The holding step comprises holding the mixture after modification of the cell-binding agent with a bifunctional crosslinking reagent, after conjugation of a cytotoxic agent to the cell-binding agents having linkers bound thereto, and/or after a purification step.

**[0029]** The holding step comprises maintaining the solution at a suitable temperature (e.g., about 2° C to about 37° C) for a suitable period of time (e.g., about 1 hour to about 1 week) to release the unstably bound linkers from the cell-binding agent while not substantially releasing the stably bound linkers from the cell-binding agent. In one embodiment, the holding step comprises maintaining the solution at a low temperature (e.g., about 2° C to about 10° C or about 4° C), at room temperature (e.g., about 20° C to about 30° C or about 20° C to about 25° C), or at an elevated temperature (e.g., about 30° C to about 37° C).

**[0030]** The duration of the holding step depends on the temperature at which the holding step is performed. For example, the duration of the holding step can be substantially reduced by performing the holding step at elevated temperature, with the maximum temperature limited by the stability of the cell-binding agent-cytotoxic agent conjugate. The holding step can comprise maintaining the solution for about 1 hour to about 1 day (e.g., about 1 hour,

about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 6 hours, about 7 hours, about 8 hours, about 9 hours, about 10 hours, about 12 hours, about 14 hours, about 16 hours, about 18 hours, about 20 hours, about 22 hours, or about 24 hours), about 5 hours to about 1 week, about 12 hours to about 1 week (e.g., about 12 hours, about 16 hours, about 20 hours, about 24 hours, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, or about 7 days), for about 12 hours to about 1 week (e.g., about 12 hours, about 16 hours, about 20 hours, about 24 hours, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, or about 7 days), or about 1 day to about 1 week.

**[0031]** In one embodiment, the holding step comprises maintaining the solution at a temperature of about 2 °C to about 8 °C for a period of at least about 12 hours for up to 1 day.

**[0032]** The pH value for the holding step preferably is about 4 to about 10. In one embodiment, the pH value for the holding step is about 4 or more, but less than about 6 (e.g., 4 to 5.9) or about 5 or more, but less than about 6 (e.g., 5 to 5.9). In another embodiment, the pH values for the holding step range from about 6 to about 10 (e.g., about 6.5 to about 9, about 6 to about 8). For example, pH values for the holding step can be about 6, about 6.5, about 7, about 7.5, about 8, about 8.5, about 9, about 9.5, or about 10.

**[0033]** The holding step can be performed before or after the cell-binding agent is conjugated to the cytotoxic agent. In one embodiment, the holding step is performed directly after the modification of the cell-binding agent with the bifunctional crosslinking reagent. For example, the inventive process comprises a holding step after modification of the cell-binding agent with a bifunctional crosslinking reagent and before conjugation. After modification of the cell-binding agent, a purification step may be performed before the hold step and/or after the hold step, but prior to the conjugation step. In another embodiment, the holding step is performed directly after conjugation of the cytotoxic agent to the cell-binding agent having linkers bound thereto and prior to purification step. In another embodiment, the holding step is performed after the conjugation and purification steps and followed by an additional purification step.

**[0034]** In specific embodiments, the holding step can comprise incubating the mixture at 4° C at a pH of about 6-7.5 for about 12 hours to about 1 week, incubating the mixture at 25° C at a pH of about 6-7.5 for about 12 hours to about 1 week, incubating the mixture at 4° C at a pH of about 4.5-5.9 for about 5 hours to about 5 days, or incubating the mixture at 25° C at a pH of about 4.5-5.9 for about 5 hours to about 1 day.

**[0035]** The invention provides a process for preparing compositions of stable conjugates comprising a cell-binding agent chemically coupled to a cytotoxic agent, wherein the compositions are substantially free of unstable conjugates. In this respect, the invention provides a process for preparing cell-binding agent-cytotoxic agent conjugate of substantially high purity and stability. Such compositions can be used for treating diseases because of the high purity and stability of the conjugates. Compositions comprising a cell-binding agent, such as an antibody, chemically coupled to a cytotoxic agent, such as a maytansinoid, are described in, for example, U.S. Patent 7,374,762. In one aspect of the invention, a cell-binding agent-cytotoxic agent conjugate of substantially high purity has one or more of the following features: (a) greater than about 90% (e.g., greater than or equal to about 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%), preferably greater than about 95%, of conjugate species are monomeric, (b) unconjugated linker level in the conjugate preparation is less than about 10% (e.g., less than or equal to about 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or 0%) (relative to total linker), (c) less than 10% of conjugate species are crosslinked (e.g., less than or equal to about 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or 0%), (d) free cytotoxic agent level in the conjugate preparation is less than about 2% (e.g., less than or equal to about 1.5%, 1.4%, 1.3%, 1.2%, 1.1%, 1.0%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, or 0%) (relative to total cytotoxic agent), and/or (e) no substantial increase in free cytotoxic agent level upon storage (e.g., after about 1 week, about 2 weeks, about 3 weeks, about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, about 1 year, about 2 years, or about 5 years). “Substantial increase” in free cytotoxic agent level means that after certain storage time, the increase in the level of free cytotoxic agent is less than about 0.1%, about 0.2%, about 0.3%, about 0.4%, about 0.5%, about 0.6%, about 0.7%, about 0.8%, about 0.9%, about 1.0%, about 1.1%, about 1.2%, about 1.3%, about 1.4%, about 1.5%, about 1.6%, about 1.7%, about 1.8%, about 1.9%, about 2.0%, about 2.2%, about 2.5%, about 2.7%, about 3.0%, about 3.2%, about 3.5%, about 3.7%, or about 4.0%.

**[0036]** As used herein, the term “unconjugated linker” refers to the cell-binding agent that is covalently linked with the bifunctional crosslinking reagent, wherein the cell-binding agent is not covalently coupled to the cytotoxic agent through the linker of the bifunctional crosslinking reagent (i.e., the “unconjugated linker” can be represented by CBA-L, wherein CBA represents the cell-binding agent and L represents the bifunctional crosslinking reagent).

In contrast, the cell-binding agent cytotoxic agent conjugate can be represented by CBA-L-D, wherein D represents the cytotoxic agent).

**[0037]** In one embodiment, the average molar ratio of the cytotoxic agent to the cell-binding agent in the cell-binding agent cytotoxic agent conjugate is about 1 to about 10, about 2 to about 7, about 3 to about 5, about 2.5 to about 4.5 (e.g., about 2.5, about 2.6, about 2.7, about 2.8, about 2.9, about 3.0, about 3.1, about 3.3, about 3.4, about 3.5, about 3.6, about 3.7, about 3.8, about 3.9, about 4.0, about 4.1, about 4.2, about 4.3, about 4.4, about 4.5), about 3.0 to about 4.0, about 3.2 to about 4.2, about 4.5 to 5.5 (e.g., about 4.5, about 4.6, about 4.7, about 4.8, about 4.9, about 5.0, about 5.1, about 5.2, about 5.3, about 5.4, about 5.5).

**[0038]** The cell-binding agent can be any suitable agent that binds to a cell, typically and preferably an animal cell (e.g., a human cell). The cell-binding agent preferably is a peptide or a polypeptide or a glycopeptide. Suitable cell-binding agents include, for example, antibodies (e.g., monoclonal antibodies and fragments thereof), interferons (e.g., .alpha., .beta., .gamma.), lymphokines (e.g., IL-2, IL-3, IL-4, IL-6), hormones (e.g., insulin, TRH (thyrotropin releasing hormone), MSH (melanocyte-stimulating hormone), steroid hormones, such as androgens and estrogens), growth factors and colony-stimulating factors such as EGF, TGF-alpha, FGF, VEGF, G-CSF, M-CSF and GM-CSF (Burgess, *Immunology Today* 5:155-158 (1984)), nutrient-transport molecules (e.g., transferrin), vitamins (e.g., folate) and any other agent or molecule that specifically binds a target molecule on the surface of a cell.

**[0039]** Where the cell-binding agent is an antibody, it binds to an antigen that is a polypeptide and may be a transmembrane molecule (e.g., receptor) or a ligand, such as a growth factor. Exemplary antigens include molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor vmc, factor IX, tissue factor (TF), and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin, such as human serum albumin; Muellerian-inhibiting substance; relaxin A-

chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT4, NT-5, or NT-6), or a nerve growth factor such as NGF- $\beta$ ; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3, TGF- $\beta$ 4, or TGF- $\beta$ 5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins, EpCAM, GD3, FLT3, PSMA, PSCA, MUC1, MUC16, STEAP, CEA, TENB2, EphA receptors, EphB receptors, folate receptor, FOLR1, mesothelin, cripto,  $\alpha_v\beta_6$ , integrins, VEGF, VEGFR, EGFR, transferrin receptor, IRTA1, IRTA2, IRTA3, IRTA4, IRTA5; CD proteins such as CD2, CD3, CD4, CD5, CD6, CD8, CD11, CD14, CD19, CD20, CD21, CD22, CD25, CD26, CD28, CD30, CD33, CD36, CD37, CD38, CD40, CD44, CD52, CD55, CD56, CD59, CD70, CD79, CD80, CD81, CD103, CD105, CD134, CD137, CD138, CD152 or an antibody which binds to one or more tumor-associated antigens or cell-surface receptors disclosed in U.S. Patent Application Publication No. 2008/0171040 or U.S. Patent Application Publication No. 2008/0305044 and are incorporated in their entirety by reference; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon, such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the HIV envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins, such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; endoglin, c-Met, IGF1R, prostate antigens such as PCA3, PSA, PSGR, NGEP, PSMA, PSCA, TMEFF2, and STEAP1; LGR5, B7H4, and fragments of any of the above-listed polypeptides.

**[0040]** Additionally, GM-CSF, which binds to myeloid cells can be used as a cell-binding agent to diseased cells from acute myelogenous leukemia. IL-2 which binds to activated T-cells can be used for prevention of transplant graft rejection, for therapy and prevention of graft-versus-host disease, and for treatment of acute T-cell leukemia. MSH, which binds to melanocytes, can be used for the treatment of melanoma, as can antibodies directed towards

melanomas. Folic acid can be used to target the folate receptor expressed on ovarian and other tumors. Epidermal growth factor can be used to target squamous cancers such as lung and head and neck. Somatostatin can be used to target neuroblastomas and other tumor types.

**[0041]** Cancers of the breast and testes can be successfully targeted with estrogen (or estrogen analogues) or androgen (or androgen analogues) respectively as cell-binding agents

**[0042]** The term “antibody,” as used herein, refers to any immunoglobulin, any immunoglobulin fragment, such as Fab, Fab’, F(ab’)<sub>2</sub>, dsFv, sFv, minibodies, diabodies, tribodies, tetrabodies (Parham, *J. Immunol.* 131: 2895-2902 (1983); Spring et al. *J. Immunol.* 113: 470-478 (1974); Nisonoff et al. *Arch. Biochem. Biophys.* 89: 230-244 (1960), Kim et al., *Mol. Cancer Ther.*, 7: 2486-2497 (2008), Carter, *Nature Revs.*, 6: 343-357 (2006)), or immunoglobulin chimera, which can bind to an antigen on the surface of a cell (e.g., which contains a complementarity determining region (CDR)). Any suitable antibody can be used as the cell-binding agent. One of ordinary skill in the art will appreciate that the selection of an appropriate antibody will depend upon the cell population to be targeted. In this regard, the type and number of cell surface molecules (i.e., antigens) that are selectively expressed in a particular cell population (typically and preferably a diseased cell population) will govern the selection of an appropriate antibody for use in the inventive composition. Cell surface expression profiles are known for a wide variety of cell types, including tumor cell types, or, if unknown, can be determined using routine molecular biology and histochemistry techniques.

**[0043]** The antibody can be polyclonal or monoclonal, but is most preferably a monoclonal antibody. As used herein, “polyclonal” antibodies refer to heterogeneous populations of antibody molecules, typically contained in the sera of immunized animals. “Monoclonal” antibodies refer to homogenous populations of antibody molecules that are specific to a particular antigen. Monoclonal antibodies are typically produced by a single clone of B lymphocytes (“B cells”). Monoclonal antibodies may be obtained using a variety of techniques known to those skilled in the art, including standard hybridoma technology (see, e.g., Köhler and Milstein, *Eur. J. Immunol.*, 5: 511-519 (1976), Harlow and Lane (eds.), *Antibodies: A Laboratory Manual*, CSH Press (1988), and C.A. Janeway et al. (eds.), *Immunobiology*, 5<sup>th</sup> Ed., Garland Publishing, New York, NY (2001)). In brief, the hybridoma method of producing monoclonal antibodies typically involves injecting any suitable animal, typically and preferably a mouse, with an antigen (i.e., an “immunogen”). The animal is



subsequently sacrificed, and B cells isolated from its spleen are fused with human myeloma cells. A hybrid cell is produced (i.e., a “hybridoma”), which proliferates indefinitely and continuously secretes high titers of an antibody with the desired specificity *in vitro*. Any appropriate method known in the art can be used to identify hybridoma cells that produce an antibody with the desired specificity. Such methods include, for example, enzyme-linked immunosorbent assay (ELISA), Western blot analysis, and radioimmunoassay. The population of hybridomas is screened to isolate individual clones, each of which secretes a single antibody species to the antigen. Because each hybridoma is a clone derived from fusion with a single B cell, all the antibody molecules it produces are identical in structure, including their antigen binding site and isotype. Monoclonal antibodies also may be generated using other suitable techniques including EBV-hybridoma technology (see, e.g., Haskard and Archer, *J. Immunol. Methods*, 74(2): 361-67 (1984), and Roder et al., *Methods Enzymol.*, 121: 140-67 (1986)), bacteriophage vector expression systems (see, e.g., Huse et al., *Science*, 246: 1275-81 (1989)), or phage display libraries comprising antibody fragments, such as Fab and scFv (single chain variable region) (see, e.g., U.S. Patents 5,885,793 and 5,969,108, and International Patent Application Publications WO 92/01047 and WO 99/06587).

**[0044]** The monoclonal antibody can be isolated from or produced in any suitable animal, but is preferably produced in a mammal, more preferably a mouse or human, and most preferably a human. Methods for producing an antibody in mice are well known to those skilled in the art and are described herein. With respect to human antibodies, one of ordinary skill in the art will appreciate that polyclonal antibodies can be isolated from the sera of human subjects vaccinated or immunized with an appropriate antigen. Alternatively, human antibodies can be generated by adapting known techniques for producing human antibodies in non-human animals such as mice (see, e.g., U.S. Patents 5,545,806, 5,569,825, and 5,714,352, and U.S. Patent Application Publication No. 2002/0197266 A1).

**[0045]** While being the ideal choice for therapeutic applications in humans, human antibodies, particularly human monoclonal antibodies, typically are more difficult to generate than mouse monoclonal antibodies. Mouse monoclonal antibodies, however, induce a rapid host antibody response when administered to humans, which can reduce the therapeutic or diagnostic potential of the antibody-cytotoxic agent conjugate. To circumvent these complications, a monoclonal antibody preferably is not recognized as “foreign” by the human immune system.

**[0046]** To this end, phage display can be used to generate the antibody. In this regard, phage libraries encoding antigen-binding variable (V) domains of antibodies can be generated using standard molecular biology and recombinant DNA techniques (see, e.g., Sambrook et al. (eds.), *Molecular Cloning, A Laboratory Manual*, 3<sup>rd</sup> Edition, Cold Spring Harbor Laboratory Press, New York (2001)). Phage encoding a variable region with the desired specificity are selected for specific binding to the desired antigen, and a complete human antibody is reconstituted comprising the selected variable domain. Nucleic acid sequences encoding the reconstituted antibody are introduced into a suitable cell line, such as a myeloma cell used for hybridoma production, such that human antibodies having the characteristics of monoclonal antibodies are secreted by the cell (see, e.g., Janeway et al., *supra*, Huse et al., *supra*, and U.S. Patent 6,265,150). Alternatively, monoclonal antibodies can be generated from mice that are transgenic for specific human heavy and light chain immunoglobulin genes. Such methods are known in the art and described in, for example, U.S. Patents 5,545,806 and 5,569,825, and Janeway et al., *supra*.

**[0047]** Most preferably the antibody is a humanized antibody. As used herein, a “humanized” antibody is one in which the complementarity-determining regions (CDR) of a mouse monoclonal antibody, which form the antigen binding loops of the antibody, are grafted onto the framework of a human antibody molecule. Owing to the similarity of the frameworks of mouse and human antibodies, it is generally accepted in the art that this approach produces a monoclonal antibody that is antigenically identical to a human antibody but binds the same antigen as the mouse monoclonal antibody from which the CDR sequences were derived. Methods for generating humanized antibodies are well known in the art and are described in detail in, for example, Janeway et al., *supra*, U.S. Patents 5,225,539, 5,585,089 and 5,693,761, European Patent No. 0239400 B1, and United Kingdom Patent No. 2188638. Humanized antibodies can also be generated using the antibody resurfacing technology described in U.S. Patent 5,639,641 and Pedersen et al., *J. Mol. Biol.*, 235: 959-973 (1994). While the antibody employed in the conjugate of the inventive composition most preferably is a humanized monoclonal antibody, a human monoclonal antibody and a mouse monoclonal antibody, as described above, are also within the scope of the invention.

**[0048]** Antibody fragments that have at least one antigen binding site, and thus recognize and bind to at least one antigen or receptor present on the surface of a target cell, also are within the scope of the invention. In this respect, proteolytic cleavage of an intact antibody molecule can produce a variety of antibody fragments that retain the ability to recognize and

bind antigens. For example, limited digestion of an antibody molecule with the protease papain typically produces three fragments, two of which are identical and are referred to as the Fab fragments, as they retain the antigen binding activity of the parent antibody molecule. Cleavage of an antibody molecule with the enzyme pepsin normally produces two antibody fragments, one of which retains both antigen-binding arms of the antibody molecule, and is thus referred to as the F(ab')<sub>2</sub> fragment. Reduction of a F(ab')<sub>2</sub> fragment with dithiothreitol or mercaptoethylamine produces a fragment referred to as a Fab' fragment. A single-chain variable region fragment (sFv) antibody fragment, which consists of a truncated Fab fragment comprising the variable (V) domain of an antibody heavy chain linked to a V domain of a light antibody chain via a synthetic peptide, can be generated using routine recombinant DNA technology techniques (see, e.g., Janeway et al., *supra*). Similarly, disulfide-stabilized variable region fragments (dsFv) can be prepared by recombinant DNA technology (see, e.g., Reiter et al., *Protein Engineering*, 7: 697-704 (1994)). Antibody fragments in the context of the invention, however, are not limited to these exemplary types of antibody fragments. Any suitable antibody fragment that recognizes and binds to a desired cell surface receptor or antigen can be employed. Antibody fragments are further described in, for example, Parham, *J. Immunol.*, 131: 2895-2902 (1983), Spring et al., *J. Immunol.*, 113: 470-478 (1974), and Nisonoff et al., *Arch. Biochem. Biophys.*, 89: 230-244 (1960). Antibody-antigen binding can be assayed using any suitable method known in the art, such as, for example, radioimmunoassay (RIA), ELISA, Western blot, immunoprecipitation, and competitive inhibition assays (see, e.g., Janeway et al., *supra*, and U.S. Patent Application Publication No. 2002/0197266 A1).

**[0049]** In addition, the antibody can be a chimeric antibody or an antigen binding fragment thereof. By "chimeric" it is meant that the antibody comprises at least two immunoglobulins, or fragments thereof, obtained or derived from at least two different species (e.g., two different immunoglobulins, such as a human immunoglobulin constant region combined with a murine immunoglobulin variable region). The antibody also can be a domain antibody (dAb) or an antigen binding fragment thereof, such as, for example, a camelid antibody (see, e.g., Desmyter et al., *Nature Struct. Biol.*, 3: 752, (1996)), or a shark antibody, such as, for example, a new antigen receptor (IgNAR) (see, e.g., Greenberg et al., *Nature*, 374: 168 (1995), and Stanfield et al., *Science*, 305: 1770-1773 (2004)).

**[0050]** Any suitable antibody can be used in the context of the invention. For example, the monoclonal antibody J5 is a murine IgG2a antibody that is specific for Common Acute

Lymphoblastic Leukemia Antigen (CALLA) (Ritz et al., *Nature*, 283: 583-585 (1980)), and can be used to target cells that express CALLA (e.g., acute lymphoblastic leukemia cells). The monoclonal antibody MY9 is a murine IgG1 antibody that binds specifically to the CD33 antigen (Griffin et al., *Leukemia Res.*, 8: 521 (1984)), and can be used to target cells that express CD33 (e.g., acute myelogenous leukemia (AML) cells).

**[0051]** Similarly, the monoclonal antibody anti-B4 (also referred to as B4) is a murine IgG1 antibody that binds to the CD19 antigen on B cells (Nadler et al., *J. Immunol.*, 131: 244-250 (1983)), and can be used to target B cells or diseased cells that express CD19 (e.g., non-Hodgkin's lymphoma cells and chronic lymphoblastic leukemia cells). N901 is a murine monoclonal antibody that binds to the CD56 (neural cell adhesion molecule) antigen found on cells of neuroendocrine origin, including small cell lung tumor, which can be used in the conjugate to target drugs to cells of neuroendocrine origin. The J5, MY9, and B4 antibodies preferably are resurfaced or humanized prior to their use as part of the conjugate. Resurfacing or humanization of antibodies is described in, for example, Roguska et al., *Proc. Natl. Acad. Sci. USA*, 91: 969-73 (1994).

**[0052]** In addition, the monoclonal antibody C242 binds to the CanAg antigen (see, e.g., U.S. Patent 5,552,293), and can be used to target the conjugate to CanAg expressing tumors, such as colorectal, pancreatic, non-small cell lung, and gastric cancers. HuC242 is a humanized form of the monoclonal antibody C242 (see, e.g., U.S. Patent 5,552,293). The hybridoma from which HuC242 is produced is deposited with ECACC identification Number 90012601. HuC242 can be prepared using CDR-grafting methodology (see, e.g., U.S. Patents 5,585,089, 5,693,761, and 5,693,762) or resurfacing technology (see, e.g., U.S. Patent 5,639,641). HuC242 can be used to target the conjugate to tumor cells expressing the CanAg antigen, such as, for example, colorectal, pancreatic, non-small cell lung, and gastric cancer cells.

**[0053]** To target ovarian cancer and prostate cancer cells, an anti-MUC1 antibody can be used as the cell-binding agent in the conjugate. Anti-MUC1 antibodies include, for example, anti-HMFG-2 (see, e.g., Taylor-Papadimitriou et al., *Int. J. Cancer*, 28: 17-21 (1981)), hCTM01 (see, e.g., van Hof et al., *Cancer Res.*, 56: 5179-5185 (1996)), and DS6. Prostate cancer cells also can be targeted with the conjugate by using an anti-prostate-specific membrane antigen (PSMA) as the cell-binding agent, such as J591 (see, e.g., Liu et al., *Cancer Res.*, 57: 3629-3634 (1997)). Moreover, cancer cells that express the Her2 antigen, such as breast, prostate, and ovarian cancers, can be targeted with the conjugate by using anti-

HER2 antibodies, e.g., trastuzumab, as the cell-binding agent. Cells that express epidermal growth factor receptor (EGFR) and variants thereof, such as the type III deletion mutant, EGFRvIII, can be targeted with the conjugate by using anti-EGFR antibodies. Anti-EGFR antibodies are described in International Patent Application Nos. PCT/US11/058385 and PCT/US11/058378. Anti-EGFRvIII antibodies are described in U.S. Patents 7,736,644 and 7,628,986, and U.S. Patent Application Publications 2010/0111979, 2009/0240038, 2009/0175887, 2009/0156790, and 2009/0155282. Anti-IGF-IR antibodies that bind to insulin-like growth factor receptor, such as those described in U.S. Patent 7,982,024, also can be used in the conjugate. Antibodies that bind to CD27L, Cripto, CD138, CD38, EphA2, integrins, CD37, folate, CD20, PSGR, NGEP, PSCA, TMEFF2, STEAP1, endoglin, and Her3 also can be used in the conjugate.

**[0054]** In one embodiment, the antibody is selected from the group consisting of huN901, huMy9-6, huB4, huC242, an anti-HER2 antibody (e.g., trastuzumab), bivatuzumab, sibrotuzumab, rituximab, huDS6, anti-mesothelin antibodies described in International Patent Application Publication WO 2010/124797 (such as MF-T), anti-cripto antibodies described in U.S. Patent Application Publication 2010/0093980 (such as huB3F6), anti-CD138 antibodies described in U.S. Patent Application Publication 2007/0183971 (such as huB-B4), anti-EGFR antibodies described in International Patent Application Nos. PCT/US11/058385 and PCT/US11/058378 (such as EGFR-7), anti-EGFRvIII antibodies described U.S. Patents 7,736,644 and 7,628,986 and U.S. Patent Application Publications 2010/0111979, 2009/0240038, 2009/0175887, 2009/0156790 and 2009/0155282, humanized EphA2 antibodies described in International Patent Application Publications WO 2011/039721 and WO 2011/039724 (such as 2H11R35R74); anti-CD38 antibodies described in International Patent Application Publication WO 2008/047242 (such as hu38SB19), anti-folate antibodies described in International Patent Application Publication WO 2011/106528, and U.S. Patent Application Publication 2012/0009181 (e.g., huMov19); anti-IGF1R antibodies described in U.S. Patents 5,958,872, 6,596,743, and 7,982,024; anti-CD37 antibodies described in U.S. Patent Application Publication 2011/0256153 (e.g., huCD37-3); anti-integrin  $\alpha_v\beta_6$  antibodies described in U.S. Patent Application Publication 2006/0127407 (e.g., CNTO95); and anti-Her3 antibodies described in International Patent Application Publication WO 2012/019024.

**[0055]** Particularly preferred antibodies are humanized monoclonal antibodies described herein. Examples include, but are not limited to, huN901, huMy9-6, huB4, huC242, a humanized monoclonal anti-Her2 antibody (e.g., trastuzumab), bivatuzumab, sibrotuzumab,

CNTO95, huDS6, and rituximab (see, e.g., U.S. Patents 5,639,641 and 5,665,357, U.S. Provisional Patent Application No. 60/424,332 (which is related to U.S. Patent 7,557,189), International (PCT) Patent Application Publication No. WO 02/16401, Pedersen et al., *supra*, Roguska et al., *supra*, Liu et al., *supra*, Nadler et al., *supra*, Colomer et al., *Cancer Invest.*, 19: 49-56 (2001), Heider et al., *Eur. J. Cancer*, 31A: 2385-2391 (1995), Welt et al., *J. Clin. Oncol.*, 12: 1193-1203 (1994), and Maloney et al., *Blood*, 90: 2188-2195 (1997)). Other humanized monoclonal antibodies are known in the art and can be used in connection with the invention.

**[0056]** In one embodiment, the cell-binding agent is an humanized anti-folate antibody or antigen binding fragment thereof that specifically binds a human folate receptor 1, wherein the antibody comprises: (a) a heavy chain CDR1 comprising GYFMN; a heavy chain CDR2 comprising RIHPYDGDTFYNQXaa<sub>1</sub>FXaa<sub>2</sub>Xaa<sub>3</sub>; and a heavy chain CDR3 comprising YDGSRAMDY; and (b) a light chain CDR1 comprising KASQSVSFAGTSLMH; a light chain CDR2 comprising RASNLEA; and a light chain CDR3 comprising QQSREYPYT; wherein Xaa<sub>1</sub> is selected from K, Q, H, and R; Xaa<sub>2</sub> is selected from Q, H, N, and R; and Xaa<sub>3</sub> is selected from G, E, T, S, A, and V. Preferably, the heavy chain CDR2 sequence comprises RIHPYDGDTFYNQKFQG.

**[0057]** In another embodiment, the anti-folate antibody is a humanized antibody or antigen binding fragment thereof that specifically binds the human folate receptor 1 comprising the heavy chain having the amino acid sequence of  
 QVQLVQSGAEVVKPGASVKISCKASGYTFTGYFMNWVKQSPGQSLEWIGRIHPYDGD  
 DTFYNQKFQGGKATLTVDKSSNTAHMELLSLTSEDFAVYYCTRYDGSRAMDYWGQG  
 TTVTSSASTKGPSVFLPAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH  
 TTPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC  
 PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE  
 VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA  
 KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPP  
 VLDSGDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK.

**[0058]** In another embodiment, the anti-folate antibody is a humanized antibody or antigen binding fragment thereof encoded by the plasmid DNA deposited with the ATCC on April 7, 2010 and having ATCC deposit nos. PTA-10772 and PTA-10773 or 10774.

**[0059]** In another embodiment, the anti-folate antibody is a humanized antibody or antigen binding fragment thereof comprising a heavy chain variable domain at least about

90%, 95%, 99% or 100% identical to

QVQLVQSGAEVVKPGASVKISCKASGYTFTGYFMNWVKQSPGQSLEWIGRIHPYDGD  
DTFYNQKFQGKATLTVDKSSNTAHMELLSLTSEDFAVYYCTRYDGSRAMDYWGQG  
TTVTVSS, and a light chain variable domain at least about 90%, 95%, 99% or 100%  
identical to

DIVLTQSPLSLAVSLGQPAIISCKASQSVSFAGTSLMHWHYHQKPGQQPRLLIYRASNL  
EAGVPDRFSGSGSKTDFTLNISPVEAEDAATYYCQQSREYPYTFGGGTKLEIKR; or  
DIVLTQSPLSLAVSLGQPAIISCKASQSVSFAGTSLMHWHYHQKPGQQPRLLIYRASNL  
EAGVPDRFSGSGSKTDFTLTISPVEAEDAATYYCQQSREYPYTFGGGTKLEIKR.

**[0060]** While the cell-binding agent preferably is an antibody, the cell-binding agent also can be a non-antibody molecule. Suitable non-antibody molecules include, for example, interferons (e.g., alpha, beta, or gamma interferon), lymphokines (e.g., interleukin 2 (IL-2), IL-3, IL-4, or IL-6), hormones (e.g., insulin), growth factors (e.g., EGF, TGF-alpha, FGF, and VEGF), colony-stimulating factors (e.g., G-CSF, M-CSF, and GM-CSF (see, e.g., Burgess, *Immunology Today*, 5: 155-158 (1984)), somatostatin, and transferrin (see, e.g., O'Keefe et al., *J. Biol. Chem.*, 260: 932-937 (1985)). For example, GM-CSF, which binds to myeloid cells, can be used as a cell-binding agent to target acute myelogenous leukemia cells. In addition, IL-2, which binds to activated T-cells, can be used for prevention of transplant graft rejection, for therapy and prevention of graft-versus-host disease, and for treatment of acute T-cell leukemia. Epidermal growth factor (EGF) can be used to target squamous cancers such as lung cancer and head and neck cancer. Somatostatin can be used to target neuroblastoma cells and other tumor cell types.

**[0061]** The conjugate can comprise any suitable cytotoxic agent. A "cytotoxic agent," as used herein, refers to any compound that results in the death of a cell, induces cell death, or decreases cell viability. Suitable cytotoxic agents include, for example, maytansinoids and conjugatable ansamitocins (see, for example, PCT/US11/059131 filed November 3, 2011), taxoids, CC-1065 and CC-1065 analogs, and dolastatin and dolastatin analogs. In a preferred embodiment of the invention, the cytotoxic agent is a maytansinoid, including maytansinol and maytansinol analogs. Maytansinoids are compounds that inhibit microtubule formation and are highly toxic to mammalian cells. Examples of suitable maytansinol analogues include those having a modified aromatic ring and those having modifications at other positions. Such maytansinoids are described in, for example, U.S. Patents 4,256,746,

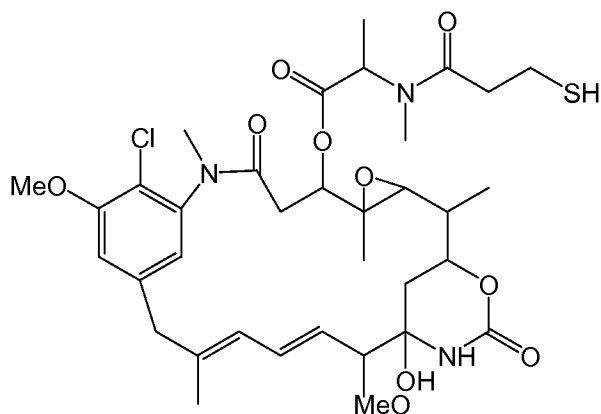
4,294,757, 4,307,016, 4,313,946, 4,315,929, 4,322,348, 4,331,598, 4,361,650, 4,362,663, 4,364,866, 4,424,219, 4,371,533, 4,450,254, 5,475,092, 5,585,499, 5,846,545, and 6,333,410.

**[0062]** Examples of maytansinol analogs having a modified aromatic ring include:

(1) C-19-dechloro (U.S. Patent 4,256,746) (prepared by LAH reduction of ansamycin P2), (2) C-20-hydroxy (or C-20-demethyl) +/-C-19-dechloro (U.S. Patents 4,361,650 and 4,307,016) (prepared by demethylation using *Streptomyces* or *Actinomyces* or dechlorination using LAH), and (3) C-20-demethoxy, C-20-acyloxy (-OCOR), +/-dechloro (U.S. Patent 4,294,757) (prepared by acylation using acyl chlorides).

**[0063]** Examples of maytansinol analogs having modifications of positions other than an aromatic ring include: (1) C-9-SH (U.S. Patent 4,424,219) (prepared by the reaction of maytansinol with H<sub>2</sub>S or P<sub>2</sub>S<sub>5</sub>), (2) C-14-alkoxymethyl (demethoxy/CH<sub>2</sub>OR) (U.S. Patent 4,331,598), (3) C-14-hydroxymethyl or acyloxymethyl (CH<sub>2</sub>OH or CH<sub>2</sub>OAc) (U.S. Patent 4,450,254) (prepared from *Nocardia*), (4) C-15-hydroxy/acyloxy (U.S. Patent 4,364,866) (prepared by the conversion of maytansinol by *Streptomyces*), (5) C-15-methoxy (U.S. Patents 4,313,946 and 4,315,929) (isolated from *Trewia nudiflora*), (6) C-18-N-demethyl (U.S. Patents 4,362,663 and 4,322,348) (prepared by the demethylation of maytansinol by *Streptomyces*), and (7) 4,5-deoxy (U.S. Patent 4,371,533) (prepared by the titanium trichloride/LAH reduction of maytansinol).

**[0064]** In a preferred embodiment of the invention, the conjugate utilizes the thiol-containing maytansinoid DM1, also known as N<sup>2'</sup>-deacetyl-N<sup>2'</sup>-(3-mercapto-1-oxopropyl)-maytansine, as the cytotoxic agent. The structure of DM1 is represented by formula (I):

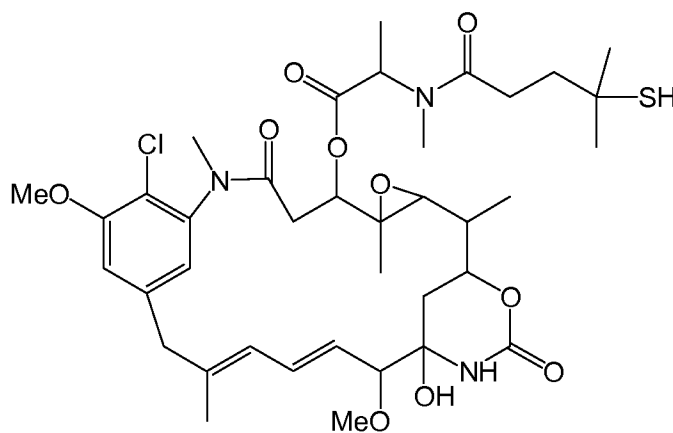


(I)

**[0065]** In another preferred embodiment of the invention, the conjugate utilizes the thiol-containing maytansinoid DM4, also known as N<sup>2'</sup>-deacetyl-N<sup>2'</sup>-(4-methyl-4-mercapto-1-



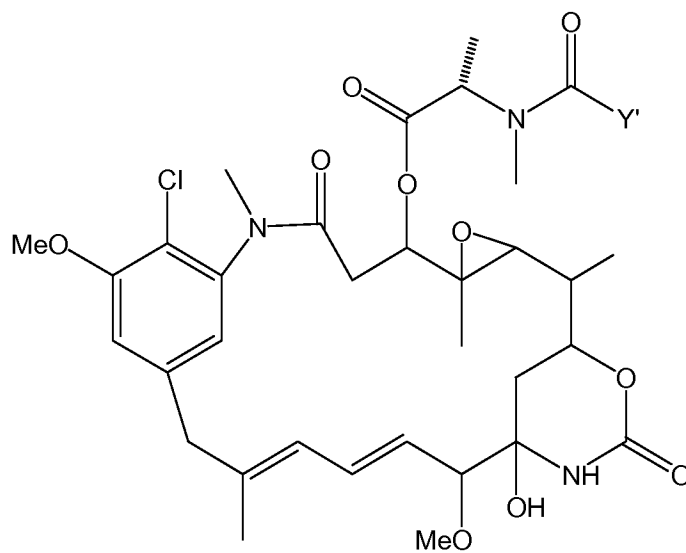
oxopentyl)-maytansine, as the cytotoxic agent. The structure of DM4 is represented by formula (II):



(II)

[0066] Other maytansinoids may be used in the context of the invention, including, for example, thiol and disulfide-containing maytansinoids bearing a mono or di-alkyl substitution on the carbon atom bearing the sulfur atom. Particularly preferred is a maytansinoid having at the C-3 position (a) C-14 hydroxymethyl, C-15 hydroxy, or C-20 desmethyl functionality, and (b) an acylated amino acid side chain with an acyl group bearing a hindered sulfhydryl group, wherein the carbon atom of the acyl group bearing the thiol functionality has one or two substituents, said substituents being CH<sub>3</sub>, C<sub>2</sub>H<sub>5</sub>, linear or branched alkyl or alkenyl having from 1 to 10 carbon atoms, cyclic alkyl or alkenyl having from 3 to 10 carbon atoms, phenyl, substituted phenyl, or heterocyclic aromatic or heterocycloalkyl radical, and further wherein one of the substituents can be H, and wherein the acyl group has a linear chain length of at least three carbon atoms between the carbonyl functionality and the sulfur atom.

[0067] Additional maytansinoids for use in the context of the invention include compounds represented by formula (III):



(III),

wherein Y' represents

$(\text{CR}_7\text{R}_8)_l(\text{CR}_9=\text{CR}_{10})_p(\text{C}\equiv\text{C})_q\text{A}_o(\text{CR}_5\text{R}_6)_m\text{D}_u(\text{CR}_{11}=\text{CR}_{12})_r(\text{C}\equiv\text{C})_s\text{B}_t(\text{CR}_3\text{R}_4)_n\text{CR}_1\text{R}_2\text{SZ}$ ,

wherein  $\text{R}_1$  and  $\text{R}_2$  are each independently  $\text{CH}_3$ ,  $\text{C}_2\text{H}_5$ , linear alkyl or alkenyl having from 1 to 10 carbon atoms, branched or cyclic alkyl or alkenyl having from 3 to 10 carbon atoms, phenyl, substituted phenyl or heterocyclic aromatic or heterocycloalkyl radical, and wherein  $\text{R}_2$  also can be H,

wherein A, B, D are cycloalkyl or cycloalkenyl having 3-10 carbon atoms, simple or substituted aryl, or heterocyclic aromatic, or heterocycloalkyl radical,

wherein  $\text{R}_3$ ,  $\text{R}_4$ ,  $\text{R}_5$ ,  $\text{R}_6$ ,  $\text{R}_7$ ,  $\text{R}_8$ ,  $\text{R}_9$ ,  $\text{R}_{10}$ ,  $\text{R}_{11}$ , and  $\text{R}_{12}$  are each independently H,  $\text{CH}_3$ ,  $\text{C}_2\text{H}_5$ , linear alkyl or alkenyl having from 1 to 10 carbon atoms, branched or cyclic alkyl or alkenyl having from 3 to 10 carbon atoms, phenyl, substituted phenyl or heterocyclic aromatic, or heterocycloalkyl radical,

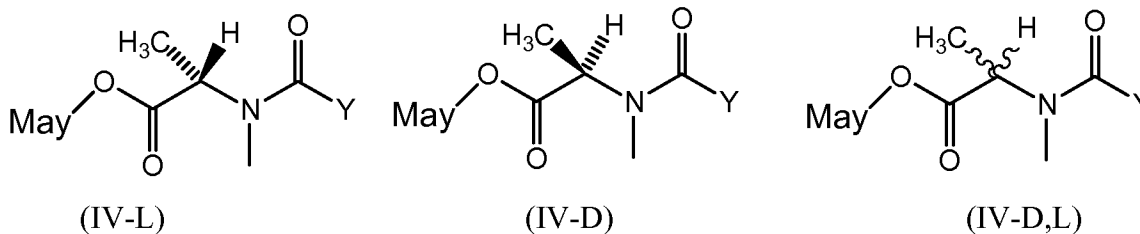
wherein l, m, n, o, p, q, r, s, and t are each independently zero or an integer from 1 to 5, provided that at least two of l, m, n, o, p, q, r, s and t are not zero at any one time, and

wherein Z is H, SR or COR, wherein R is linear alkyl or alkenyl having from 1 to 10 carbon atoms, branched or cyclic alkyl or alkenyl having from 3 to 10 carbon atoms, or simple or substituted aryl or heterocyclic aromatic, or heterocycloalkyl radical.

**[0068]** Preferred embodiments of formula (III) include compounds of formula (III)

wherein (a)  $\text{R}_1$  is H,  $\text{R}_2$  is methyl and Z is H, (b)  $\text{R}_1$  and  $\text{R}_2$  are methyl and Z is H, (c)  $\text{R}_1$  is H,  $\text{R}_2$  is methyl, and Z is  $-\text{SCH}_3$ , and (d)  $\text{R}_1$  and  $\text{R}_2$  are methyl, and Z is  $-\text{SCH}_3$ .

**[0069]** Such additional maytansinoids also include compounds represented by formula (IV-L), (IV-D), or (IV-D,L):



wherein Y represents  $(CR_7R_8)_l(CR_5R_6)_m(CR_3R_4)_nCR_1R_2SZ$ ,

wherein  $R_1$  and  $R_2$  are each independently  $CH_3$ ,  $C_2H_5$ , linear alkyl, or alkenyl having from 1 to 10 carbon atoms, branched or cyclic alkyl or alkenyl having from 3 to 10 carbon atoms, phenyl, substituted phenyl, or heterocyclic aromatic or heterocycloalkyl radical, and wherein  $R_2$  also can be H,

wherein  $R_3$ ,  $R_4$ ,  $R_5$ ,  $R_6$ ,  $R_7$ , and  $R_8$  are each independently H,  $CH_3$ ,  $C_2H_5$ , linear alkyl or alkenyl having from 1 to 10 carbon atoms, branched or cyclic alkyl or alkenyl having from 3 to 10 carbon atoms, phenyl, substituted phenyl, or heterocyclic aromatic or heterocycloalkyl radical,

wherein l, m, and n are each independently an integer of from 1 to 5, and in addition n can be zero,

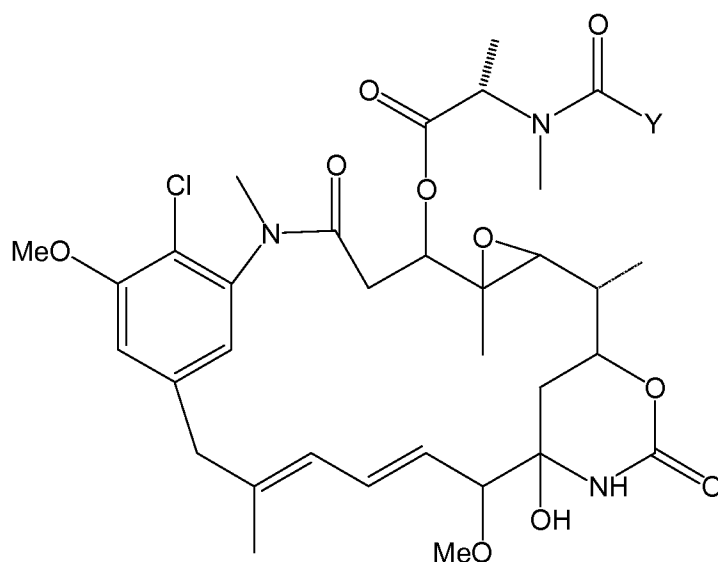
wherein Z is H, SR, or COR wherein R is linear or branched alkyl or alkenyl having from 1 to 10 carbon atoms, cyclic alkyl or alkenyl having from 3 to 10 carbon atoms, or simple or substituted aryl or heterocyclic aromatic or heterocycloalkyl radical, and

wherein May represents a maytansinoid which bears the side chain at C-3, C-14 hydroxymethyl, C-15 hydroxy, or C-20 desmethyl.

**[0070]** Preferred embodiments of formulas (IV-L), (IV-D) and (IV-D,L) include compounds of formulas (IV-L), (IV-D) and (IV-D,L) wherein (a)  $R_1$  is H,  $R_2$  is methyl,  $R_5$ ,  $R_6$ ,  $R_7$ , and  $R_8$  are each H, l and m are each 1, n is 0, and Z is H, (b)  $R_1$  and  $R_2$  are methyl,  $R_5$ ,  $R_6$ ,  $R_7$ ,  $R_8$  are each H, l and m are 1, n is 0, and Z is H, (c)  $R_1$  is H,  $R_2$  is methyl,  $R_5$ ,  $R_6$ ,  $R_7$ , and  $R_8$  are each H, l and m are each 1, n is 0, and Z is  $-SCH_3$ , or (d)  $R_1$  and  $R_2$  are methyl,  $R_5$ ,  $R_6$ ,  $R_7$ ,  $R_8$  are each H, l and m are 1, n is 0, and Z is  $-SCH_3$ .

**[0071]** Preferably the cytotoxic agent is represented by formula (IV-L).

**[0072]** Additional preferred maytansinoids also include compounds represented by formula (V):



(V)

wherein Y represents  $(CR_7R_8)_l(CR_5R_6)_m(CR_3R_4)_nCR_1R_2SZ$ ,

wherein  $R_1$  and  $R_2$  are each independently  $CH_3$ ,  $C_2H_5$ , linear alkyl, or alkenyl having from 1 to 10 carbon atoms, branched or cyclic alkyl or alkenyl having from 3 to 10 carbon atoms, phenyl, substituted phenyl or heterocyclic aromatic or heterocycloalkyl radical, and wherein  $R_2$  also can be H,

wherein  $R_3$ ,  $R_4$ ,  $R_5$ ,  $R_6$ ,  $R_7$ , and  $R_8$  are each independently H,  $CH_3$ ,  $C_2H_5$ , linear alkyl or alkenyl having from 1 to 10 carbon atoms, branched or cyclic alkyl or alkenyl having from 3 to 10 carbon atoms, phenyl, substituted phenyl, or heterocyclic aromatic or heterocycloalkyl radical,

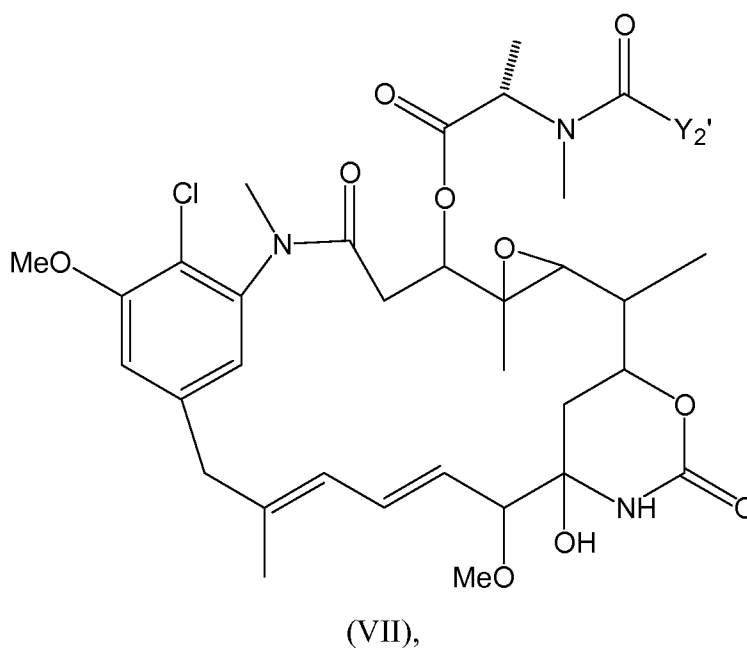
wherein l, m, and n are each independently an integer of from 1 to 5, and in addition n can be zero, and

wherein Z is H, SR or COR, wherein R is linear alkyl or alkenyl having from 1 to 10 carbon atoms, branched or cyclic alkyl or alkenyl having from 3 to 10 carbon atoms, or simple or substituted aryl or heterocyclic aromatic or heterocycloalkyl radical.

**[0073]** Preferred embodiments of formula (V) include compounds of formula (V)

wherein (a)  $R_1$  is H,  $R_2$  is methyl,  $R_5$ ,  $R_6$ ,  $R_7$ , and  $R_8$  are each H; l and m are each 1; n is 0; and Z is H, (b)  $R_1$  and  $R_2$  are methyl;  $R_5$ ,  $R_6$ ,  $R_7$ ,  $R_8$  are each H, l and m are 1; n is 0; and Z is H, (c)  $R_1$  is H,  $R_2$  is methyl,  $R_5$ ,  $R_6$ ,  $R_7$ , and  $R_8$  are each H, l and m are each 1, n is 0, and Z is  $-SCH_3$ , or (d)  $R_1$  and  $R_2$  are methyl,  $R_5$ ,  $R_6$ ,  $R_7$ ,  $R_8$  are each H, l and m are 1, n is 0, and Z is  $-SCH_3$ .





wherein  $Y_2'$  represents

$(CR_7R_8)_l(CR_9=CR_{10})_p(C\equiv C)_qA_o(CR_5R_6)_mD_u(CR_{11}=CR_{12})_r(C\equiv C)_sB_t(CR_3R_4)_nCR_1R_2SZ_2$ ,

wherein  $R_1$  and  $R_2$  are each independently  $CH_3$ ,  $C_2H_5$ , linear branched or alkyl or alkenyl having from 1 to 10 carbon atoms, cyclic alkyl or alkenyl having from 3 to 10 carbon atoms, phenyl, substituted phenyl or heterocyclic aromatic or heterocycloalkyl radical, and in addition  $R_2$  can be H,

wherein A, B, and D each independently is cycloalkyl or cycloalkenyl having 3 to 10 carbon atoms, simple or substituted aryl, or heterocyclic aromatic or heterocycloalkyl radical,

wherein  $R_3$ ,  $R_4$ ,  $R_5$ ,  $R_6$ ,  $R_7$ ,  $R_8$ ,  $R_9$ ,  $R_{10}$ ,  $R_{11}$ , and  $R_{12}$  are each independently H,  $CH_3$ ,  $C_2H_5$ , linear alkyl or alkenyl having from 1 to 10 carbon atoms, branched or cyclic alkyl or alkenyl having from 3 to 10 carbon atoms, phenyl, substituted phenyl or heterocyclic aromatic or heterocycloalkyl radical,

wherein l, m, n, o, p, q, r, s, and t are each independently zero or an integer of from 1 to 5, provided that at least two of l, m, n, o, p, q, r, s and t are not zero at any one time, and

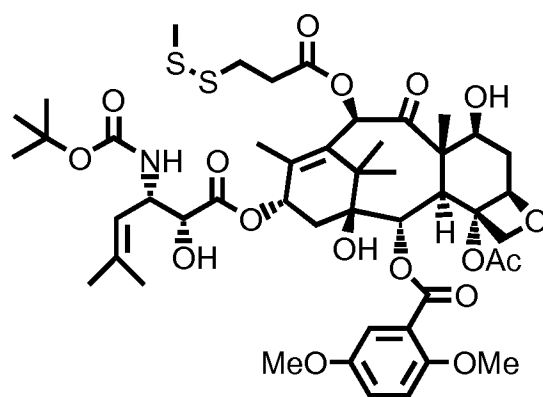
wherein  $Z_2$  is SR or -COR, wherein R is linear alkyl or alkenyl having from 1 to 10 carbon atoms, branched or cyclic alkyl or alkenyl having from 3 to 10 carbon atoms, or simple or substituted aryl or heterocyclic aromatic or heterocycloalkyl radical.

**[0076]** Preferred embodiments of formula (VII) include compounds of formula (VII), wherein  $R_1$  is H and  $R_2$  is methyl.

**[0077]** In addition to maytansinoids, the cytotoxic agent used in the conjugate can be a taxane or derivative thereof. Taxanes are a family of compounds that includes paclitaxel

(Taxol®), a cytotoxic natural product, and docetaxel (Taxotere®), a semi-synthetic derivative, which are both widely used in the treatment of cancer. Taxanes are mitotic spindle poisons that inhibit the depolymerization of tubulin, resulting in cell death. While docetaxel and paclitaxel are useful agents in the treatment of cancer, their antitumor activity is limited because of their non-specific toxicity towards normal cells. Further, compounds like paclitaxel and docetaxel themselves are not sufficiently potent to be used in conjugates of cell-binding agents.

**[0078]** A preferred taxane for use in the preparation of a cytotoxic conjugate is the taxane of formula (VIII):



(VIII)

**[0079]** Methods for synthesizing taxanes that can be used in the context of the invention, along with methods for conjugating taxanes to cell-binding agents such as antibodies, are described in detail in U.S. Patents 5,416,064, 5,475,092, 6,340,701, 6,372,738, 6,436,931, 6,596,757, 6,706,708, 6,716,821, and 7,390,898.

**[0080]** The cytotoxic also can be CC-1065 or a derivative thereof. CC-1065 is a potent anti-tumor antibiotic isolated from the culture broth of *Streptomyces zelensis*. CC-1065 is about 1000-fold more potent *in vitro* than commonly used anti-cancer drugs, such as doxorubicin, methotrexate, and vincristine (Bhuyan et al., *Cancer Res.*, 42: 3532-3537 (1982)). CC-1065 and its analogs are disclosed in U.S. Patents 5,585,499, 5,846,545, 6,340,701, and 6,372,738. The cytotoxic potency of CC-1065 has been correlated with its alkylating activity and its DNA-binding or DNA-intercalating activity. These two activities reside in separate parts of the molecule. In this respect, the alkylating activity is contained in the cyclopropylpyrroloindole (CPI) subunit and the DNA-binding activity resides in the two pyrroloindole subunits of CC-1065.

**[0081]** Several CC-1065 analogs are known in the art and also can be used as the cytotoxic agent in the conjugate (see, e.g., Warpehoski et al., *J. Med. Chem.*, 31: 590-603 (1988)). A series of CC-1065 analogs has been developed in which the CPI moiety is replaced by a cyclopropabenzindole (CBI) moiety (Boger et al., *J. Org. Chem.*, 55: 5823-5833 (1990), and Boger et al., *Bioorg. Med. Chem. Lett.*, 1: 115-120 (1991)). These CC-1065 analogs maintain the high *in vitro* potency of the parental drug, without causing delayed toxicity in mice. Like CC-1065, these compounds are alkylating agents that covalently bind to the minor groove of DNA to cause cell death.

**[0082]** The therapeutic efficacy of CC-1065 analogs can be greatly improved by changing the *in vivo* distribution through targeted delivery to a tumor site, resulting in lower toxicity to non-targeted tissues, and thus, lower systemic toxicity. To this end, conjugates of analogs and derivatives of CC-1065 with cell-binding agents that specifically target tumor cells have been generated (see, e.g., U.S. Patents 5,475,092, 5,585,499, and 5,846,545). These conjugates typically display high target-specific cytotoxicity *in vitro*, and anti-tumor activity in human tumor xenograft models in mice (see, e.g., Chari et al., *Cancer Res.*, 55: 4079-4084 (1995)).

**[0083]** Methods for synthesizing CC-1065 analogs are described in detail in U.S. Patents 5,475,092, 5,585,499, 5,846,545, 6,534,660, 6,586,618, 6,756,397, and 7,329,760.

**[0084]** Drugs such as methotrexate, daunorubicin, doxorubicin, vincristine, vinblastine, melphalan, mitomycin C, chlorambucil, calicheamicin, tubulysin and tubulysin analogs, duocarmycin and duocarmycin analogs, dolastatin and dolastatin analogs also can be used as the cytotoxic agents of the invention. Doxorubicin and daunorubicin compounds (see, e.g., U.S. Patent 6,630,579) can also be used as the cytotoxic agent.

**[0085]** The cell-binding agent cytotoxic agent conjugates may be prepared by *in vitro* methods. In order to link a cytotoxic agent to the antibody, a linking group is used. Suitable linking groups are well known in the art and include disulfide groups, acid labile groups, photolabile groups, peptidase labile groups, and esterase labile groups, as well as noncleavable linking groups.

**[0086]** In accordance with the invention, the cell-binding agent is modified by reacting a bifunctional crosslinking reagent with the cell-binding agent, thereby resulting in the covalent attachment of a linker molecule to the cell-binding agent. As used herein, a "bifunctional crosslinking reagent" refers to a reagent that possesses two reactive groups; one of which is capable of reacting with a cell-binding agent, while the other one is capable of reacting with



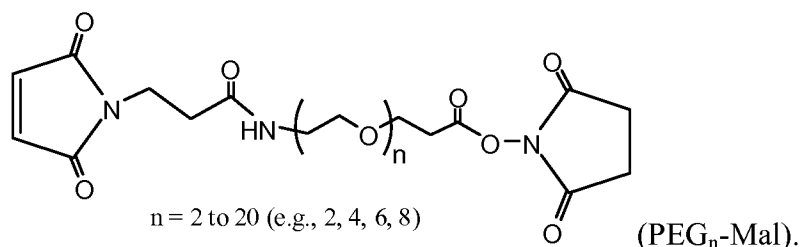
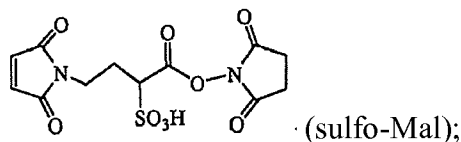
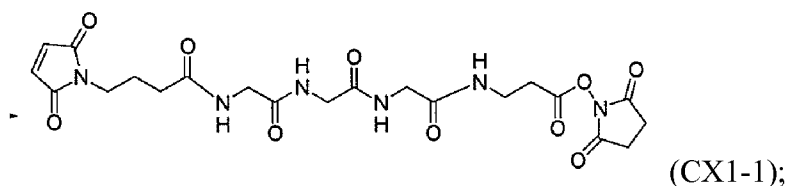
the cytotoxic agent to link the cell-binding agent with the cytotoxic agent, thereby forming a conjugate.

**[0087]** Any suitable bifunctional crosslinking reagent can be used in connection with the invention, so long as the linker reagent provides for retention of the therapeutic, e.g., cytotoxicity, and targeting characteristics of the cytotoxic agent and the cell-binding agent, respectively, while providing an acceptable toxicity profile. Preferably, the linker molecule joins the cytotoxic agent to the cell-binding agent through chemical bonds (as described above), such that the cytotoxic agent and the cell-binding agent are chemically coupled (e.g., covalently bonded) to each other.

**[0088]** In one embodiment, the bifunctional crosslinking reagent comprises non-cleavable linkers. A non-cleavable linker is any chemical moiety that is capable of linking a cytotoxic agent, such as a maytansinoid, a taxane, or a CC-1065 analog, to a cell-binding agent in a stable, covalent manner. Thus, non-cleavable linkers are substantially resistant to acid-induced cleavage, light-induced cleavage, peptidase-induced cleavage, esterase-induced cleavage, and disulfide bond cleavage, at conditions under which the cytotoxic agent or the cell-binding agent remains active.

**[0089]** Suitable crosslinking reagents that form non-cleavable linkers between a cytotoxic agent and the cell-binding agent are well known in the art. In one embodiment, the cytotoxic agent is linked to the cell-binding agent through a thioether bond. Examples of non-cleavable linkers include linkers having a maleimido- or haloacetyl-based moiety for reaction with the cytotoxic agent. Such bifunctional crosslinking agents are well known in the art (see U.S. Patent Application Publication Nos. 2010/0129314, 2009/0274713, 2008/0050310, 2005/0169933, and Pierce Biotechnology Inc. P.O. Box 117, Rockland, IL 61105, USA) and include, but not limited to, N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC), N-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxy-(6-amidocaproate), which is a "long chain" analog of SMCC (LC-SMCC),  $\kappa$ -maleimidoundecanoic acid N-succinimidyl ester (KMUA),  $\gamma$ -maleimidobutyric acid N-succinimidyl ester (GMBS),  $\epsilon$ -maleimidocaproic acid N-hydroxysuccinimide ester (EMCS), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-( $\alpha$ -maleimidoacetoxy)-succinimide ester (AMAS), succinimidyl-6-( $\beta$ -maleimidopropionamido)hexanoate (SMPH), N-succinimidyl 4-(p-maleimidophenyl)-butyrate (SMPB), and N-(p-maleimidophenyl)isocyanate (PMPI). Cross-linking reagents comprising a haloacetyl-based moiety include N-succinimidyl-4-(iodoacetyl)-aminobenzoate (SIAB), N-succinimidyl

iodoacetate (SIA), N-succinimidyl bromoacetate (SBA), and N-succinimidyl 3-(bromoacetamido)propionate (SBAP), bis-maleimidopolyethyleneglycol (BMPEO), BM(PEO)<sub>2</sub>, BM(PEO)<sub>3</sub>, N-(β-maleimidopropoxy)succinimide ester (BMPS), 5-maleimidovaleric acid NHS, HBVS, 4-(4-N-maleimidophenyl)-butyric acid hydrazide•HCl (MPBH), Succinimidyl-(4-vinylsulfonyl)benzoate (SVSB), dithiobis-maleimidoethane (DTME), 1,4-bis-maleimidobutane (BMB), 1,4 bismaleimidyl-2,3-dihydroxybutane (BMDB), bis-maleimidohexane (BMH), bis-maleimidoethane (BMOE), sulfosuccinimidyl 4-(N-maleimido-methyl)cyclohexane-1-carboxylate (sulfo-SMCC), sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (sulfo-SIAB), m-Maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBS), N-(γ-maleimidobutyloxy)sulfosuccinimide ester (sulfo-GMBS), N-(ε-maleimidocaproxyloxy)sulfosuccinimide ester (sulfo-EMCS), N-(κ-maleimidoundecanoyloxy)sulfosuccinimide ester (sulfo-KMUS) and sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate (sulfo-SMPB) CX1-1, sulfo-Mal and PEG<sub>n</sub>-Mal. Preferably, the bifunctional crosslinking reagent is SMCC.

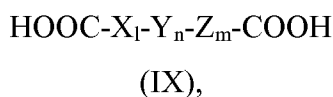


**[0090]** In one embodiment, the linking reagent is a cleavable linker. Examples of suitable cleavable linkers include disulfide linkers, acid labile linkers, photolabile linkers, peptidase labile linkers, and esterase labile linkers. Disulfide containing linkers are linkers cleavable through disulfide exchange, which can occur under physiological conditions. Acid labile linkers are linkers cleavable at acid pH. For example, certain intracellular compartments, such as endosomes and lysosomes, have an acidic pH (pH 4-5), and provide conditions suitable to cleave acid labile linkers. Photo labile linkers are useful at the body

surface and in many body cavities that are accessible to light. Furthermore, infrared light can penetrate tissue. Peptidase labile linkers can be used to cleave certain peptides inside or outside cells (see e.g., Trouet et al., *Proc. Natl. Acad. Sci. USA*, 79: 626-629 (1982), and Umemoto et al., *Int. J. Cancer*, 43: 677-684 (1989)). In one embodiment, the cleavable linker is cleaved under mild conditions, i.e., conditions within a cell under which the activity of the cytotoxic agent is not affected.

[0091] In one embodiment, the cytotoxic agent is linked to a cell-binding agent through a disulfide bond. The linker molecule comprises a reactive chemical group that can react with the cell-binding agent. Preferred reactive chemical groups for reaction with the cell-binding agent are *N*-succinimidyl esters and *N*-sulfo succinimidyl esters. Additionally the linker molecule comprises a reactive chemical group, preferably a dithiopyridyl group, that can react with the cytotoxic agent to form a disulfide bond. Bifunctional crosslinking reagents that enable the linkage of the cell-binding agent with the cytotoxic agent via disulfide bonds are known in the art and include, for example, *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (see, e.g., Carlsson et al., *Biochem. J.*, 173: 723-737 (1978)), *N*-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB) (see, e.g., U.S. Patent 4,563,304), *N*-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP) (see, e.g., CAS Registry number 341498-08-6), and *N*-succinimidyl-4-(2-pyridyldithio)2-sulfo butanoate (sulfo-SPDB) (see, e.g., U.S. Patent Application Publication No. 2009/0274713). Other bifunctional crosslinking reagents that can be used to introduce disulfide groups are known in the art and are described in U.S. Patents 6,913,748, 6,716,821 and U.S. Patent Application Publications 2009/0274713 and 2010/0129314, all of which are incorporated herein in its entirety by reference.

[0092] Other crosslinking reagents lacking a sulfur atom that form non-cleavable linkers can also be used in the inventive method. Such linkers can be derived from dicarboxylic acid based moieties. Suitable dicarboxylic acid based moieties include, but are not limited to,  $\alpha,\omega$ -dicarboxylic acids of the general formula (IX):



wherein X is a linear or branched alkyl, alkenyl, or alkynyl group having 2 to 20 carbon atoms, Y is a cycloalkyl or cycloalkenyl group bearing 3 to 10 carbon atoms, Z is a substituted or unsubstituted aromatic group bearing 6 to 10 carbon atoms, or a substituted or

unsubstituted heterocyclic group wherein the hetero atom is selected from N, O or S, and wherein l, m, and n are each 0 or 1, provided that l, m, and n are all not zero at the same time.

**[0093]** Many of the non-cleavable linkers disclosed herein are described in detail in U.S. Patent Application Publication No. 2005/0169933 A1.

**[0094]** The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

#### Example 1

**[0095]** This example demonstrates a processes for manufacturing cell-binding agent-cytotoxic agent conjugates of improved homogeneity comprising performing the modification reaction at a lower temperature.

**[0096]** Humanized CD37-3 antibody (huCD37-3) was reacted with the heterobifunctional crosslinking reagent SMCC (N-succinimidyl-4-(maleimidomethyl)cyclohexanecarboxylate) and the maytansinoid DM1 using a previously described process, as well as the improved process that is the subject of the present application.

**[0097]** For the previously described process, Process A (see, e.g., Chari et al., U.S. 5,208,020), huCD37-3 (15 mg/mL) first was reacted with SMCC (6.0-fold molar excess relative to the amount of antibody, dissolved in DMA, dimethylacetamide) to form the modified antibody. The modification reaction was performed at 20° C in 50 mM sodium phosphate buffer (pH 6.7) containing 2 mM EDTA (ethylenediaminetetraacetic acid) and 10% DMA for 180 minutes. The reaction was quenched with 1 M acetate to adjust the pH to 4.5 and the modified antibody was purified using a column of Sephadex G-25F resin equilibrated and eluted in 20 mM sodium acetate (pH 4.5) containing 2mM EDTA. After purification, the modified antibody (at 5 mg/mL) was adjusted to pH 5.0 with potassium phosphate tribasic buffer and was reacted with the maytansinoid DM1 (7.2-fold molar excess relative to the amount of antibody, dissolved in DMA) to form the conjugated antibody. The conjugation reaction was performed at 20° C in 20 mM sodium acetate buffer (pH 5.0) containing 2 mM EDTA and 5% DMA for approximately 20 hours. The reaction mixture was then purified using a column of Sephadex G-25F resin equilibrated and eluted in 10 mM sodium succinate (pH 5.0).

**[0098]** For Process B (involving performing the modification step at high pH and room temperature), huCD37-3 (15 mg/mL) first was reacted with SMCC (6.0-fold molar excess relative to the amount of antibody, dissolved in DMA) to form the modified antibody. The

modification reaction was performed at 20° C in 50 mM sodium phosphate buffer (pH 7.5) containing 2 mM EDTA and 10% DMA for 50 minutes. The reaction was quenched with 1 M acetic acid to adjust the pH to 4.5 and the modified antibody was purified using a column of Sephadex G-25F resin equilibrated and eluted in 20 mM sodium acetate (pH 4.5) containing 2 mM EDTA. After purification, the modified antibody (at 5 mg/mL) was adjusted to pH 5.0 with potassium phosphate tribasic buffer and was reacted with the maytansinoid DM1 (7.2-fold molar excess relative to the amount of antibody, dissolved in DMA) to form the conjugated antibody. The conjugation reaction was performed at 20° C in 20 mM sodium acetate buffer (pH 5.0) containing 2 mM EDTA and 5% DMA for approximately 20 hours. The reaction mixture was then purified using a column of Sephadex G-25F resin equilibrated and eluted in 10 mM sodium succinate (pH 5.0).

**[0099]** For the inventive process, Process C (involving performing the modification step at high pH and low temperature), huCD37-3 (15 mg/mL) first was reacted with SMCC (6.0-fold molar excess relative to the amount of antibody, dissolved in DMA) to form the modified antibody. The modification reaction was performed at 10° C in 50 mM sodium phosphate buffer (pH 7.5) containing 2 mM EDTA and 10% DMA for 50 minutes. The reaction was quenched with 1 M acetic acid to adjust the pH to 4.5 and the modified antibody was purified using a column of Sephadex G-25F resin equilibrated and eluted in 20mM sodium acetate (pH 4.5) containing 2mM EDTA. After purification, the modified antibody (at 5 mg/mL) was adjusted to pH 5.0 with potassium phosphate tribasic buffer and was reacted with the maytansinoid DM1 (7.2-fold molar excess relative to the amount of antibody, dissolved in DMA) to form the conjugated antibody. The conjugation reaction was performed at 20° C in 20 mM sodium acetate buffer (pH 5.0) containing 2 mM EDTA and 5% DMA for approximately 20 hours. The reaction mixture was then purified using a column of Sephadex G-25F resin equilibrated and eluted in 10 mM sodium succinate (pH 5.0).

**[0100]** Conjugate derived from the three processes was analyzed by: UV spectroscopy for conjugate concentration and Maytansinoid to Antibody Ratio (MAR); Free Maytansinoid by Dual Column reversed phase chromatography; Mass Spectrometry for determination of unconjugated linker level; reduced SDS PAGE electrophoresis for determination of level of non-reducible species; non-reduced SDS PAGE electrophoresis for determination of level of fragments; and SEC-HPLC for determination of conjugate monomer.

**[0101]** Concentration and Maytansinoid to Antibody Ratio were determined by measuring the absorbance of the conjugate at 252 and 280 nm in a UV-VIS spectrophotometer and using the molar extinction coefficients of DM1 and antibody at the two wavelengths to calculate the molar concentrations of antibody and DM1.

**[0102]** The un-conjugated linker level of the conjugates was analyzed by mass spectrometry: peak areas of individual conjugate species (including conjugates with or without un-conjugated linkers) were measured; the un-conjugated linker level was calculated by the ratio of the sum of areas containing un-conjugated linkers (weighted by the number of linkers) to the sum of areas of all conjugate species (also weighted by the number of linkers).

**[0103]** The non-reducible species level of the conjugates was analyzed by reduced SDS gel electrophoresis: peak areas of individual reduced conjugate species (including reduced light chain, reduced heavy chain, cross-linked light-light chains, cross-linked light-heavy chains, etc.) were measured; the non-reducible species level was calculated by the ratio of the sum of areas of non-reducible species to the sum of areas of all species.

**[0104]** The monomer level of the conjugates was analyzed by size exclusion HPLC: peak areas of monomer, dimer, aggregates and low molecular weight species were measured using an absorbance detector set to a wavelength of 252 nm or 280 nm; the monomer level was calculated by the ratio of the monomer area to the total area.

**[0105]** The amount of free maytansinoid present in the conjugate was analyzed by dual column (HiSep and C18 columns) HPLC: peak areas of total free maytansinoid species (eluted in the gradient and identified by comparison of elution time with known standards) were measured using an absorbance detector set to a wavelength of 252 nm; the amount of free maytansinoid was calculated using a standard curve generated by the peak areas of known amount of standards.

**[0106]** As shown in Table 1 below, conjugate manufactured using the inventive process (Process C) was superior to that manufactured using the previously described process, Process A, with respect to unconjugated linker, non-reducible species and monomer, as well as the Process B, involving performing the modification step at high pH and room temperature.

Table 1. Comparison of key properties of the conjugate manufactured by the inventive process compared to other processes

	Process A Modification at pH 6.7, 20° C	Process B Modification at pH 7.5, 20° C	Process C Modification at pH 7.5, 10° C
Concentration (mg/mL)	3.2	3.1	3.2
MAR	3.7	3.8	3.6
Monomer (SEC HPLC)	95.2%	94.8%	97.8%
Non-reducible species (Reduced Gel Chip)	11.4%	10.9%	4.4%
Un-conjugated linker (MDP)	14%	16%	7%
Free Maytansinoid	0.5%	0.4%	0.4%
Fragmentation (Non-reduced Gel Chip)	3.6%	3.0%	3.6%

**[0107]** The results of the experiments described in this example demonstrate that performing the modification step at a low temperature (e.g., 10° C) produces a conjugate that is superior to conjugate manufactured using the previously described process. In addition, the results of the experiments described in this example demonstrate that performing the modification step at a high pH (e.g., 7.5) produces a conjugate of superior quality only when the modification step is performed at a low temperature (e.g., 10° C).

#### Example 2

**[0108]** This example demonstrates a processes for manufacturing cell-binding agent-cytotoxic agent conjugates of improved homogeneity comprising performing the modification reaction at a lower temperature and a higher pH.

**[0109]** A humanized antibody was reacted with the heterobifunctional crosslinking reagent SMCC and the maytansinoid DM1 to make a conjugate with a MAR (maytansinoid to antibody ratio, also known as drug to antibody ratio) of approximately 3.5.

**[0110]** The reaction was performed using a previously described process (see, e.g., U.S. Patent Application Publications 2011/0166319 and 2006/0182750), as well as the inventive

process comprising performing the modification reaction at a higher pH and a lower temperature.

**[0111]** Using the previously described process, the humanized antibody (15 mg/mL) first was reacted with SMCC (7.5-fold molar excess relative to the amount of antibody) to form the modified antibody. The modification reaction was performed at 21° C in 50 mM sodium phosphate buffer (pH 6.7) containing 2 mM EDTA and 5% DMA for 120 minutes. The reaction was quenched with 0.5 M citrate to adjust the pH to 5.0, and the modified antibody was purified using a column of Sephadex G25F. After purification, the modified antibody (at 5 mg/mL) was reacted with the maytansinoid DM1 (5.4-fold molar excess relative to the amount of antibody; 1.3-fold excess relative to the measured amount of linker on the antibody) to form the conjugated antibody. The conjugation reaction was performed at ambient temperature in 20 mM citrate buffer (pH 5.0) containing 2 mM EDTA and 5% DMA for approximately 17 hours. The reaction mixture was then purified using a column of Sephadex G25F resin equilibrated and eluted in 10 mM sodium succinate (pH 5.0).

**[0112]** In the inventive process, the humanized antibody (3 mg/mL) first was reacted with SMCC (6.0-fold molar excess relative to the amount of antibody) to form the modified antibody. The modification reaction was performed at 0° C in 50 mM sodium phosphate buffer (pH 8.2) containing 2 mM EDTA and 5% DMA for 117 minutes. The reaction was quenched with 0.5 M citrate to adjust the pH to 5.0, and the modified antibody was purified using a column of Sephadex G25F. After purification, the modified antibody (2.5 mg/mL) was reacted with the maytansinoid DM1 (5.2-fold molar excess relative to the amount of antibody; 1.3-fold excess relative to the measured amount of linker on the antibody) to form the conjugated antibody. The conjugation reaction was performed at ambient temperature in 20 mM citrate buffer (pH 5.0) containing 2 mM EDTA and 5% DMA for approximately 20 hours. The reaction mixture was then purified using a column of Sephadex G25F resin equilibrated and eluted in 10 mM sodium succinate (pH 5.0).

**[0113]** Conjugate derived from the two processes was analyzed by: Mass Spectrometry for determination of unconjugated linker level; reduced SDS PAGE electrophoresis for determination of level of non-reducible species; and SEC-HPLC for determination of conjugate monomer.

**[0114]** As shown in Table 2 below, conjugate manufactured using the inventive process was superior to conjugate manufactured using the previously described process with respect to unconjugated linker and non-reducible species.



Table 2. Comparison of key properties of conjugate manufactured by the inventive process compared to previous process

	Previous Process Modification at pH 6.7, Room Temperature	Inventive Process Modification at pH 8.2, 0° C
MAR	3.6	3.1
Monomer% (SEC HPLC)	96.8%	97.5%
Non-reducible species (Reduced Gel Chip)	12.9%	6.8%
Un-conjugated linker% (MDP)	12.3%	7.6%
Total Free Maytansinoid %	0.2%	0.1%

[0115] The results of the experiments described in this example demonstrate that performing the modification step at a low temperature (e.g., 0° C) and high pH (e.g., pH 8.2) produces a conjugate that is superior to conjugate manufactured using the previously described process, wherein the modification step is performed at room temperature and a lower pH (e.g., pH 6.7).

### Example 3

[0116] This example illustrates a large-scale process for manufacturing cell-binding agent-cytotoxic agent conjugates of improved homogeneity comprising performing the modification reaction at a lower temperature and a higher pH.

[0117] A humanized antibody is reacted with the heterobifunctional crosslinking reagent SMCC and the maytansinoid DM1 to prepare a stable humanized antibody-SMCC-DM1 conjugate.

[0118] In particular, using the inventive process described herein, a humanized antibody is reacted with SMCC to form the modified antibody. The modification reaction is performed for 40 minutes using a molar excess of SMCC over antibody of 5.7 at about 10° C in a buffer having a pH of about 7.8 in 50 mM sodium phosphate, 2 mM EDTA, with 7% (v/v) DMA. After modification, the pH of the reaction mixture is adjusted to 4.5 with 1 M acetic acid, and the modified antibody is purified using TFF. After purification, the modified antibody is reacted with the maytansinoid DM1 (about 1.2 fold molar excess over bound

linker) to form the conjugated antibody. The conjugation reaction is performed for 16 hours at ambient temperature at a pH of about 5.0 in 20 mM sodium acetate, 2.0 mM EDTA, with 5.0% (v/v) DMA. The reaction mixture is then purified using TFF.

**[0119]** Analysis of the conjugate can be conducted by: Mass Spectrometry for determination of unconjugated linker level; reduced SDS PAGE electrophoresis for determination of level of non-reducible species; and SEC-HPLC for determination of conjugate monomer. The results of the analysis demonstrate that conjugate prepared by the inventive process is superior to conjugate manufactured using previously described processes (see, e.g., U.S. Patent Application Publications 2011/0166319 and 2006/0182750).

**[0120]** All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

**[0121]** The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

**[0122]** Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as

specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

## CLAIMS:

1. A process for preparing a cell-binding agent having a linker bound thereto, which process comprises contacting a cell-binding agent with a bifunctional crosslinking reagent at a temperature of about 15° C or less to covalently attach a linker to the cell-binding agent and thereby prepare a mixture comprising cell-binding agents having linkers bound thereto.
2. The process of claim 1, wherein the contacting occurs in a solution having a pH of about 7.5 to about 9.
3. The process of claim 2, wherein the pH is about 7.8.
4. The process of claim 1 or 2, wherein the solution comprises a buffering agent selected from a citrate buffer, an acetate buffer, a succinate buffer, and a phosphate buffer.
5. The process of claim 1 or 2, wherein the solution comprises a buffering agent selected from the group consisting of HEPPSO (N-(2-Hydroxyethyl)piperazine-N'-(2-hydroxypropanesulfonic acid)), POPSO (Piperazine-1,4-bis-(2-hydroxy-propane-sulfonic acid) dehydrate), HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), HEPPS (EPPS) (4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid), TES (N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid), and a combination thereof.
6. The process of any one of claims 1-5, wherein the contacting occurs at a temperature of about -10° C to about 15° C.
7. The process of claim 6, wherein the temperature is about 10° C.
8. The process of any one of claims 1-7, wherein the cell-binding agent is selected from the group consisting of antibodies, interferons, interleukin 2 (IL-2), interleukin 3 (IL-3), interleukin 4 (IL-4), interleukin 6 (IL-6), insulin, EGF, TGF- $\alpha$ , FGF, G-CSF, VEGF, MCSF, GM-CSF, and transferrin.
9. The process of claim 8, wherein the cell-binding agent is an antibody.
10. The process of claim 9, wherein the antibody is a monoclonal antibody.

11. The process of claim 10, wherein the antibody is a humanized monoclonal antibody.
12. The process of any one of claims 9-11, wherein the antibody is selected from the group consisting of huN901, huMy9-6, huB4, huC242, trastuzumab, bivatuzumab, sibrotuzumab, CNTO95, huDS6, rituximab, anti-CD27L, anti-Her2, anti-EGFR, anti-EGFRvIII, Cripto, anti-CD138, anti-CD38, anti-EphA2, integrin targeting antibody, anti-CD37, anti-folate, anti-Her3, and anti-IGFIR.
13. The process of any one of claims 1-12, wherein the bifunctional crosslinking reagent comprises an N-succinimidyl ester moiety, an N-sulfosuccinimidyl ester moiety, a maleimido-based moiety, or a haloacetyl-based moiety.
14. The process of claim 13, wherein the bifunctional crosslinking reagent comprises a maleimido-based moiety.
15. The process of claim 14, wherein the bifunctional crosslinking reagent is selected from the group consisting of N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC), N-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxy-(6-amidocaproate) (LC-SMCC),  $\kappa$ -maleimidoundecanoic acid N-succinimidyl ester (KMUA),  $\gamma$ -maleimidobutyric acid N-succinimidyl ester (GMBS),  $\beta$ -maleimidopropoxy-succinimidyl ester (BMPS),  $\epsilon$ -maleimidocaproic acid N-hydroxysuccinimide ester (EMCS), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-( $\alpha$ -maleimidoacetoxy)-succinimide ester (AMAS), succinimidyl-6-( $\beta$ -maleimidopropionamido)hexanoate (SMPH), N-succinimidyl 4-(p-maleimidophenyl)-butyrate (SMPB), and N-(p-maleimidophenyl)isocyanate (PMPI), sulfo-Mal, PEG<sub>4</sub>-Mal and CX1-1.
16. The process of claim 15, wherein the bifunctional crosslinking reagent is N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC).
17. A process for preparing a conjugate comprising a cell-binding agent chemically coupled to a cytotoxic agent, which process comprises:

- (a) contacting a cell-binding agent with a bifunctional crosslinking reagent at a temperature of about 15° C or less to covalently attach a linker to the cell-binding agent and thereby prepare a first mixture comprising cell-binding agents having linkers bound thereto,
- (b) subjecting the first mixture to tangential flow filtration, selective precipitation, non-adsorptive chromatography, adsorptive filtration, adsorptive chromatography, or a combination thereof and thereby prepare a purified first mixture of cell-binding agents having linkers bound thereto,
- (c) conjugating a cytotoxic agent to the cell-binding agents having linkers bound thereto in the purified first mixture by reacting the cell-binding agents having linkers bound thereto with a cytotoxic agent in a solution having a pH of about 4 to about 9 to prepare a second mixture comprising (i) cell-binding agent chemically coupled through the linker to the cytotoxic agent, (ii) free cytotoxic agent, and (iii) reaction by-products, and
- (d) subjecting the second mixture to tangential flow filtration, selective precipitation, non-adsorptive chromatography, adsorptive filtration, adsorptive chromatography, or a combination thereof to purify the cell-binding agents chemically coupled through the linkers to the cytotoxic agent from the other components of the second mixture and thereby prepare a purified second mixture of cell-binding agents chemically coupled through the linkers to the cytotoxic agent.

18. The process of claim 17, wherein the contacting in step (a) occurs in a solution having a pH of about 7.5 to about 9.

19. The process of claim 18, wherein the solution comprises a buffering agent selected from the group consisting of a citrate buffer, an acetate buffer, a succinate buffer, and a phosphate buffer.

20. The process of claim 18, wherein the solution comprises a buffering agent selected from the group consisting of HEPPSO (N-(2-Hydroxyethyl)piperazine-N'-(2-hydroxypropanesulfonic acid)), POPSO (Piperazine-1,4-bis-(2-hydroxy-propane-sulfonic acid) dehydrate), HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), HEPPS (EPPS) (4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid), TES (N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid), and a combination thereof.

21. The process of claim 18, wherein the pH is about 7.8.

22. The process of any one of claims 17-21, wherein the contacting in step (a) occurs at a temperature of about -10° C to about 15° C.
23. The process of claim 22, wherein the temperature is about 10° C.
24. The process of any one of claims 17-23, wherein the non-adsorptive chromatography is selected from the group consisting of SEPHADEX™ resins, SEPHACRYL™ resins, SUPERDEX™ resins, and BIO-GEL® resins.
25. The process of any one of claims 17-23, wherein the adsorptive chromatography is selected from the group consisting of hydroxyapatite chromatography, hydrophobic charge induction chromatography (HCIC), hydrophobic interaction chromatography (HIC), ion exchange chromatography, mixed mode ion exchange chromatography, immobilized metal affinity chromatography (IMAC), dye ligand chromatography, affinity chromatography, reversed phase chromatography, and combinations thereof.
26. The process of any one of claims 17-23, wherein tangential flow filtration is utilized in steps (b) and (d).
27. The process of any one of claims 17-23, wherein adsorptive chromatography is utilized in steps (b) and (d).
28. The process of any one of claims 17-23, wherein non-adsorptive chromatography is utilized in steps (b) and (d).
29. The process of any one of claims 17-23, wherein tangential flow filtration is utilized in step (b) and adsorptive chromatography is utilized in step (d).
30. The process of any one of claims 17-23, wherein adsorptive chromatography is utilized in step (b) and tangential flow filtration is utilized in step (d).
31. The process of any one of claims 17-30, wherein the cell-binding agent is selected from the group consisting of antibodies, interferons, interleukin 2 (IL-2), interleukin 3 (IL-3), interleukin 4 (IL-4), interleukin 6 (IL-6), insulin, EGF, TGF- $\alpha$ , FGF, G-CSF, VEGF, MCSF, GM-CSF, and transferrin.

32. The process of claim 31, wherein the cell-binding agent is an antibody.
33. The process of claim 32, wherein the antibody is a monoclonal antibody.
34. The process of claim 33, wherein the antibody is a humanized monoclonal antibody.
35. The process of any one of claims 32-34, wherein the antibody is selected from the group consisting of huN901, huMy9-6, huB4, huC242, trastuzumab, bivatuzumab, sibrotuzumab, CNTO95, huDS6, rituximab, anti-CD27L, anti-Her2, anti-EGFR, anti-EGFRvIII, Cripto, anti-CD138, anti-CD38, anti-EphA2, integrin targeting antibody, anti-CD37, anti-folate, anti-Her3 and anti-IGFIR.
36. The process of any one of claims 17-35, wherein the cytotoxic agent is selected from the group consisting of maytansinoids, taxanes, CC1065, and analogs of the foregoing.
37. The process of claim 36, wherein the cytotoxic agent is a maytansinoid.
38. The process of claim 37, wherein the maytansinoid comprises a thiol group.
39. The process of claim 38, wherein the maytansinoid is DM1.
40. The process of claim 38, wherein the maytansinoid is DM4.
41. The process of any one of claims 17-40, wherein the cell-binding agent is chemically coupled to the cytotoxic agent via chemical bonds selected from the group consisting of disulfide bonds, acid labile bonds, photolabile bonds, peptidase labile bonds, thioether bonds, and esterase labile bonds.
42. The process of any one of claims 17-40, wherein the bifunctional crosslinking reagent comprises an N-succinimidyl ester moiety, an N-sulfosuccinimidyl ester moiety, a maleimido-based moiety, or a haloacetyl-based moiety.
43. The process of claim 42, wherein the bifunctional crosslinking reagent comprises a maleimido-based moiety.



44. The process of claim 43, wherein the bifunctional crosslinking reagent is selected from the group consisting of N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC), N-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxy-(6-amidocaproate) (LC-SMCC),  $\kappa$ -maleimidoundecanoic acid N-succinimidyl ester (KMUA),  $\gamma$ -maleimidobutyric acid N-succinimidyl ester (GMBS),  $\beta$ -maleimidopropoxy-succinimidyl ester (BMPS),  $\epsilon$ -maleimidocaproic acid N-hydroxysuccinimide ester (EMCS), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-( $\alpha$ -maleimidoacetoxy)-succinimide ester (AMAS), succinimidyl-6-( $\beta$ -maleimidopropionamido)hexanoate (SMPH), N-succinimidyl 4-(p-maleimidophenyl)-butyrate (SMPB), N-(p-maleimidophenyl)isocyanate (PMPI), sulfo-Mal, PEG<sub>4</sub>-Mal and CX1-1.

45. The process of claim 44, wherein the bifunctional crosslinking reagent is N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC).

46. The process of any one of claims 17-45, wherein the solution in step (c) comprises sucrose.

47. The process of any one of claims 17-46, wherein the solution in step (c) comprises a buffering agent selected from the group consisting of a citrate buffer, an acetate buffer, a succinate buffer, and a phosphate buffer.

48. The process of any one of claims 17-46, wherein the solution in step (c) comprises a buffering agent selected from the group consisting of HEPPSO (N-(2-Hydroxyethyl)piperazine-N'-(2-hydroxypropanesulfonic acid)), POPSO (Piperazine-1,4-bis-(2-hydroxy-propane-sulfonic acid) dehydrate), HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), HEPPS (EPPS) (4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid), TES (N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid), and a combination thereof.

49. The process of any one of claims 17-48, further comprising  
(e) holding the mixture between at least one of steps a-b, steps b-c, and steps c-d to release the unstably bound linkers from the cell-binding agent.

50. A process for preparing a conjugate comprising a cell-binding agent chemically coupled to a cytotoxic agent, which process comprises:

(a) contacting a cell-binding agent with a bifunctional crosslinking reagent at a temperature of about 15° C or less to covalently attach a linker to the cell-binding agent and thereby prepare a first mixture comprising cell-binding agents having linkers bound thereto,

(b) conjugating a cytotoxic agent to the cell-binding agents having linkers bound thereto in the first mixture by reacting the cell-binding agents having linkers bound thereto with a cytotoxic agent in a solution having a pH of about 4 to about 9 to prepare a second mixture comprising (i) cell-binding agent chemically coupled through the linker to the cytotoxic agent, (ii) free cytotoxic agent, and (iii) reaction by-products, and

(c) subjecting the second mixture to tangential flow filtration, selective precipitation, non-adsorptive chromatography, adsorptive filtration, adsorptive chromatography, or a combination thereof, to purify the cell binding agents chemically coupled through the linkers to the cytotoxic agent from the other components of the second mixture and thereby prepare a purified second mixture of cell binding agents chemically coupled through the linkers to the cytotoxic agent.

51. The process of claim 50, wherein the contacting in step (a) occurs in a solution having a pH of about 7.5 to about 9.

52. The process of claim 51, wherein the solution comprises a buffering agent selected from a citrate buffer, an acetate buffer, a succinate buffer, and a phosphate buffer.

53. The process of claim 51, wherein the solution comprises a buffering agent selected from the group consisting of HEPPSO (N-(2-Hydroxyethyl)piperazine-N'-(2-hydroxypropanesulfonic acid)), POPSO (Piperazine-1,4-bis-(2-hydroxy-propane-sulfonic acid) dehydrate), HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), HEPPS (EPPS) (4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid), TES (N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid), and a combination thereof.

54. The process of any one of claims 51-53, wherein the pH is about 7.8.

55. The process of any one of claims 50-54, wherein the contacting in step (a) occurs at a temperature of about -10° C to about 15° C.

56. The process of claim 55, wherein the temperature is about 10° C.
57. The process of any one of claims 50-56, wherein the non-adsorptive chromatography is selected from the group consisting of SEPHADEX™ resins, SEPHACRYL™ resins, SUPERDEX™ resins, and BIO-GEL® resins.
58. The process of any one of claims 50-56, wherein the adsorptive chromatography is selected from the group consisting of hydroxyapatite chromatography, hydrophobic charge induction chromatography (HCIC), hydrophobic interaction chromatography (HIC), ion exchange chromatography, mixed mode ion exchange chromatography, immobilized metal affinity chromatography (IMAC), dye ligand chromatography, affinity chromatography, reversed phase chromatography, and combinations thereof.
59. The process of any one of claims 50-58, wherein the cell binding agent is selected from the group consisting of antibodies, interferons, interleukin 2 (IL-2), interleukin 3 (IL-3), interleukin 4 (IL-4), interleukin 6 (IL-6), insulin, EGF, TGF- $\alpha$ , FGF, G-CSF, VEGF, MCSF, GM-CSF, and transferrin.
60. The process of claim 59, wherein the cell binding agent is an antibody.
61. The process of claim 60, wherein the antibody is a monoclonal antibody.
62. The process of claim 61, wherein the antibody is a humanized monoclonal antibody.
63. The process of any one of claims 60-62, wherein the antibody is selected from the group consisting of huN901, huMy9-6, huB4, huC242, trastuzumab, bivatuzumab, sibrotuzumab, CNTO95, huDS6, rituximab, anti-CD27L, anti-Her2, anti-EGFR, anti-EGFRvIII, Cripto, anti-CD138, anti-CD38, anti-EphA2, integrin targeting antibody, anti-CD37, anti-folate, anti-Her3, and anti-IGFIR.
64. The process of any one of claims 50-63, wherein the cytotoxic agent is selected from the group consisting of maytansinoids, taxanes, CC1065, and analogs of the foregoing.
65. The process of claim 64, wherein the cytotoxic agent is a maytansinoid.

66. The process of claim 65, wherein the maytansinoid comprises a thiol group.
67. The process of claim 66, wherein the maytansinoid is DM1.
68. The process of claim 66, wherein the maytansinoid is DM4.
69. The process of any one of claims 50-68, wherein the cell binding agent is chemically coupled to the cytotoxic agent via chemical bonds selected from the group consisting of disulfide bonds, acid labile bonds, photolabile bonds, peptidase labile bonds, thioether bonds, and esterase labile bonds.
70. The process of any one of claims 50-69, wherein the bifunctional crosslinking reagent comprises an N-succinimidyl ester moiety, an N-sulfosuccinimidyl ester moiety, a maleimido-based moiety, or a haloacetyl-based moiety.
71. The process of claim 70, wherein the bifunctional crosslinking reagent comprises a maleimido-based moiety.
72. The process of claim 71, wherein the bifunctional crosslinking reagent is selected from the group consisting of N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC), N-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxy-(6-amidocaproate) (LC-SMCC),  $\kappa$ -maleimidoundecanoic acid N-succinimidyl ester (KMUA),  $\gamma$ -maleimidobutyric acid N-succinimidyl ester (GMBS),  $\beta$ -maleimidopropoxy-succinimidyl ester (BMPS),  $\epsilon$ -maleimidocaproic acid N-hydroxysuccinimide ester (EMCS), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-( $\alpha$ -maleimidoacetoxy)-succinimide ester (AMAS), succinimidyl-6-( $\beta$ -maleimidopropionamido)hexanoate (SMPH), N-succinimidyl 4-(p-maleimidophenyl)-butyrate (SMPB), and N-(p-maleimidophenyl)isocyanate (PMPI), sulfo-Mal, PEG<sub>4</sub>-Mal and CX1-1.
73. The process of claim 72, wherein the bifunctional crosslinking reagent is N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC).
74. The process of any one of claims 50-73, wherein the solution in step (b) comprises sucrose.

75. The process of any one of claims 50-74, wherein the solution in step (b) comprises a buffering agent selected from the group consisting of a citrate buffer, an acetate buffer, a succinate buffer, and a phosphate buffer.

76. The process of any one of claims 50-74, wherein the solution in step (b) comprises a buffering agent selected from the group consisting of HEPPSO (N-(2-Hydroxyethyl)piperazine-N'-(2-hydroxypropanesulfonic acid)), POPSO (Piperazine-1,4-bis-(2-hydroxy-propane-sulfonic acid) dehydrate), HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), HEPPS (EPPS) (4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid), TES (N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid), and a combination thereof.

77. The process of any one of claims 50-76, further comprising  
(d) holding the mixture between at least one of steps a-b and steps b-c to release the unstably bound linkers from the cell-binding agent.