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(54) Title: RECOMBINANT LECTINS, BINDING-SITE MODIFIED LECTINS AND USES THEREOF

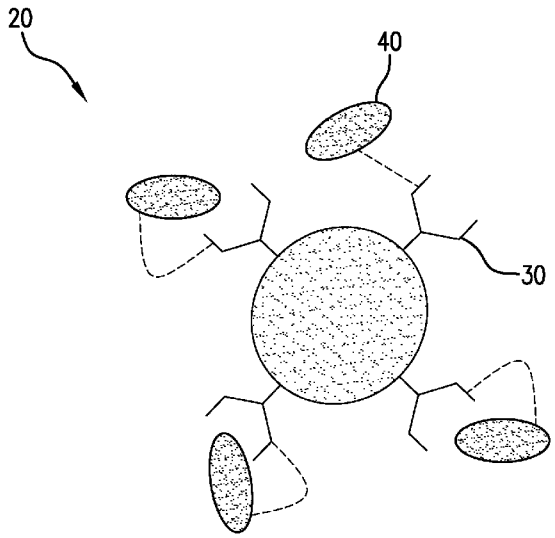


FIG.23

(57) Abstract: In one aspect, the disclosure provides cross-linked materials that include multivalent lectins with at least two binding sites for glucose, wherein the lectins include at least one affinity ligand which is capable of competing with glucose for binding with at least one of said binding sites and is covalently linked to a cysteine residue of the lectins; and conjugates that include two or more separate affinity ligands bound to a conjugate framework, wherein the two or more affinity ligands compete with glucose for binding with the lectins at said binding sites and wherein conjugates are cross-linked within the material as a result of non-covalent interactions between lectins and affinity ligands on different conjugates. These materials are designed to release amounts of conjugate in response to desired concentrations of glucose. Depending on the end application, in various embodiments, the conjugates may also include a drug and/or a detectable label.

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RECOMBINANT LECTINS, BINDING-SITE MODIFIED LECTINS AND USES THEREOF

BACKGROUND

The majority of "controlled-release" drug delivery systems known in the prior art (e.g., U.S. Patent No. 4,145,410 to Sears which describes drug release from capsules which are enzymatically labile) are incapable of releasing drugs at intervals and concentrations which are in direct proportion to the amount of a molecular indicator (e.g., a metabolite) present in the human body. The delivery or release of drug in these prior art systems is thus not literally "controlled," but simply a slow release which is independent of external or internal factors.

The treatment of diabetes mellitus with injectable insulin is a well-known and studied example where uncontrolled, slow release of insulin is undesirable. In fact, it is apparent that the simple replacement of the hormone is not sufficient to prevent the pathological sequelae associated with this disease. The development of these sequelae is believed to reflect an inability to provide exogenous insulin proportional to varying blood glucose concentrations experienced by the patient. To solve this problem several biological and bioengineering approaches to develop a more physiological insulin delivery system have been suggested (e.g., see U.S. Patent No. 4,348,387 to Brownlee et al.; U.S. Patent Nos. 5,830,506, 5,902,603, and 6,410,053 to Taylor et al. and U.S. Patent Application Publication No. 2004-0202719 to Zion et al.).

In certain embodiments of the Zion system multivalent glucose-binding molecules are combined with a glycosylated polymer-insulin conjugate. The glycosylated polymer contains multiple saccharide binding groups and forms insoluble hydrogels or particles in the presence of the glucose-binding molecule. The gel releases the glycosylated polymer-insulin conjugate in response to increases in glucose concentration. The Zion system has been demonstrated using the lectin concanavalin A (Con A) as an exemplary multivalent glucose-binding molecule. Unfortunately, Con A and many of the other readily available lectins have the potential to stimulate lymphocyte proliferation. By binding to carbohydrate receptors on the surfaces of certain types of lymphocytes, these so-called "mitogenic" lectins can potentially induce the mitosis of lymphocytes and thereby cause them to proliferate. Most mitogenic lectins including Con A are selective T-cell mitogens. A few lectins are less selective and stimulate both T-cells and B-cells. Local or systemic *in vivo* exposure to mitogenic lectins can result in inflammation, cytotoxicity, macrophage digestion, and allergic reactions including anaphylaxis. In addition,

plant lectins are known to be particularly immunogenic, giving rise to the production of high titers of anti-lectin specific antibodies. It will be appreciated that mitogenic lectins cannot therefore be used in their native form for *in vivo* methods and devices unless great care is taken to prevent their release. For example, in U.S. Patent No. 5,830,506, Taylor highlights the toxic risks that are involved in using Con A and emphasizes the importance and difficulty of containing Con A within a drug delivery device that also requires glucose and insulin molecules to diffuse freely in and out of the device.

The risks and difficulties that are involved with these and other *in vivo* uses of lectins could be avoided if a method existed for reducing the mitogenicity of lectins without interfering with their ability to function as cross-linking agents within a Zion system which responds to useful concentrations of glucose.

SUMMARY

In one aspect, the disclosure provides cross-linked materials that include multivalent lectins with at least two binding sites for glucose, wherein the lectins include at least one affinity ligand which is capable of competing with glucose for binding with at least one of said binding sites and is covalently linked to a cysteine residue of the lectins; and conjugates that include two or more separate affinity ligands bound to a conjugate framework, wherein the two or more affinity ligands compete with glucose for binding with the lectins at said binding sites and wherein conjugates are cross-linked within the material as a result of non-covalent interactions between lectins and affinity ligands on different conjugates. These materials are designed to release amounts of conjugate in response to desired concentrations of glucose. Depending on the end application, in various embodiments, the conjugates may also include a drug and/or a detectable label. The drug, detectable label and affinity ligands may be covalently or non-covalently bound to the conjugate framework. The disclosure also provides methods of using these materials and methods of making these materials. In another aspect, the disclosure provides exemplary recombinant lectins (unmodified or modified with an affinity ligand) for use in glucose responsive materials instead of native lectins such as Con A.

DEFINITIONS

Definitions of specific functional groups, chemical terms, and general terms used throughout the specification are described in more detail below. For purposes of this invention,

the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, Handbook of Chemistry and Physics, 75th Ed., inside cover, and specific functional groups are generally defined as described therein. Additionally, general principles of organic chemistry, as well as specific functional moieties and reactivity, are described in Organic Chemistry, Thomas Sorrell, University Science Books, Sausalito, 1999; Smith and March March's Advanced Organic Chemistry, 5th Edition, John Wiley & Sons, Inc., New York, 2001; Larock, Comprehensive Organic Transformations, VCH Publishers, Inc., New York, 1989; Carruthers, Some Modern Methods of Organic Synthesis, 3rd Edition, Cambridge University Press, Cambridge, 1987.

Acyl – As used herein, the term "acyl," refers to a group having the general formula –$C(=O)R^{X1}$, –$C(=O)OR^{X1}$, –$C(=O)–O–C(=O)R^{X1}$, –$C(=O)SR^{X1}$, –$C(=O)N(R^{X1})_2$, –$C(=S)R^{X1}$, –$C(=S)N(R^{X1})_2$, and –$C(=S)S(R^{X1})$, –$C(=NR^{X1})R^{X1}$, –$C(=NR^{X1})OR^{X1}$, –$C(=NR^{X1})SR^{X1}$, and –$C(=NR^{X1})N(R^{X1})_2$, wherein R^{X1} is hydrogen; halogen; substituted or unsubstituted hydroxyl; substituted or unsubstituted thiol; substituted or unsubstituted amino; substituted or unsubstituted acyl; cyclic or acyclic, substituted or unsubstituted, branched or unbranched aliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched heteroaliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched alkyl; cyclic or acyclic, substituted or unsubstituted, branched or unbranched alkenyl; substituted or unsubstituted alkynyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, aliphaticoxy, heteroaliphaticoxy, alkyloxy, heteroalkyloxy, aryloxy, heteroaryloxy, aliphaticthioxy, heteroaliphaticthioxy, alkylthioxy, heteroalkylthioxy, arylthioxy, heteroarylthioxy, mono– or di– aliphaticamino, mono– or di– heteroaliphaticamino, mono– or di– alkylamino, mono– or di– heteroalkylamino, mono– or di– arylamino, or mono– or di– heteroarylamino; or two R^{X1} groups taken together form a 5– to 6– membered heterocyclic ring. Exemplary acyl groups include aldehydes (–CHO), carboxylic acids (–CO_2H), ketones, acyl halides, esters, amides, imines, carbonates, carbamates, and ureas. Acyl substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety (e.g., aliphatic, alkyl, alkenyl, alkynyl, heteroaliphatic, heterocyclic, aryl, heteroaryl, acyl, oxo, imino, thiooxo, cyano, isocyano, amino, azido, nitro, hydroxyl, thiol, halo, aliphaticamino, heteroaliphaticamino, alkylamino, heteroalkylamino, arylamino, heteroarylamino, alkylaryl, arylalkyl, aliphaticoxy, heteroaliphaticoxy, alkyloxy, heteroalkyloxy, aryloxy, heteroaryloxy, aliphaticthioxy,

heteroaliphaticthioxy, alkylthioxy, heteroalkylthioxy, arylthioxy, heteroarylthioxy, acyloxy, and the like, each of which may or may not be further substituted).

Aliphatic – As used herein, the term "aliphatic" or "aliphatic group" denotes an optionally substituted hydrocarbon moiety that may be straight–chain (i.e., unbranched), branched, or cyclic ("carbocyclic") and may be completely saturated or may contain one or more units of unsaturation, but which is not aromatic. Unless otherwise specified, aliphatic groups contain 1-12 carbon atoms. In some embodiments, aliphatic groups contain 1-6 carbon atoms. In some embodiments, aliphatic groups contain 1-4 carbon atoms, and in yet other embodiments aliphatic groups contain 1-3 carbon atoms. Suitable aliphatic groups include, but are not limited to, linear or branched, alkyl, alkenyl, and alkynyl groups, and hybrids thereof such as (cycloalkyl)alkyl, (cycloalkenyl)alkyl or (cycloalkyl)alkenyl.

Alkenyl – As used herein, the term "alkenyl" denotes an optionally substituted monovalent group derived from a straight– or branched–chain aliphatic moiety having at least one carbon–carbon double bond by the removal of a single hydrogen atom. In certain embodiments, the alkenyl group employed in the invention contains 2-6 carbon atoms. In certain embodiments, the alkenyl group employed in the invention contains 2-5 carbon atoms. In some embodiments, the alkenyl group employed in the invention contains 2-4 carbon atoms. In another embodiment, the alkenyl group employed contains 2-3 carbon atoms. Alkenyl groups include, for example, ethenyl, propenyl, butenyl, 1–methyl–2–buten–1–yl, and the like.

Alkyl – As used herein, the term "alkyl" refers to optionally substituted saturated, straight– or branched–chain hydrocarbon radicals derived from an aliphatic moiety containing between 1-6 carbon atoms by removal of a single hydrogen atom. In some embodiments, the alkyl group employed in the invention contains 1-5 carbon atoms. In another embodiment, the alkyl group employed contains 1-4 carbon atoms. In still other embodiments, the alkyl group contains 1-3 carbon atoms. In yet another embodiments, the alkyl group contains 1-2 carbons. Examples of alkyl radicals include, but are not limited to, methyl, ethyl, n–propyl, isopropyl, n–butyl, iso–butyl, sec–butyl, sec–pentyl, iso–pentyl, tert–butyl, n–pentyl, neopentyl, n–hexyl, sec–hexyl, n–heptyl, n–octyl, n–decyl, n–undecyl, dodecyl, and the like.

Alkynyl – As used herein, the term "alkynyl" refers to an optionally substituted monovalent group derived from a straight– or branched–chain aliphatic moiety having at least one carbon–carbon triple bond by the removal of a single hydrogen atom. In certain embodiments, the alkynyl group employed in the invention contains 2-6 carbon atoms. In certain

embodiments, the alkynyl group employed in the invention contains 2-5 carbon atoms. In some embodiments, the alkynyl group employed in the invention contains 2-4 carbon atoms. In another embodiment, the alkynyl group employed contains 2-3 carbon atoms. Representative alkynyl groups include, but are not limited to, ethynyl, 2–propynyl (propargyl), 1–propynyl, and the like.

Aryl – As used herein, the term "aryl" used alone or as part of a larger moiety as in "aralkyl", "aralkoxy", or "aryloxyalkyl", refers to an optionally substituted monocyclic and bicyclic ring systems having a total of five to 10 ring members, wherein at least one ring in the system is aromatic and wherein each ring in the system contains three to seven ring members. The term "aryl" may be used interchangeably with the term "aryl ring". In certain embodiments of the present invention, "aryl" refers to an aromatic ring system which includes, but not limited to, phenyl, biphenyl, naphthyl, anthracyl and the like, which may bear one or more substituents.

Arylalkyl – As used herein, the term "arylalkyl" refers to an alkyl group substituted with an aryl group (e.g., an aromatic or heteroaromatic group).

Bivalent hydrocarbon chain – As used herein, the term "bivalent hydrocarbon chain" (also referred to as a "bivalent alkylene group") is a polymethylene group, i.e., $-(CH_2)_z-$, wherein z is a positive integer from 1 to 30, from 1 to 20, from 1 to 12, from 1 to 8, from 1 to 6, from 1 to 4, from 1 to 3, from 1 to 2, from 2 to 30, from 2 to 20, from 2 to 10, from 2 to 8, from 2 to 6, from 2 to 4, or from 2 to 3. A substituted bivalent hydrocarbon chain is a polymethylene group in which one or more methylene hydrogen atoms are replaced with a substituent. Suitable substituents include those described below for a substituted aliphatic group.

Carbonyl – As used herein, the term "carbonyl" refers to a monovalent or bivalent moiety containing a carbon-oxygen double bond. Non-limiting examples of carbonyl groups include aldehydes, ketones, carboxylic acids, ester, amide, enones, acyl halides, anhydrides, ureas, carbamates, carbonates, thioesters, lactones, lactams, hydroxamates, isocyanates, and chloroformates.

Cycloaliphatic – As used herein, the terms "cycloaliphatic", "carbocycle", or "carbocyclic", used alone or as part of a larger moiety, refer to an optionally substituted saturated or partially unsaturated cyclic aliphatic monocyclic or bicyclic ring systems, as described herein, having from 3 to 10 members. Cycloaliphatic groups include, without limitation, cyclopropyl, cyclobutyl, cyclopentyl, cyclopentenyl, cyclohexyl, cyclohexenyl, cycloheptyl, cycloheptenyl,

cyclooctyl, cyclooctenyl, and cyclooctadienyl. In some embodiments, the cycloalkyl has 3-6 carbons.

Halogen – As used herein, the terms "halo" and "halogen" refer to an atom selected from fluorine (fluoro, –F), chlorine (chloro, –Cl), bromine (bromo, –Br), and iodine (iodo, –I).

Heteroaliphatic – As used herein, the terms "heteroaliphatic" or "heteroaliphatic group", denote an optionally substituted hydrocarbon moiety having, in addition to carbon atoms, from one to five heteroatoms, that may be straight–chain (i.e., unbranched), branched, or cyclic ("heterocyclic") and may be completely saturated or may contain one or more units of unsaturation, but which is not aromatic. Unless otherwise specified, heteroaliphatic groups contain 1-6 carbon atoms wherein 1-3 carbon atoms are optionally and independently replaced with heteroatoms selected from oxygen, nitrogen and sulfur. In some embodiments, heteroaliphatic groups contain 1-4 carbon atoms, wherein 1-2 carbon atoms are optionally and independently replaced with heteroatoms selected from oxygen, nitrogen and sulfur. In yet other embodiments, heteroaliphatic groups contain 1-3 carbon atoms, wherein 1 carbon atom is optionally and independently replaced with a heteroatom selected from oxygen, nitrogen and sulfur. Suitable heteroaliphatic groups include, but are not limited to, linear or branched, heteroalkyl, heteroalkenyl, and heteroalkynyl groups.

Heteroaralkyl – As used herein, the term "heteroaralkyl" refers to an alkyl group substituted by a heteroaryl, wherein the alkyl and heteroaryl portions independently are optionally substituted.

Heteroaryl – As used herein, the term "heteroaryl" used alone or as part of a larger moiety, e.g., "heteroaralkyl", or "heteroaralkoxy", refers to an optionally substituted group having 5 to 10 ring atoms, preferably 5, 6, or 9 ring atoms; having 6, 10, or 14 π electrons shared in a cyclic array; and having, in addition to carbon atoms, from one to five heteroatoms. Heteroaryl groups include, without limitation, thienyl, furanyl, pyrrolyl, imidazolyl, pyrazolyl, triazolyl, tetrazolyl, oxazolyl, isoxazolyl, oxadiazolyl, thiazolyl, isothiazolyl, thiadiazolyl, pyridyl, pyridazinyl, pyrimidinyl, pyrazinyl, indolizinyl, purinyl, naphthyridinyl, and pteridinyl. The terms "heteroaryl" and "heteroar–", as used herein, also include groups in which a heteroaromatic ring is fused to one or more aryl, carbocyclic, or heterocyclic rings, where the radical or point of attachment is on the heteroaromatic ring. Non limiting examples include indolyl, isoindolyl, benzothienyl, benzofuranyl, dibenzofuranyl, indazolyl, benzimidazolyl, benzthiazolyl, quinolyl, isoquinolyl, cinnolinyl, phthalazinyl, quinazolinyl, quinoxalinyl, 4*H*–

quinolizinyl, carbazolyl, acridinyl, phenazinyl, phenothiazinyl, phenoxazinyl, tetrahydroquinolinyl, and tetrahydroisoquinolinyl. A heteroaryl group may be mono– or bicyclic. The term "heteroaryl" may be used interchangeably with the terms "heteroaryl ring", "heteroaryl group", or "heteroaromatic", any of which terms include rings that are optionally substituted.

Heteroatom – As used herein, the term "heteroatom" refers to nitrogen, oxygen, or sulfur, and includes any oxidized form of nitrogen or sulfur, and any quaternized form of a basic nitrogen. The term "nitrogen" also includes a substituted nitrogen.

Heterocyclic – As used herein, the terms "heterocycle", "heterocyclyl", "heterocyclic radical", and "heterocyclic ring" are used interchangeably and refer to a stable optionally substituted 5- to 7-membered monocyclic or 7- to 10-membered bicyclic heterocyclic moiety that is either saturated or partially unsaturated, and having, in addition to carbon atoms, one or more heteroatoms, as defined above. A heterocyclic ring can be attached to its pendant group at any heteroatom or carbon atom that results in a stable structure and any of the ring atoms can be optionally substituted. Examples of such saturated or partially unsaturated heterocyclic radicals include, without limitation, tetrahydrofuranyl, tetrahydrothienyl, pyrrolidinyl, pyrrolidonyl, piperidinyl, pyrrolinyl, tetrahydroquinolinyl, tetrahydroisoquinolinyl, decahydroquinolinyl, oxazolidinyl, piperazinyl, dioxanyl, dioxolanyl, diazepinyl, oxazepinyl, thiazepinyl, morpholinyl, and quinuclidinyl. The terms "heterocycle", "heterocyclyl", "heterocyclyl ring", "heterocyclic group", "heterocyclic moiety", and "heterocyclic radical", are used interchangeably herein, and also include groups in which a heterocyclyl ring is fused to one or more aryl, heteroaryl, or carbocyclic rings, such as indolinyl, 3*H*–indolyl, chromanyl, phenanthridinyl, or tetrahydroquinolinyl, where the radical or point of attachment is on the heterocyclyl ring. A heterocyclyl group may be mono– or bicyclic. The term "heterocyclylalkyl" refers to an alkyl group substituted by a heterocyclyl, wherein the alkyl and heterocyclyl portions independently are optionally substituted.

Unsaturated – As used herein, the term "unsaturated", means that a moiety has one or more double or triple bonds.

Partially unsaturated – As used herein, the term "partially unsaturated" refers to a ring moiety that includes at least one double or triple bond. The term "partially unsaturated" is intended to encompass rings having multiple sites of unsaturation, but is not intended to include aryl or heteroaryl moieties, as herein defined.

Optionally substituted – As described herein, compounds of the invention may contain "optionally substituted" moieties. In general, the term "substituted", whether preceded by the term "optionally" or not, means that one or more hydrogens of the designated moiety are replaced with a suitable substituent. Unless otherwise indicated, an "optionally substituted" group may have a suitable substituent at each substitutable position of the group, and when more than one position in any given structure may be substituted with more than one substituent selected from a specified group, the substituent may be either the same or different at every position. Combinations of substituents envisioned by this invention are preferably those that result in the formation of stable or chemically feasible compounds. The term "stable", as used herein, refers to compounds that are not substantially altered when subjected to conditions to allow for their production, detection, and, in certain embodiments, their recovery, purification, and use for one or more of the purposes disclosed herein.

Suitable monovalent substituents on a substitutable carbon atom of an "optionally substituted" group are independently halogen; $-(CH_2)_{0-4}R^\circ$; $-(CH_2)_{0-4}OR^\circ$; $-O-(CH_2)_{0-4}C(O)OR^\circ$; $-(CH_2)_{0-4}CH(OR^\circ)_2$; $-(CH_2)_{0-4}SR^\circ$; $-(CH_2)_{0-4}Ph$, which may be substituted with R°; $-(CH_2)_{0-4}O(CH_2)_{0-1}Ph$ which may be substituted with R°; –CH=CHPh, which may be substituted with R°; $-NO_2$; –CN; $-N_3$; $-(CH_2)_{0-4}N(R^\circ)_2$; $-(CH_2)_{0-4}N(R^\circ)C(O)R^\circ$; $-N(R^\circ)C(S)R^\circ$; $-(CH_2)_{0-4}N(R^\circ)C(O)NR^\circ_2$; $-N(R^\circ)C(S)NR^\circ_2$; $-(CH_2)_{0-4}N(R^\circ)C(O)OR^\circ$; $-N(R^\circ)N(R^\circ)C(O)R^\circ$; $-N(R^\circ)N(R^\circ)C(O)NR^\circ_2$; $-N(R^\circ)N(R^\circ)C(O)OR^\circ$; $-(CH_2)_{0-4}C(O)R^\circ$; $-C(S)R^\circ$; $-(CH_2)_{0-4}C(O)OR^\circ$; $-(CH_2)_{0-4}C(O)SR^\circ$; $-(CH_2)_{0-4}C(O)OSiR^\circ_3$; $-(CH_2)_{0-4}OC(O)R^\circ$; $-OC(O)(CH_2)_{0-4}SR-$, SC(S)SR°; $-(CH_2)_{0-4}SC(O)R^\circ$; $-(CH_2)_{0-4}C(O)NR^\circ_2$; $-C(S)NR^\circ_2$; $-C(S)SR^\circ$; –SC(S)SR°, $-(CH_2)_{0-4}OC(O)NR^\circ_2$; $-C(O)N(OR^\circ)R^\circ$; $-C(O)C(O)R^\circ$; $-C(O)CH_2C(O)R^\circ$; $-C(NOR^\circ)R^\circ$; $-(CH_2)_{0-4}SSR^\circ$; $-(CH_2)_{0-4}S(O)_2R^\circ$; $-(CH_2)_{0-4}S(O)_2OR^\circ$; $-(CH_2)_{0-4}OS(O)_2R^\circ$; $-S(O)_2NR^\circ_2$; $-(CH_2)_{0-4}S(O)R^\circ$; $-N(R^\circ)S(O)_2NR^\circ_2$; $-N(R^\circ)S(O)_2R^\circ$; $-N(OR^\circ)R^\circ$; $-C(NH)NR^\circ_2$; $-P(O)_2R^\circ$; $-P(O)R^\circ_2$; $-OP(O)R^\circ_2$; $-OP(O)(OR^\circ)_2$; SiR°_3; $-(C_{1-4}$ straight or branched alkylene)$O-N(R^\circ)_2$; or $-(C_{1-4}$ straight or branched alkylene)$C(O)O-N(R^\circ)_2$, wherein each R° may be substituted as defined below and is independently hydrogen, C_{1-6} aliphatic, $-CH_2Ph$, $-O(CH_2)_{0-1}Ph$, or a 5–6–membered saturated, partially unsaturated, or aryl ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur, or, notwithstanding the definition above, two independent occurrences of R°, taken together with their intervening atom(s), form a 3–12–membered saturated, partially unsaturated, or aryl mono– or bicyclic ring having 0–4

heteroatoms independently selected from nitrogen, oxygen, or sulfur, which may be substituted as defined below.

Suitable monovalent substituents on R° (or the ring formed by taking two independent occurrences of R° together with their intervening atoms), are independently halogen, $-(CH_2)_{0-2}R^{\bullet}$, $-(haloR^{\bullet})$, $-(CH_2)_{0-2}OH$, $-(CH_2)_{0-2}OR^{\bullet}$, $-(CH_2)_{0-2}CH(OR^{\bullet})_2$; $-O(haloR^{\bullet})$, $-CN$, $-N_3$, $-(CH_2)_{0-2}C(O)R^{\bullet}$, $-(CH_2)_{0-2}C(O)OH$, $-(CH_2)_{0-2}C(O)OR^{\bullet}$, $-(CH_2)_{0-2}SR^{\bullet}$, $-(CH_2)_{0-2}SH$, $-(CH_2)_{0-2}NH_2$, $-(CH_2)_{0-2}NHR^{\bullet}$, $-(CH_2)_{0-2}NR^{\bullet}_2$, $-NO_2$, $-SiR^{\bullet}_3$, $-OSiR^{\bullet}_3$, $-C(O)SR^{\bullet}$, $-(C_{1-4}$ straight or branched alkylene)$C(O)OR^{\bullet}$, or $-SSR^{\bullet}$ wherein each $R^{\bullet}$ is unsubstituted or where preceded by "halo" is substituted only with one or more halogens, and is independently selected from C_{1-4} aliphatic, $-CH_2Ph$, $-O(CH_2)_{0-1}Ph$, or a 5–6–membered saturated, partially unsaturated, or aryl ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. Suitable divalent substituents on a saturated carbon atom of R° include =O and =S.

Suitable divalent substituents on a saturated carbon atom of an "optionally substituted" group include the following: =O, =S, $=NNR^*_2$, $=NNHC(O)R^*$, $=NNHC(O)OR^*$, $=NNHS(O)_2R^*$, $=NR^*$, $=NOR^*$, $-O(C(R^*_2))_{2-3}O-$, or $-S(C(R^*_2))_{2-3}S-$, wherein each independent occurrence of R^* is selected from hydrogen, C_{1-6} aliphatic which may be substituted as defined below, or an unsubstituted 5–6–membered saturated, partially unsaturated, or aryl ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. Suitable divalent substituents that are bound to vicinal substitutable carbons of an "optionally substituted" group include: $-O(CR^*_2)_{2-3}O-$, wherein each independent occurrence of R^* is selected from hydrogen, C_{1-6} aliphatic which may be substituted as defined below, or an unsubstituted 5–6–membered saturated, partially unsaturated, or aryl ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

Suitable substituents on the aliphatic group of R^* include halogen, $-R^{\bullet}$, $-(haloR^{\bullet})$, $-OH$, $-OR^{\bullet}$, $-O(haloR^{\bullet})$, $-CN$, $-C(O)OH$, $-C(O)OR^{\bullet}$, $-NH_2$, $-NHR^{\bullet}$, $-NR^{\bullet}_2$, or $-NO_2$, wherein each $R^{\bullet}$ is unsubstituted or where preceded by "halo" is substituted only with one or more halogens, and is independently C_{1-4} aliphatic, $-CH_2Ph$, $-O(CH_2)_{0-1}Ph$, or a 5–6–membered saturated, partially unsaturated, or aryl ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

Suitable substituents on a substitutable nitrogen of an "optionally substituted" group include $-R^{\dagger}$, $-NR^{\dagger}_2$, $-C(O)R^{\dagger}$, $-C(O)OR^{\dagger}$, $-C(O)C(O)R^{\dagger}$, $-C(O)CH_2C(O)R^{\dagger}$, $-S(O)_2R^{\dagger}$, $-S(O)_2NR^{\dagger}_2$, $-C(S)NR^{\dagger}_2$, $-C(NH)NR^{\dagger}_2$, or $-N(R^{\dagger})S(O)_2R^{\dagger}$; wherein each $R^{\dagger}$ is independently

hydrogen, C_{1-6} aliphatic which may be substituted as defined below, unsubstituted –OPh, or an unsubstituted 5–6–membered saturated, partially unsaturated, or aryl ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur, or, notwithstanding the definition above, two independent occurrences of $R^{\dagger}$, taken together with their intervening atom(s) form an unsubstituted 3–12–membered saturated, partially unsaturated, or aryl mono– or bicyclic ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

Suitable substituents on the aliphatic group of $R^{\dagger}$ are independently halogen, $-R^{\bullet}$, –(haloR$^{\bullet}$), –OH, $-OR^{\bullet}$, –O(haloR$^{\bullet}$), –CN, –C(O)OH, $-C(O)OR^{\bullet}$, $-NH_2$, $-NHR^{\bullet}$, $-NR^{\bullet}_2$, or $-NO_2$, wherein each $R^{\bullet}$ is unsubstituted or where preceded by "halo" is substituted only with one or more halogens, and is independently C_{1-4} aliphatic, $-CH_2Ph$, $-O(CH_2)_{0-1}Ph$, or a 5–6–membered saturated, partially unsaturated, or aryl ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

Suitable protecting group – As used herein, the term "suitable protecting group," refers to amino protecting groups or hydroxyl protecting groups depending on its location within the compound and includes those described in detail in *Protecting Groups in Organic Synthesis*, T. W. Greene and P. G. M. Wuts, 3rd edition, John Wiley & Sons, 1999.

Suitable amino–protecting groups include methyl carbamate, ethyl carbamante, 9–fluorenylmethyl carbamate (Fmoc), 9–(2–sulfo)fluorenylmethyl carbamate, 9–(2,7–dibromo)fluoroenylmethyl carbamate, 2,7–di–*t*–butyl–[9–(10,10–dioxo–10,10,10,10–tetrahydrothioxanthyl)]methyl carbamate (DBD–Tmoc), 4–methoxyphenacyl carbamate (Phenoc), 2,2,2–trichloroethyl carbamate (Troc), 2–trimethylsilylethyl carbamate (Teoc), 2–phenylethyl carbamate (hZ), 1–(1–adamantyl)–1–methylethyl carbamate (Adpoc), 1,1–dimethyl–2–haloethyl carbamate, 1,1–dimethyl–2,2–dibromoethyl carbamate (DB–*t*–BOC), 1,1–dimethyl–2,2,2–trichloroethyl carbamate (TCBOC), 1–methyl–1–(4–biphenylyl)ethyl carbamate (Bpoc), 1–(3,5–di–*t*–butylphenyl)–1–methylethyl carbamate (*t*–Bumeoc), 2–(2'– and 4'–pyridyl)ethyl carbamate (Pyoc), 2–(*N,N*–dicyclohexylcarboxamido)ethyl carbamate, *t*–butyl carbamate (BOC), 1–adamantyl carbamate (Adoc), vinyl carbamate (Voc), allyl carbamate (Alloc), 1–isopropylallyl carbamate (Ipaoc), cinnamyl carbamate (Coc), 4–nitrocinnamyl carbamate (Noc), 8–quinolyl carbamate, *N*–hydroxypiperidinyl carbamate, alkyldithio carbamate, benzyl carbamate (Cbz), *p*–methoxybenzyl carbamate (Moz), *p*–nitobenzyl carbamate, *p*–bromobenzyl carbamate, *p*–chlorobenzyl carbamate, 2,4–dichlorobenzyl carbamate, 4–methylsulfinylbenzyl carbamate (Msz), 9–anthrylmethyl carbamate, diphenylmethyl carbamate, 2–methylthioethyl carbamate, 2–

methylsulfonylethyl carbamate, 2–(*p*–toluenesulfonyl)ethyl carbamate, [2–(1,3–dithianyl)]methyl carbamate (Dmoc), 4–methylthiophenyl carbamate (Mtpc), 2,4–dimethylthiophenyl carbamate (Bmpc), 2–phosphonioethyl carbamate (Peoc), 2–triphenylphosphonioisopropyl carbamate (Ppoc), 1,1–dimethyl–2–cyanoethyl carbamate, *m*–chloro–*p*–acyloxybenzyl carbamate, *p*–(dihydroxyboryl)benzyl carbamate, 5–benzisoxazolylmethyl carbamate, 2–(trifluoromethyl)–6–chromonylmethyl carbamate (Tcroc), *m*–nitrophenyl carbamate, 3,5–dimethoxybenzyl carbamate, *o*–nitrobenzyl carbamate, 3,4–dimethoxy–6–nitrobenzyl carbamate, phenyl(*o*–nitrophenyl)methyl carbamate, phenothiazinyl–(10)–carbonyl derivative, *N'*–*p*–toluenesulfonylaminocarbonyl derivative, *N'*–phenylaminothiocarbonyl derivative, *t*–amyl carbamate, *S*–benzyl thiocarbamate, *p*–cyanobenzyl carbamate, cyclobutyl carbamate, cyclohexyl carbamate, cyclopentyl carbamate, cyclopropylmethyl carbamate, *p*–decyloxybenzyl carbamate, 2,2–dimethoxycarbonylvinyl carbamate, *o*–(*N,N*–dimethylcarboxamido)benzyl carbamate, 1,1–dimethyl–3–(*N,N*–dimethylcarboxamido)propyl carbamate, 1,1–dimethylpropynyl carbamate, di(2–pyridyl)methyl carbamate, 2–furanylmethyl carbamate, 2–iodoethyl carbamate, isoborynl carbamate, isobutyl carbamate, isonicotinyl carbamate, *p*–(*p'*–methoxyphenylazo)benzyl carbamate, 1–methylcyclobutyl carbamate, 1–methylcyclohexyl carbamate, 1–methyl–1–cyclopropylmethyl carbamate, 1–methyl–1–(3,5–dimethoxyphenyl)ethyl carbamate, 1–methyl–1–(*p*–phenylazophenyl)ethyl carbamate, 1–methyl–1–phenylethyl carbamate, 1–methyl–1–(4–pyridyl)ethyl carbamate, phenyl carbamate, *p*–(phenylazo)benzyl carbamate, 2,4,6–tri–*t*–butylphenyl carbamate, 4–(trimethylammonium)benzyl carbamate, 2,4,6–trimethylbenzyl carbamate, formamide, acetamide, chloroacetamide, trichloroacetamide, trifluoroacetamide, phenylacetamide, 3–phenylpropanamide, picolinamide, 3–pyridylcarboxamide, *N*–benzoylphenylalanyl derivative, benzamide, *p*–phenylbenzamide, *o*–nitophenylacetamide, *o*–nitrophenoxyacetamide, acetoacetamide, (*N'*–dithiobenzyloxycarbonylamino)acetamide, 3–(*p*–hydroxyphenyl)propanamide, 3–(*o*–nitrophenyl)propanamide, 2–methyl–2–(*o*–nitrophenoxy)propanamide, 2–methyl–2–(*o*–phenylazophenoxy)propanamide, 4–chlorobutanamide, 3–methyl–3–nitrobutanamide, *o*–nitrocinnamide, *N*–acetylmethionine derivative, *o*–nitrobenzamide, *o*–(benzoyloxymethyl)benzamide, 4,5–diphenyl–3–oxazolin–2–one, *N*–phthalimide, *N*–dithiasuccinimide (Dts), *N*–2,3–diphenylmaleimide, *N*–2,5–dimethylpyrrole, *N*–1,1,4,4–tetramethyldisilylazacyclopentane adduct (STABASE), 5–substituted 1,3–dimethyl–1,3,5–triazacyclohexan–2–one, 5–substituted 1,3–dibenzyl–1,3,5–

triazacyclohexan–2–one, 1–substituted 3,5–dinitro–4–pyridone, *N*–methylamine, *N*–allylamine, *N*–[2–(trimethylsilyl)ethoxy]methylamine (SEM), *N*–3–acetoxypropylamine, *N*–(1–isopropyl–4–nitro–2–oxo–3–pyroolin–3–yl)amine, quaternary ammonium salts, *N*–benzylamine, *N*–di(4–methoxyphenyl)methylamine, *N*–5–dibenzosuberylamine, *N*–triphenylmethylamine (Tr), *N*–[(4–methoxyphenyl)diphenylmethyl]amine (MMTr), *N*–9–phenylfluorenylamine (PhF), *N*–2,7–dichloro–9–fluorenylmethyleneamine, *N*–ferrocenylmethylamino (Fcm), *N*–2–picolylamino *N'*–oxide, *N*–1,1–dimethylthiomethyleneamine, *N*–benzylideneamine, *N*–*p*–methoxybenzylideneamine, *N*–diphenylmethyleneamine, *N*–[(2–pyridyl)mesityl]methyleneamine, *N*–(*N',N'*–dimethylaminomethylene)amine, *N,N'*–isopropylidenediamine, *N*–*p*–nitrobenzylideneamine, *N*–salicylideneamine, *N*–5–chlorosalicylideneamine, *N*–(5–chloro–2–hydroxyphenyl)phenylmethyleneamine, *N*–cyclohexylideneamine, *N*–(5,5–dimethyl–3–oxo–1–cyclohexenyl)amine, *N*–borane derivative, *N*–diphenylborinic acid derivative, *N*–[phenyl(pentacarbonylchromium– or tungsten)carbonyl]amine, *N*–copper chelate, *N*–zinc chelate, *N*–nitroamine, *N*–nitrosoamine, amine *N*–oxide, diphenylphosphinamide (Dpp), dimethylthiophosphinamide (Mpt), diphenylthiophosphinamide (Ppt), dialkyl phosphoramidates, dibenzyl phosphoramidate, diphenyl phosphoramidate, benzenesulfenamide, *o*–nitrobenzenesulfenamide (Nps), 2,4–dinitrobenzenesulfenamide, pentachlorobenzenesulfenamide, 2–nitro–4–methoxybenzenesulfenamide, triphenylmethylsulfenamide, 3–nitropyridinesulfenamide (Npys), *p*–toluenesulfonamide (Ts), benzenesulfonamide, 2,3,6,–trimethyl–4–methoxybenzenesulfonamide (Mtr), 2,4,6–trimethoxybenzenesulfonamide (Mtb), 2,6–dimethyl–4–methoxybenzenesulfonamide (Pme), 2,3,5,6–tetramethyl–4–methoxybenzenesulfonamide (Mte), 4–methoxybenzenesulfonamide (Mbs), 2,4,6–trimethylbenzenesulfonamide (Mts), 2,6–dimethoxy–4–methylbenzenesulfonamide (iMds), 2,2,5,7,8–pentamethylchroman–6–sulfonamide (Pmc), methanesulfonamide (Ms), β–trimethylsilylethanesulfonamide (SES), 9–anthracenesulfonamide, 4–(4',8'–dimethoxynaphthylmethyl)benzenesulfonamide (DNMBS), benzylsulfonamide, trifluoromethylsulfonamide, and phenacylsulfonamide.

Suitable hydroxyl protecting groups include methyl, methoxylmethyl (MOM), methylthiomethyl (MTM), *t*–butylthiomethyl, (phenyldimethylsilyl)methoxymethyl (SMOM), benzyloxymethyl (BOM), *p*–methoxybenzyloxymethyl (PMBM), (4–methoxyphenoxy)methyl (*p*–AOM), guaiacolmethyl (GUM), *t*–butoxymethyl, 4–pentenyloxymethyl (POM), siloxymethyl, 2–methoxyethoxymethyl (MEM), 2,2,2–trichloroethoxymethyl, bis(2–

chloroethoxy)methyl, 2–(trimethylsilyl)ethoxymethyl (SEMOR), tetrahydropyranyl (THP), 3–bromotetrahydropyranyl, tetrahydrothiopyranyl, 1–methoxycyclohexyl, 4–methoxytetrahydropyranyl (MTHP), 4–methoxytetrahydrothiopyranyl, 4–methoxytetrahydrothiopyranyl S,S–dioxide, 1–[(2–chloro–4–methyl)phenyl]–4–methoxypiperidin–4–yl (CTMP), 1,4–dioxan–2–yl, tetrahydrofuranyl, tetrahydrothiofuranyl, 2,3,3a,4,5,6,7,7a–octahydro–7,8,8–trimethyl–4,7–methanobenzofuran–2–yl, 1–ethoxyethyl, 1–(2–chloroethoxy)ethyl, 1–methyl–1–methoxyethyl, 1–methyl–1–benzyloxyethyl, 1–methyl–1–benzyloxy–2–fluoroethyl, 2,2,2–trichloroethyl, 2–trimethylsilylethyl, 2–(phenylselenyl)ethyl, *t*–butyl, allyl, *p*–chlorophenyl, *p*–methoxyphenyl, 2,4–dinitrophenyl, benzyl, *p*–methoxybenzyl, 3,4–dimethoxybenzyl, *o*–nitrobenzyl, *p*–nitrobenzyl, *p*–halobenzyl, 2,6–dichlorobenzyl, *p*–cyanobenzyl, *p*–phenylbenzyl, 2–picolyl, 4–picolyl, 3–methyl–2–picolyl *N*–oxido, diphenylmethyl, *p,p'*–dinitrobenzhydryl, 5–dibenzosuberyl, triphenylmethyl, α–naphthyldiphenylmethyl, *p*–methoxyphenyldiphenylmethyl, di(*p*–methoxyphenyl)phenylmethyl, tri(*p*–methoxyphenyl)methyl, 4–(4'–bromophenacyloxyphenyl)diphenylmethyl, 4,4',4''–tris(4,5–dichlorophthalimidophenyl)methyl, 4,4',4''–tris(levulinoyloxyphenyl)methyl, 4,4',4''–tris(benzoyloxyphenyl)methyl, 3–(imidazol–1–yl)bis(4',4''–dimethoxyphenyl)methyl, 1,1–bis(4–methoxyphenyl)–1'–pyrenylmethyl, 9–anthryl, 9–(9–phenyl)xanthenyl, 9–(9–phenyl–10–oxo)anthryl, 1,3–benzodithiolan–2–yl, benzisothiazolyl S,S–dioxido, trimethylsilyl (TMS), triethylsilyl (TES), triisopropylsilyl (TIPS), dimethylisopropylsilyl (IPDMS), diethylisopropylsilyl (DEIPS), dimethylthexylsilyl, *t*–butyldimethylsilyl (TBDMS), *t*–butyldiphenylsilyl (TBDPS), tribenzylsilyl, tri–*p*–xylylsilyl, triphenylsilyl, diphenylmethylsilyl (DPMS), *t*–butylmethoxyphenylsilyl (TBMPS), formate, benzoylformate, acetate, chloroacetate, dichloroacetate, trichloroacetate, trifluoroacetate, methoxyacetate, triphenylmethoxyacetate, phenoxyacetate, *p*–chlorophenoxyacetate, 3–phenylpropionate, 4–oxopentanoate (levulinate), 4,4–(ethylenedithio)pentanoate (levulinoyldithioacetal), pivaloate, adamantoate, crotonate, 4–methoxycrotonate, benzoate, *p*–phenylbenzoate, 2,4,6–trimethylbenzoate (mesitoate), alkyl methyl carbonate, 9–fluorenylmethyl carbonate (Fmoc), alkyl ethyl carbonate, alkyl 2,2,2–trichloroethyl carbonate (Troc), 2–(trimethylsilyl)ethyl carbonate (TMSEC), 2–(phenylsulfonyl) ethyl carbonate (Psec), 2–(triphenylphosphonio) ethyl carbonate (Peoc), alkyl isobutyl carbonate, alkyl vinyl carbonate alkyl allyl carbonate, alkyl *p*–nitrophenyl carbonate, alkyl benzyl carbonate, alkyl *p*–methoxybenzyl carbonate, alkyl 3,4–dimethoxybenzyl carbonate, alkyl *o*–nitrobenzyl carbonate, alkyl *p*–nitrobenzyl carbonate, alkyl *S*–benzyl thiocarbonate, 4–ethoxy–1–

napththyl carbonate, methyl dithiocarbonate, 2–iodobenzoate, 4–azidobutyrate, 4–nitro–4–methylpentanoate, *o*–(dibromomethyl)benzoate, 2–formylbenzenesulfonate, 2–(methylthiomethoxy)ethyl, 4–(methylthiomethoxy)butyrate, 2–(methylthiomethoxymethyl)benzoate, 2,6–dichloro–4–methylphenoxyacetate, 2,6–dichloro–4–(1,1,3,3–tetramethylbutyl)phenoxyacetate, 2,4–bis(1,1–dimethylpropyl)phenoxyacetate, chlorodiphenylacetate, isobutyrate, monosuccinoate, (*E*)–2–methyl–2–butenoate, *o*–(methoxycarbonyl)benzoate, α–naphthoate, nitrate, alkyl *N,N,N',N'*–tetramethylphosphorodiamidate, alkyl *N*–phenylcarbamate, borate, dimethylphosphinothioyl, alkyl 2,4–dinitrophenylsulfenate, sulfate, methanesulfonate (mesylate), benzylsulfonate, and tosylate (Ts). For protecting 1,2– or 1,3–diols, the protecting groups include methylene acetal, ethylidene acetal, 1–*t*–butylethylidene ketal, 1–phenylethylidene ketal, (4–methoxyphenyl)ethylidene acetal, 2,2,2–trichloroethylidene acetal, acetonide, cyclopentylidene ketal, cyclohexylidene ketal, cycloheptylidene ketal, benzylidene acetal, *p*–methoxybenzylidene acetal, 2,4–dimethoxybenzylidene ketal, 3,4–dimethoxybenzylidene acetal, 2–nitrobenzylidene acetal, methoxymethylene acetal, ethoxymethylene acetal, dimethoxymethylene ortho ester, 1–methoxyethylidene ortho ester, 1–ethoxyethylidine ortho ester, 1,2–dimethoxyethylidene ortho ester, α–methoxybenzylidene ortho ester, 1–(*N,N*–dimethylamino)ethylidene derivative, α–(*N,N'*–dimethylamino)benzylidene derivative, 2–oxacyclopentylidene ortho ester, di–*t*–butylsilylene group (DTBS), 1,3–(1,1,3,3–tetraisopropyldisiloxanylidene) derivative (TIPDS), tetra–*t*–butoxydisiloxane–1,3–diylidene derivative (TBDS), cyclic carbonates, cyclic boronates, ethyl boronate, and phenyl boronate.

Agglutinated – When two or more cells are "agglutinated" by a cross-linking agent as described herein, they are each physically associated with the cross-linking agent in a cell-agent-cell complex. Typically, agglutination only occurs once the cross-linking agent concentration reaches a threshold concentration. This concentration is referred to as the minimum agglutination concentration (MAC). The MAC for a given cross-linking agent is commonly measured using a spectrophotometric plate reader that can quantify changes in solution absorbance.

Associated – As used herein, two entities are physically "associated" with one another when they are bound by direct non-covalent interactions. Desirable non-covalent interactions include those of the type which occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific, for example, ionic interactions, hydrogen bonds, van der

Waals interactions, hydrophobic interactions, etc. The strength, or affinity of the physical association can be expressed in terms of the dissociation constant (K_d) of the interaction, wherein a smaller K_d represents a greater affinity. For example, the association properties of a selected cross-linking agent and target molecule can be quantified using methods well known in the art.

Biodegradable – As used herein, the term "biodegradable" refers to molecules that degrade (i.e., lose at least some of their covalent structure) under physiological or endosomal conditions. Biodegradable molecules are not necessarily hydrolytically degradable and may require enzymatic action to degrade.

Biomolecule – As used herein, the term "biomolecule" refers to molecules (e.g., polypeptides, amino acids, polynucleotides, nucleotides, polysaccharides, sugars, lipids, nucleoproteins, glycoproteins, lipoproteins, steroids, metabolites, etc.) whether naturally-occurring or artificially created (e.g., by synthetic or recombinant methods) that are commonly found in cells and tissues. Specific classes of biomolecules include, but are not limited to, enzymes, receptors, neurotransmitters, hormones, cytokines, cell response modifiers such as growth factors and chemotactic factors, antibodies, vaccines, haptens, toxins, interferons, ribozymes, anti-sense agents, plasmids, DNA, and RNA.

Drug – As used herein, the term "drug" refers to small molecules or biomolecules that alter, inhibit, activate, or otherwise affect a biological event. For example, drugs may include, but are not limited to, anti-AIDS substances, anti-cancer substances, antibiotics, anti-diabetic substances, immunosuppressants, anti-viral substances, enzyme inhibitors, neurotoxins, opioids, hypnotics, anti-histamines, lubricants, tranquilizers, anti-convulsants, muscle relaxants and anti-Parkinson substances, anti-spasmodics and muscle contractants including channel blockers, miotics and anti-cholinergics, anti-glaucoma compounds, anti-parasite and/or anti-protozoal compounds, modulators of cell-extracellular matrix interactions including cell growth inhibitors and anti-adhesion molecules, vasodilating agents, inhibitors of DNA, RNA or protein synthesis, anti-hypertensives, analgesics, anti-pyretics, steroidal and non-steroidal anti-inflammatory agents, anti-angiogenic factors, anti-secretory factors, anticoagulants and/or anti-thrombotic agents, local anesthetics, ophthalmics, prostaglandins, anti-depressants, anti-psychotic substances, anti-emetics, and imaging agents. A more complete listing of exemplary drugs suitable for use in the present invention may be found in "Pharmaceutical Substances: Syntheses, Patents, Applications" by Axel Kleemann and Jurgen Engel, Thieme Medical Publishing, 1999; the "Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals",

edited by Susan Budavari *et al.*, CRC Press, 1996, and the United States Pharmacopeia-25/National Formulary-20, published by the United States Pharmcopeial Convention, Inc., Rockville MD, 2001.

Hyperbranched – As used herein, a "hyperbranched" structure is a covalent structure that includes at least one branched branch (e.g., a dendrimeric structure). A hyperbranched structure may include polymeric and/or non-polymeric substructures.

Lectin – As used herein, a "lectin" is a protein that binds with specificity to saccharides and polysaccharides. A lectin can be of any origin (e.g., plant, animal or other). In certain embodiments a lectin can be isolated from a natural source. In other embodiments a lectin can be produced synthetically or recombinantly. A lectin can be composed of one or more subunits under physiological conditions. In preferred embodiments a lectin is composed of two or more subunits under physiological conditions (e.g., four subunits). The subunits may be the same or different.

Mitogenic lectin – A "mitogenic lectin" is a lectin that stimulates the proliferation of T-cells as measured by a thymidine uptake assay using peripheral blood mononuclear cells (PBMC) from one or more healthy patients. Generally a mitogenic lectin will produce a detectable level of thymidine uptake at concentrations of 1 ug/ml. Exemplary mitogenic lectins include, but are not limited to, artocarpus integrifolia agglutinin (Jacalin), bauhinia purpurea agglutinin (BPA), concanavalin A (Con A), succinyl-concanavalin A (s-Con A), erythrina corallodendron agglutinin (ECorA), euonymus europaeus agglutinin (EEA), glycine max agglutinin (SBA), Lens culinaris agglutinin (LcH), maackia amurensis agglutinin (MAA), phaseolus vulgaris agglutinin (PHA), pokeweed mitogen (PWM), wheat germ agglutinin (WGA), and vicia faba agglutinin (VFA) all of which are available from Sigma-Aldrich of St. Louis, MO. It is to be understood that the terms "mitogenic lectin" include derivatives of native lectins that retain the ability to stimulate the proliferation of T-cells (e.g., derivatives that include amino acid substitutions, deletions or additions). Exemplary derivatives are those into which amino acid residues have been introduced by site-directed mutagenesis (e.g., in order to provide additional reactive groups for chemical modification). Generally, suitable derivatives will have at least 90% sequence homology with a native lectin as determined using standard methods known in the art (e.g., using Blast with default settings). Preferably the derivatives will have at least 95% sequence homology, more preferably 99% sequence homology with a native lectin. Without limitation, exemplary derivatives may induce a level of T-cell proliferation that is at

least 90% that of their native counterparts. More preferably, the level is at least 95%, even more preferably at least 99%.

Native lectin – As used herein, a "native lectin" is a protein with the chemical composition of a lectin that is found in nature.

Percentage homology – As used herein, the terms "percentage homology" refer to the percentage of sequence identity between two sequences after optimal alignment as defined in the present disclosure. For example, two nucleotide sequences are said to be "identical" if the sequence of nucleotides in the two sequences is the same when aligned for maximum correspondence as described below. Sequence comparisons between two nucleotide sequences are typically performed by comparing sequences of two optimally aligned sequences over a region or "comparison window" to identify and compare regions of sequence similarity. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman, *Ad. App. Math.* 2:482 (1981), by the homology alignment algorithm of Neddleman and Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444 (1988), by computerized implementation of these algorithms, or by visual inspection.

Percentage of sequence identity – "Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, where the portion of the nucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleotide residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. This definition of sequence identity given above is the definition that would be used by one of ordinary skill in the art. The definition by itself does not need the help of any algorithm. The algorithms are only helpful to facilitate the optimal alignments of sequences, rather than calculate sequence identity. From this definition, it follows that there is a well defined and only one value for the sequence identity between two compared sequences which value corresponds to the value obtained for the optimal alignment.

Physiological conditions – As used herein, "physiological conditions" are those conditions that are found in the arterial blood of a typical patient. Generally, the patient is a

mammal, e.g., a human, dog, cat, mouse, etc. In human patients, the pH under physiological conditions is typically between about 7.35 and about 7.45 (preferably about 7.40). Human physiological temperatures range from about 36.4 to about 37.4 C (preferably about 36.9 C).

Polymer – As used herein, a "polymer" or "polymeric structure" is a structure that includes a string of covalently bound monomers. A polymer can be made from one type of monomer or more than one type of monomer. The term "polymer" therefore encompasses copolymers, including block-copolymers in which different types of monomer are grouped separately within the overall polymer. A polymer can be linear or branched.

Polynucleotide – As used herein, a "polynucleotide" is a polymer of nucleotides. The terms "polynucleotide", "nucleic acid", and "oligonucleotide" may be used interchangeably. The polymer may include natural nucleosides (i.e., adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine), nucleoside analogs (e.g., 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, 4-acetylcytidine, 5-(carboxyhydroxymethyl)uridine, dihydrouridine, methylpseudouridine, 1-methyl adenosine, 1-methyl guanosine, N6-methyl adenosine, and 2-thiocytidine), chemically modified bases, biologically modified bases (e.g., methylated bases), intercalated bases, modified sugars (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, 2′-O-methylcytidine, arabinose, and hexose), or modified phosphate groups (e.g., phosphorothioates and 5' -N-phosphoramidite linkages).

Polypeptide – As used herein, a "polypeptide" is a polymer of amino acids. The terms "polypeptide", "protein", "oligopeptide", and "peptide" may be used interchangeably. Polypeptides may contain natural amino acids, non-natural amino acids (i.e., compounds that do not occur in nature but that can be incorporated into a polypeptide chain) and/or amino acid analogs as are known in the art. Also, one or more of the amino acid residues in a polypeptide may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation, functionalization, or other modification, etc. These modifications may include cyclization of the peptide, the incorporation of D-amino acids, etc.

Polysaccharide – As used herein, a "polysaccharide" is a polymer of saccharides. The terms "polysaccharide", "carbohydrate", and "oligosaccharide", may be used interchangeably.

The polymer may include natural saccharides (e.g., arabinose, lyxose, ribose, xylose, ribulose, xylulose, allose, altrose, galactose, glucose, gulose, idose, mannose, talose, fructose, psicose, sorbose, tagatose, mannoheptulose, sedoheptulose, octolose, and sialose) and/or modified saccharides (e.g., 2′-fluororibose, 2′-deoxyribose, and hexose). Exemplary disaccharides include sucrose, lactose, maltose, trehalose, gentiobiose, isomaltose, kojibiose, laminaribiose, mannobiose, melibiose, nigerose, rutinose, and xylobiose.

Small molecule – As used herein, the term "small molecule" refers to molecules, whether naturally-occurring or artificially created (e.g., via chemical synthesis), that have a relatively low molecular weight. Typically, small molecules are monomeric and have a molecular weight of less than about 1500 g/mol. Preferred small molecules are biologically active in that they produce a local or systemic effect in animals, preferably mammals, more preferably humans. In certain preferred embodiments, the small molecule is a drug. Preferably, though not necessarily, the drug is one that has already been deemed safe and effective for use by the appropriate governmental agency or body. For example, drugs for human use listed by the FDA under 21 C.F.R. §§ 330.5, 331 through 361, and 440 through 460; drugs for veterinary use listed by the FDA under 21 C.F.R. §§ 500 through 589, are all considered acceptable for use in accordance with the present invention.

Treat – As used herein, the term "treat" (or "treating", "treated", "treatment", etc.) refers to the administration of a material of the present disclosure to a subject in need thereof with the purpose to alleviate, relieve, alter, ameliorate, improve or affect a condition (e.g., diabetes), a symptom or symptoms of a condition (e.g., hyperglycemia), or the predisposition toward a condition.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Comparison between RP-HPLC chromatograms obtained for (a) exemplary conjugate synthesized using TSAT-C6 as the scaffold, AEM as the affinity ligand, and NH2-B1-BOC2(A1,B29)-insulin as the drug and (b) an insulin-glycogen conjugate synthesized according to Example 32.

Figure 2: Accelerated stability testing (AST) aggregation assay for Conjugate 1 (□), Conjugate 2 (△), and RHI (◆) in PBS buffer. The conjugates demonstrate greatly enhanced stability over pharmaceutical grade RHI.

Figure 3(a): Accelerated stability testing (AST) chemical stability results (a) RP-HPLC AST conjugate stability.

Figure 3(b): Accelerated stability testing (AST) chemical stability results (b) LC/MS data on AST conjugates.

Figure 4: *In vivo* bioactivity in (n=4) non-diabetic, male Sprague-Dawley (SD) rats for fresh conjugate (▲) and 72 hr AST conjugate (○). The 72 hr AST conjugate bioactivity was indistinguishable from that of the fresh conjugate ($p > 0.21$ for all timepoints).

Figure 5: Blood glucose depression profile in non-diabetic, male SD rats (n = 3) for subcutaneously injected (▲) insulin-dextran (70 K) at a dose of ~ 20 U of insulin equivalents/kg.

Figure 6: Blood glucose depression profile in non-diabetic, male SD rats (n = 3) for subcutaneously injected (■) insulin-glycogen (Type II oyster) at a dose of ~ 2.5 U of insulin equivalents/kg.

Figure 7: Blood glucose levels resulting from a 3.5 U equivalent insulin/kg subcutaneous dose of (◆) TSAT-C6-AEM-2 insulin conjugate and (□) soluble recombinant human insulin (RHI) in male non-diabetic SD rats. Each set of data represents the average and standard deviation for n = 6 rats.

Figure 8: Serum insulin concentrations resulting from a 3.5 U equivalent insulin/kg subcutaneous dose of (◆) TSAT-C6-AEM-2 insulin conjugate and (□) soluble recombinant human insulin (RHI) in male non-diabetic SD rats. Each set of data represents the average and standard deviation for n = 6 rats.

Figure 9: Plot of (◆) serum insulin and (○) blood glucose levels following subcutaneous injection in non-diabetic, male SD rats at time 0 with TSAT-C6-AEM-2 (B29-substituted) insulin conjugate (5 U/kg). Data represents the average and standard deviation for n = 3 rats.

Figure 10: Chemical structures of AEG, AEM, AEBM and AETM. The affinity of these sugar based affinity ligands for Con A increases as shown.

Figure 11: Chemical structures of some exemplary non-dendrimeric conjugates.

Figure 12: Plot of serum insulin (left) and blood glucose (right) levels following subcutaneous injection in non-diabetic, male SD rats at time 0 with TSAT-C6-AEM-2 insulin conjugate (◆), soluble recombinant human insulin, (○) and insulin lispro (Δ) (all 3.5 U/kg). Data represents the average and standard deviation for n = 6 rats.

Figure 13: Plot of blood glucose levels following subcutaneous injection in non-diabetic, male SD rats (n=3 for each formulation) at time 0 with TSAT-C6 based insulin conjugates with the different affinity ligands as shown. The glucose lowering response decreases as the affinity of the affinity ligand increases.

Figure 14: Plot of serum insulin (left) and blood glucose (right) levels following subcutaneous injection in non-diabetic, male SD rats (n=3) at time 0 with TSAT-C6-AEM-2 conjugate (3.5 U/kg).

Figure 15: Plot of serum insulin (left) and blood glucose (right) levels following subcutaneous injection in non-diabetic, male SD rats (n=3) at time 0 with TSAT-C6-AEBM-2 conjugate (5 U/kg).

Figure 16: Plot of serum insulin (left) and blood glucose (right) levels following subcutaneous injection in non-diabetic, male SD rats (n=3) at time 0 with TSAT-C6-AEBM-1 AETM-1 conjugate (5 U/kg).

Figure 17: SDS PAGE analysis of partially purified SCI-3 to test for soluble protein. Lane 1 = Ladder; Lane 2 = 10 ul Native Con A control; Lane 3 = 10 ul supernatant from wash #1; Lane 4 = 20 ul supernatant from wash #1; Lane 5 = 10 ul supernatant from wash #2; Lane 6 = 20 ul supernatant from wash #2; Lane 7 = 10 ul supernatant from wash #3; Lane 8 = 20 ul supernatant from wash #3; Lane 9 = Blowthrough SCI-3 (10X concentrated); and Lane 10 = Blowthrough SCI-3 (40X concentrated).

Figure 18: SDS PAGE analysis of partially purified SCI-4 to test for soluble protein. Lane 1 = Ladder; Lane 2 = 10 ul Native Con A control; Lane 3 = 10 ul supernatant from wash #1; Lane 4 = 20 ul supernatant from wash #1; Lane 5 = 10 ul supernatant from wash #2; Lane 6 = 20 ul supernatant from wash #2; Lane 7 = 10 ul supernatant from wash #3; and Lane 8 = 20 ul supernatant from wash #3. The amount of soluble SCI-4 was estimated to be between 75-125 mg protein (actual soluble protein 62 mg).

Figure 19: Standard curve obtained using Measure-iT™ thiol quantitation standards in thiol assay measurements.

Figure 20: Results from a Measure-iT™ thiol quantitation assay that was used to measure the extent of reaction between an exemplary sugar affinity ligand and an exemplary recombinant Con A (rCon A) of the present disclosure. An increased ratio of sugar affinity ligand to rCon A was found to correlate with a decrease in unreacted disulfide bonds.

Figure 21: Comparison of minimum agglutination concentration (MAC) for exemplary Con A samples. (A) Native Con A, (B) Acetyl Con A, (C) rCon A SCI-3 reacted to AEM-LC-SMPH, (D) rCon A SCI-4 reacted to AEM-LC-SMPH, (E) rCon A SCI-3 reacted to AEM-LC-SMPH with degassed solvent, and (F) rCon A SCI-4 reacted to AEM-LC-SMPH with degassed solvent. Columns 1 through 12 correspond to the Con A or rCon A protein concentration in solution varying from 1,000 to 0.5 ug/mL (left to right).

Figure 22: Confirmation of comparison of minimum agglutination concentration (MAC) for exemplary Con A samples. (A) Native Con A, (B) Acetyl Con A, (C) rCon A SCI-3 reacted to AEM-LC-SMPH, (D) rCon A SCI-4 reacted to AEM-LC-SMPH, (E) rCon A SCI-3 reacted to AEM-LC-SMPH with degassed solvent, and (F) rCon A SCI-4 reacted to AEM-LC-SMPH with degassed solvent. Columns 1 through 12 correspond to the Con A or rCon A protein concentration in solution varying from 1,000 to 0.5 ug/mL (left to right).

Figure 23: Schematic of a multivalent lectin 20 with at least two binding sites 30 for glucose, wherein the lectin 20 includes at least one affinity ligand 40 which is capable of competing with glucose for binding with at least one of said binding sites 30 and is covalently linked to a cysteine residue of the lectin 20.

Figure 24: Schematic of a cross-linked material 10 that includes multivalent lectins 20 of Figure 23 (for simplicity the at least one covalently linked affinity ligand 40 which is capable of competing with glucose for binding with at least one of said binding sites 30 is not shown in the main schematic of Figure 24); and conjugates 50 that include two or more separate affinity ligands 60 bound to a conjugate framework 70, wherein the two or more affinity ligands 60 compete with glucose for binding with the lectins 20 at said binding sites 30 and wherein conjugates 50 are cross-linked within the material 10 as a result of non-covalent interactions between lectins 20 and affinity ligands 60 on different conjugates 50.

Figure 25: Structure of wild-type human insulin with A-peptide (SEQ ID NO:22) and B-peptide (SEQ ID NO:23) linked via three disulfide bridges.

DETAILED DESCRIPTION OF VARIOUS EMBODIMENTS

This application refers to a number of documents including patent and non-patent documents. The entirety of each of these documents is incorporated herein by reference.

In one aspect and as shown in Figures 23 and 24, the disclosure provides a cross-linked material 10 that includes multivalent lectins 20 with at least two binding sites 30 for glucose,

wherein the lectins 20 include at least one affinity ligand 40 which is capable of competing with glucose for binding with at least one of said binding sites 30 and is covalently linked to a cysteine residue of the lectins 20; and conjugates 50 that include two or more separate affinity ligands 60 bound to a conjugate framework 70, wherein the two or more affinity ligands 60 compete with glucose for binding with the lectins 20 at said binding sites 30 and wherein conjugates 50 are cross-linked within the material 10 as a result of non-covalent interactions between lectins 20 and affinity ligands 60 on different conjugates 50. These materials are designed to release amounts of conjugate in response to desired concentrations of glucose. Depending on the end application, in various embodiments, the conjugates may also include a drug and/or a detectable label. The drug, detectable label and affinity ligands may be covalently or non-covalently bound to the conjugate framework. The disclosure also provides methods of using these materials and methods of making these materials. In another aspect, the disclosure provides exemplary recombinant lectins for use in glucose responsive materials instead of native lectins such as Con A.

The lectins of the present disclosure bind glucose and are multivalent. The conjugates include a conjugate framework with two or more separate affinity ligands that compete with glucose for binding with the lectins. When lectins and conjugates are combined in the absence of glucose, a non-covalently cross-linked material is formed. When the material is placed in the presence of free glucose these compete for the interactions between the lectins and the conjugates. Above a certain concentration of free glucose, the level of competition becomes such that the material begins to degrade by releasing conjugates. As a result, conjugates are released from the material in a manner which is directly tied to the local concentration of glucose.

Multivalent lectins

Lectins in a cross-linked material of the present disclosure include at least two binding sites for glucose (i.e., they are multivalent). In addition, the lectins include at least one covalently linked affinity ligand which is capable of associating with one of these binding sites. The at least one affinity ligand is covalently linked to a cysteine residue of the lectins. In various embodiments, the lectins may include just one covalently linked affinity ligand. In various embodiments, the lectins may include one covalently linked affinity ligand per binding site. Typically a multivalent lectin will include 2 or 4 binding sites (e.g., a dimer or tetramer of a

monovalent lectin) but the present disclosure also encompasses lectins with 3, 5 or more binding sites. The present disclosure also encompasses lectins with more than one covalently linked affinity ligand per binding site. The present disclosure further encompasses materials which include a mixture of lectins that include different numbers of covalently linked affinity ligands and/or that include unmodified lectins.

Lectins

The methods of the present disclosure may be applied to any lectin that includes a cysteine residue (whether present naturally or introduced recombinantly via site-directed mutagenesis). Lectins have been isolated from a variety of natural sources including seeds, roots, bark, fungi, bacteria, seaweed, sponges, mollusks, fish eggs, body fluids of invertebrates and lower vertebrates, and mammalian cell membranes (e.g., see *The Lectins: Properties, Functions, and Applications in Biology and Medicine*, Edited by Liener et al., Academic Press, 1986). A number of lectins have also been produced recombinantly (e.g., see Streicher and Sharon, *Methods Enzymol.* 363:47-77, 2003 and U.S. Patent Publication No. 20060247154). As noted above, lectins bind saccharides and polysaccharides with a high degree of specificity. For example, some lectins will bind only to mannose or glucose residues, while others only recognize galactose residues. Some lectins require that the particular residue be in a terminal position, while others bind to residues within a polysaccharide chain. Some lectins require specific anomeric structures and yet others recognize specific sugar sequences. The structures and properties of lectins have been extensively described in the literature. For recent reviews and a list of lectins see *Lectins*, Edited by Sharon and Lis, Kluwer Academic Publishers, 2003; *Handbook of Animal Lectins: Properties and Biomedical Applications*, Edited by Kilpatrick, Wiley, 2000; and *Handbook of Plant Lectins: Properties and Biomedical Applications*, Edited by Van Damme et al., Wiley, 1998.

Exemplary glucose-binding lectins include calnexin, calreticulin, N-acetylglucosamine receptor, selectin, asialoglycoprotein receptor, collectin (mannose-binding lectin), mannose receptor, aggrecan, versican, pisum sativum agglutinin (PSA), vicia faba lectin, lens culinaris lectin, soybean lectin, peanut lectin, lathyrus ochrus lectin, sainfoin lectin, sophora japonica lectin, bowringia milbraedii lectin, concanavalin A (Con A), and pokeweed mitogen. In various embodiments, human analogs of plant lectins may be used. These include, without limitation, human mannan binding protein (MBP, also called mannan binding lectin, Sheriff et al.,

Structural Biology, 1:789-794 (1994); Dumestre-Perard et al., *Molecular Immunology*, 39:465-473 (2002)), human pulmonary surfactant protein A (SP-A, Allen, et al., *Infection and Immunity*, 67:4563-4569 (1999)), human pulmonary surfactant protein D (SP-D, Persson et al., *The Journal of Biological Chemistry*, 265:5755-5760 (1990)), CL-43 (a human serum protein), and conglutinin.

Lectins in accordance with the present disclosure may be produced by any available means. Lectins can be expressed in cells using recombinant techniques, such as through the use of an expression vector (Sambrook *et al., Molecular Cloning: A Laboratory Manual*, CSHL Press, 1989). Recombinant lectins may be produced, for example, by utilizing a host cell system engineered to express the recombinant lectin encoded by an expression vector (e.g., bacteria, yeast, plant cells, mammalian cells, etc.). In some embodiments, an expression vector is a bacterial expression vector. In certain embodiments, lectins are expressed in bacterial cells that allow for high-efficiency protein expression of genes (e.g., *E. coli* BL21 cells). In some embodiments, lectins expressed using recombinant techniques are subsequently isolated and/or purified.

In general, the lectins of the present disclosure include at least one cysteine residue. In certain embodiments, the lectins of present disclosure include a single cysteine residue. In certain embodiments, the lectins of present disclosure include two cysteine residues. In certain embodiments, the at least one cysteine residue is present in the wild-type sequence of the lectin. In certain embodiments, the wild-type sequence of the lectin includes one or more cysteine residues that are mutated or deleted and one or more cysteine residues are introduced elsewhere in the sequence (e.g., closer to the glucose binding site). In certain embodiments, the at least one cysteine residue is introduced into the lectin sequence by site-directed mutagenesis. In some embodiments, a cysteine residue is introduced by replacing another residue in a wild-type lectin sequence (i.e., amino acid substitution). In some embodiments, a cysteine residue is introduced by adding a cysteine residue to a wild-type lectin sequence (i.e., amino acid addition).

In certain embodiments, the at least one cysteine residue is introduced at a location which is exposed on the surface of the lectin when the lectin is folded under physiological conditions. In some embodiments, the at least one cysteine residue is introduced at a location which is within or in close proximity to the glucose binding site when the lectin is folded under physiological conditions. It is to be understood that there may be some variability in how close the cysteine is to the glucose binding site depending on the nature of the affinity ligand that is ultimately

covalently attached via the cysteine residue. For example, as discussed in more detail herein, affinity ligands with longer and/or more flexible reactive linkers may be covalently attached via a cysteine residue that is located further from the glucose binding site than affinity ligands with shorter and/or more rigid reactive linkers.

In certain embodiments, the at least one cysteine residue is introduced by substituting an amino acid in the wild-type sequence that forms part of a binding site for glucose.

In certain embodiments, the at least one cysteine residue is introduced at a location within the wild-type sequence that is within 1-5 amino acids (as measured in the primary amino acid sequence) of an amino acid that forms part of a binding site for glucose. In certain embodiments, the separation (in the primary amino acid sequence) between the at least one cysteine residue and an amino acid that forms part of a binding site for glucose sequence is in the range of 1-2, 1-3, 1-4 amino acids, e.g., 1, 2, 3, 4 or 5 amino acids.

In certain embodiments, the at least one cysteine residue is introduced at a location within the wild-type sequence such that it is separated by at least 1, 2, 3, 4 or 5 amino acids (as measured in the primary amino acid sequence) from any amino acid that forms part of a binding site for glucose.

In some of these embodiments, the lectins of the present disclosure are generated by mutating wild-type Con A as described above, wherein wild-type Con A has the following amino acid sequence (SEQ ID NO:1):

```
ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQNGKVGTAHIIYN
SVGKRLSAVVSYPNADSATVSYDVDLDNVLPEWVRVGLSASTGLYKETNTILSWS
FTSKLKSNSTHETNALHFMFNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSP
QGSSVGRALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPADGIAFFISNIDSS
IPSGSTGRLLGLFPDAN
```

and wherein one or more of the amino acids at position 12, 14, 99, 100, 208 and 228 form part of the binding site for glucose. In certain embodiments, the lectins of the present disclosure are generated by mutating wild-type Con A as described above wherein each of the amino acids at position 12, 14, 99, 100, 208 and 228 of SEQ ID NO:1 forms part of the binding site for glucose.

In certain embodiments, a lectin of the present disclosure includes at least one cysteine and has an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a wild-type lectin sequence (e.g., the wild-type sequence of Con A that is set forth in SEQ ID NO:1). In certain embodiments, amino acid substitutions beyond

substitutions that introduce a cysteine into a lectin sequence may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. In certain embodiments, a substitution may be conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and tyrosine, phenylalanine. In certain embodiments, the hydrophobic index of amino acids may be considered in choosing suitable mutations. The importance of the hydrophobic amino acid index in conferring interactive biological function on a polypeptide is generally understood in the art. Alternatively, the substitution of like amino acids can be made effectively on the basis of hydrophilicity. The importance of hydrophilicity in conferring interactive biological function of a polypeptide is generally understood in the art. The use of the hydrophobic index or hydrophilicity in designing polypeptides is further discussed in U.S. Patent No. 5,691,198.

In certain embodiments, a lectin of the present disclosure has an amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16:

SEQ ID NO:	Amino acid sequence
2	ADTIVAXXXXXXXXXXXXXPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLXXXXXXXXXXXXILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPXXXXXXXXXXIS NIDSSIPXXXXXXXXXXPDAN
3	ADTIVAXXXXXXXXXXXXXPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASTGLYKETNTILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPADGIAFFIS NIDSSIPSGSTGRLLGLFPDAN

SEQ ID NO:	Amino acid sequence
4	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLXXXXXXXXXXXXILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPADGIAFFIS NIDSSIPSGSTGRLLGLFPDAN
5	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASTGLYKETNTILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPXXXXXXXXXXXIS NIDSSIPSGSTGRLLGLFPDAN
6	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASTGLYKETNTILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPADGIAFFIS NIDSSIPXXXXXXXXXXXPDAN
7	ADTIVAVELDTXXXTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASTXXXKETNTILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPXXGIAFFIS NIDSSIPSGSTXXLLGLFPDAN
8	ADTIVAVELDTXXXTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASTGLYKETNTILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPADGIAFFIS NIDSSIPSGSTGRLLGLFPDAN
9	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASTXXXKETNTILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPADGIAFFIS NIDSSIPSGSTGRLLGLFPDAN

SEQ ID NO:	Amino acid sequence
10	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASTGLYKETNTILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPXXGIAFFIS NIDSSIPSGSTGRLLGLFPDAN
11	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASTGLYKETNTILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPADGIAFFIS NIDSSIPSGSTXXLLGLFPDAN
12	ADTIVAVELDTXPXTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASTGXXKETNTILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPAXGIAFFIS NIDSSIPSGSTXLLLGLFPDAN
13	ADTIVAVELDTXPXTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASTGLYKETNTILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPADGIAFFIS NIDSSIPSGSTGRLLGLFPDAN
14	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASTGXXKETNTILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPADGIAFFIS NIDSSIPSGSTGRLLGLFPDAN

SEQ ID NO:	Amino acid sequence
15	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASTGLYKETNTILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPAXGIAFFIS NIDSSIPSGSTGRLLGLFPDAN
16	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASTGLYKETNTILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPADGIAFFIS NIDSSIPSGSTXLLLGLFPDAN

with the proviso that in each of these sequences each X is a codable amino acid and at least one X is a cysteine. As used herein, a "codable amino acid" is any one of the 20 amino acids that are directly encoded for polypeptide synthesis by the standard genetic code.

In certain embodiments each X in these sequences that is not a cysteine is the corresponding amino acid from SEQ ID NO:1 (i.e., the amino acid sequence at that position in wild-type Con A). Thus, it is to be understood that the present disclosure encompasses lectins with amino acid sequences of SEQ ID NO:2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 where any one of the X positions is a cysteine and all other X positions are the corresponding amino acid from SEQ ID NO:1 (i.e., the amino acid sequence at that position in wild-type Con A).

In certain embodiments, a lectin of the present disclosure has an amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 where the lectin includes a single cysteine. In certain embodiments, a lectin of the present disclosure has an amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 where just one of the X positions is a cysteine and all other X positions are the corresponding amino acid from SEQ ID NO:1 (i.e., the amino acid sequence at that position in wild-type Con A). While each of these exemplary amino acid sequences is not reproduced herein for brevity it is to be understood that it is encompassed by the present disclosure.

In certain embodiments, a lectin of the present disclosure has an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the

amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 with the proviso that in each of these sequences each X is a codable amino acid and at least one X is a cysteine.

In certain embodiments, a lectin of the present disclosure has an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 where just one of the X positions is a cysteine and all other X positions are codable amino acids.

In certain embodiments, a lectin of the present disclosure has an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:17:

ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQNGKVGTAHIIYN
SVGKRLSAVVSYPNADSATVSYDVDLDNVLPEWVRVGLSASTGLXXETNTILSWS
FTSKLKSNSTHETNALHFMFNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSP
QGSSVGRALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPADGIAFFISNIDSS
IPSGSTGRLLGLFPDAN

with the proviso that at least one X is a cysteine. In some of these embodiments the lectin includes a single cysteine. In some embodiments, a lectin of the present disclosure has an amino acid sequence of SEQ ID NO:17 with the proviso that at least one X is a cysteine. In certain embodiments, a lectin of the present disclosure has an amino acid sequence of SEQ ID NO:17 where X at position 100 is a cysteine. In some of these embodiments, X at position 101 is a lysine. In certain embodiments, a lectin of the present disclosure has an amino acid sequence of SEQ ID NO:17 where X at position 101 is a cysteine. In some of these embodiments, X at position 100 is a tyrosine.

In certain embodiments, a lectin of the present disclosure has an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:18:

ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQNGKVGTAHIIYN
SVNKRLSAVVSYPNADSATVSYDVDLDNVLPEWVRVGLSASGLYKETNTILSWSF
TSKLKSXSTXETNALHFMFNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQ
GSSVGRALFYAPVHIWESSAVVASFQATFTFLIKSPDSHPADGIAFFISNIDSSI
PSGSTGRLLGLFPDAN

with the proviso that at least one X is a cysteine. In some of these embodiments the lectin includes a single cysteine. In some embodiments, a lectin of the present disclosure has an amino acid sequence of SEQ ID NO:18 with the proviso that at least one X is a cysteine. In certain embodiments, a lectin of the present disclosure has an amino acid sequence of SEQ ID NO:18 where X at position 117 is a cysteine. In some of these embodiments, X at position 120 is a histidine. In certain embodiments, a lectin of the present disclosure has an amino acid sequence of SEQ ID NO:18 where X at position 120 is a cysteine. In some of these embodiments, X at position 117 is asparagine. In certain embodiments, a lectin of the present disclosure has an amino acid sequence of SEQ ID NO:18 where X at position 117 is a cysteine and X at position 120 is a cysteine.

In certain embodiments, a lectin of the present disclosure has an amino acid sequence selected from those presented in the table below.

Construct ID	Amino acid sequence
SCI-2	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVNKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASGLYKETNTILSWSFTSKLKSCSTCETNALHFMF NQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGRA LFYAPVHIWESSAVVASFQATFTFLIKSPDSHPADGIAFFISN IDSSIPSGSTGRLLGLFPDAN (SEQ ID NO:19)
SCI-3	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASTGLCKETNTILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPADGIAFFIS NIDSSIPSGSTGRLLGLFPDAN (SEQ ID NO:20)
SCI-4	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQ NGKVGTAHIIYNSVGKRLSAVVSYPNADSATVSYDVDLDNVLP EWVRVGLSASTGLYCETNTILSWSFTSKLKSNSTHETNALHFM FNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGR ALFYAPVHIWESSAVVASFEATFTFLIKSPDSHPADGIAFFIS NIDSSIPSGSTGRLLGLFPDAN (SEQ ID NO:21)

In certain embodiments, a lectin of the present disclosure binds glucose with substantially the same affinity as the wild-type lectin from which it was derived. In certain embodiments, a lectin of the present disclosure binds glucose with an affinity that is equal to at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of the affinity of the wild-type lectin for glucose.

In certain embodiments, a lectin of the present disclosure forms multimers under physiological conditions.

In certain embodiments, a lectin of the present disclosure forms a gel in the presence of glycogen. In some of these embodiments, gel formation may be assessed using the following assay. Briefly, unmodified Oyster glycogen Type II (Sigma Aldrich, St. Louis, MO) is dissolved at 10 mg/ml in pH 7, 200 mM BES buffer (Sigma Aldrich, St. Louis, MO). 50 µL of the glycogen solution is then pipetted into a well of a 96-well microtiter plate (VWR Scientific, Bridgeport, NJ). Lectins are dissolved from a lyophilized powder at 10 mg/ml in pH 7, 100 mM BES buffer containing 1 mM manganese chloride and 1 mM calcium chloride. 50 µL of the lectin solution is then added to the 50 µL glycogen solution in the microtiter plate well, and the plate is gently vibrated to ensure adequate mixing of the two liquids. Similarly, a negative control well is made by adding 100 µL of the 10 mg/ml glycogen solution to one separate well on the microtiter plate, and one positive control well is made by mixing 50 µL of the glycogen solution with 50 µL of a 10 mg/ml wild-type (e.g., native) lectin solution in pH 7, 100 mM BES buffer containing 1000 mM sodium chloride and 1 mM manganese chloride and 1 mM calcium chloride. The plate is allowed to develop at room temperature for 10 minutes, after which time the turbidity of each plate well is assayed using a commercially available microplate reader (SpectraMAX, Molecular Devices, Mountain View, CA) at a wavelength of 490 nm (OD490). The OD490 value is then compared to the positive (wild-type lectin plus glycogen) control and negative control (glycogen only) to determine the relative amount of glycogen precipitation for a given lectin. In certain embodiments, a lectin of the present disclosure leads to a relative amount of glycogen precipitation that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of the relative amount of glycogen precipitation that the wild-type lectin produces when both are tested in this assay under the same conditions.

Generating multivalent cross-linking agents

Some lectins are multivalent, e.g., as a result of forming multimers under physiological conditions. Multivalent lectins can also be generated by covalently or non-covalently linking

two or more monovalent lectins into a single construct. Typically, two or more lectins (which may have the same or different sequences) may be linked directly to one another (e.g., via a coupling agent) or indirectly through a framework. In various embodiments 2, 3, 4 or more monovalent lectins may be combined into a single construct. In various embodiments the 2, 3, 4 or more monovalent lectins may have the same sequence. It will be appreciated that either one of these approaches may require the lectins to be chemically modified (e.g., to include pendant reactive groups) prior to coupling. It will also be appreciated that the multivalent cross-linking agents of the present disclosure are not limited to a particular coupling reaction or framework (e.g., they can be prepared using frameworks that include polymeric and/or non-polymeric structures). It will further be appreciated that the frameworks may be linear, branched, dendrimeric and/or a combination of these. Exemplary frameworks and coupling chemistries are described below in the context of the conjugates.

In various embodiments the monovalent lectins are covalently linked to each other or a framework. In such embodiments, the lectins can be directly linked (i.e., with no intervening chemical groups) or indirectly linked through a spacer (e.g., a coupling agent or covalent chain that provides some physical separation between the lectins or between the lectins and framework). As discussed below in the context of the conjugates it is to be understood that lectins may be covalently linked to each other or a framework through any number of chemical linkages, including but not limited to amide, ester, ether, isourea, and imine bonds.

In various embodiments, two or more monovalent lectins can be non-covalently linked to each other or to a framework. In certain embodiments, the dissociation constant (K_d) of the non-covalent linkage in human serum is less than 1 pmol/L. For example, lectins may be non-covalently linked to each other or a framework via a non-covalent ligand-receptor pair as is well known in the art (e.g., without limitation a biotin-avidin based pair). In such an embodiment, one member of the ligand receptor-pair is covalently linked to one lectin while the other member of the pair is covalently linked to the other lectin or framework. When the lectins (or lectins and framework) are combined, the strong non-covalent interaction between the ligand and its receptor causes the ligands to become non-covalently bound to each other (or the framework). Typical ligand/receptor pairs include protein/co-factor and enzyme/substrate pairs. Besides the commonly used biotin/avidin pair, these include without limitation, biotin/streptavidin, digoxigenin/anti-digoxigenin, FK506/FK506-binding protein (FKBP), rapamycin/FKBP, cyclophilin/cyclosporin and glutathione/glutathione transferase pairs. Other suitable

ligand/receptor pairs would be recognized by those skilled in the art, e.g., monoclonal antibodies paired with a epitope tag such as, without limitation, glutathione-S-transferase (GST), c-myc, FLAG® and further those described in Kessler pp. 105-152 of *Advances in Mutagenesis"* Ed. by Kessler, Springer-Verlag, 1990; "*Affinity Chromatography: Methods and Protocols (Methods in Molecular Biology)*" Ed. by Pascal Baillon, Humana Press, 2000; and "*Immobilized Affinity Ligand Techniques*" by Hermanson et al., Academic Press, 1992.

Affinity ligands

Any affinity ligand can be used as long as it can associate with a binding site of the lectin once covalently linked to the lectin. Typically an affinity ligand will include a recognition element which interacts with the lectin binding site and a reactive linker which enables the affinity ligand to become covalently attached to a cysteine residue in the lectin.

Recognition element

Any recognition element that can compete for binding with the lectin's cognate ligand (e.g., glucose or mannose in the case of Con A) could be used in an affinity ligand of the present disclosure. In various embodiments, the recognition element includes a saccharide. In certain embodiments the saccharide is a natural saccharide (e.g., glucose, fructose, galactose, mannose, arabinose, ribose, xylose, etc.). In certain embodiments the saccharide is a modified saccharide (e.g., 2'-fluororibose, 2'-deoxyribose, hexose, etc.). In certain embodiments the recognition element is glucose, sucrose, maltose, mannose, derivatives of these (e.g., glucosamine, mannosamine, methylglucose, methylmannose, ethylglucose, ethylmannose, etc.) and/or higher order combinations of these (e.g., a bimannose, a linear and/or branched trimannose, etc.). Other exemplary saccharides will be recognized by those skilled in the art. In particular, it is to be understood that depending on the application any one of the saccharides that are described below in the context of the conjugate affinity ligands may be used (e.g., any one of the saccharides of formula (IIIa) or (IIIb) that are described below in the context of conjugates). In certain embodiments, the recognition element includes a monosaccharide. In certain embodiments, the recognition element includes a disaccharide. In certain embodiments, the recognition element includes a trisaccharide. In some embodiments, the recognition element includes a saccharide and one or more amine groups. In some embodiments, the recognition element is aminoethylglucose (AEG). In some embodiments, the recognition element is

aminoethylmannose (AEM). In some embodiments, the recognition element is aminoethylbimannose (AEBM). In some embodiments, the recognition element is aminoethyltrimannose (AETM). In some embodiments, the recognition element is β-aminoethyl-N-acetylglucosamine (AEGA). In some embodiments, the recognition element is aminoethylfucose (AEF). In other embodiments, the recognition element is D-glucosamine (GA).

In various embodiments, the recognition element includes a polysaccharide, glycopeptide or glycolipid. In certain embodiments, the recognition element includes from 2-10 saccharide moieties, e.g., 2, 3, 4, 5, 6, 7, 8, 9 or 10 moieties. The terminal and/or internal residues of the polysaccharide, glycopeptide or glycolipid may be selected based on the saccharide specificity of the lectin in question (e.g., see Goldstein et al., *Biochem. Biophys. Acta* 317:500-504, 1973 and Lis et al., *Ann. Rev. Biochem.* 55:35-67, 1986).

As is well known in the art, certain polysaccharides can be prepared synthetically (e.g., see Lee et al., *J. Biol. Chem.* 258:199-202, 1983). Polysaccharides can also be prepared from natural sources (e.g., other polysaccharides, glycoproteins, glycolipids, etc.). For example, in certain embodiments, polysaccharides can be prepared by enzymatic cleavage of glycoproteins using endoglycosidases such as endoglycosidase D, endoglycosidase F, endoglycosidase H and/or N-endoglycosidase F (also called N-glycanase) (e.g., see Hirani et al., *Anal. Biochem.* 162:485-492, 1987). Endoglycosidases can be obtained from any source, including commercial sources (e.g., from QA-Bio, ProZyme, Roche, Sigma-Aldrich, New England Biolabs, Glyko, etc.). Alternatively or additionally, endoglycosidases can be isolated and/or purified from a cellular source (e.g., bacteria, yeast, plant, etc.). Polysaccharides that are linked to a glycoprotein via alkaline borohydride-labile bonds (O-glycosidic linkages) can be cleaved from the glycoprotein by treatment with 0.1 N NaOH containing 0.8 M $NaBH_4$ at 37 C for 68 hours according to the method of Spiro et al., *J. Biol. Chem.* 249:5704-5717, 1974. Polysaccharides can also be released by hydrazinolysis using standard chemical methods described by Takasaki et al., *Methods Enzymol.* 83:263-268, 1982.

It will also be appreciated that prior to or after cleavage from a glycoprotein any of these polysaccharides can be further trimmed using one or more exoglycosidases (e.g., sialidases, galactosidases, hexosaminidases, fucosidases, and mannosidases). One skilled in the art can readily determine procedures for removal of undesired terminal saccharide moieties in order to expose the desired terminal saccharide moieties appropriate for various lectins. Alternatively, in

certain embodiments it may be advantageous to enzymatically add a desired terminal saccharide moiety. For example, without limitation, the enzyme UDP-galactose: N-acetyl glucosamine-β-1,4-galactosyltransferase is capable of transferring galactose from UDP-galactose to N-acetyl-D-glucosamine or to other polysaccharides with a terminal N-acetyl-D-glucosamine. Addition of galactose or other saccharide residues to polysaccharides may also be accomplished synthetically, e.g., as described by Lee et al., *Methods Enzymol.* 138:424-429, 1987.

In various embodiments, the recognition element for a particular lectin may be selected empirically. According to such embodiments one or more recognition elements are screened based on their relative binding affinities for the lectin as compared to glucose. In certain embodiments a library of saccharides and/or polysaccharides are screened in this manner. A suitable recognition element will exhibit a detectable level of competition with glucose but will not compete so strongly that it prevents all binding between the lectin and glucose. In certain embodiments, different recognition elements may be screened by testing the effect of different affinity ligands on relevant lectin properties (e.g., based on their ability to inhibit agglutination and/or their material set points as discussed in more detail below and in the Examples). In certain embodiments, the recognition element will be selected in view of the conjugate that the modified lectin is to be combined with (e.g., so that the conjugate is able to displace the recognition element from the binding site and thereby form a cross-linked material).

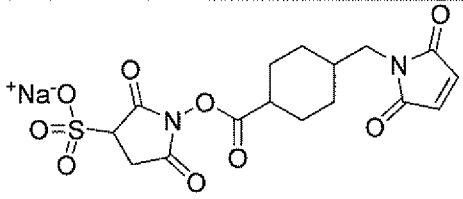
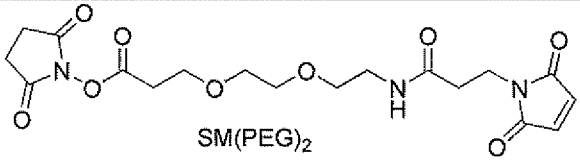
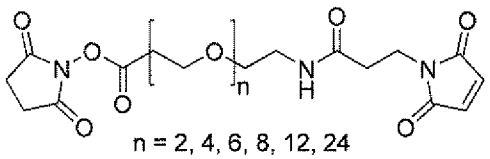
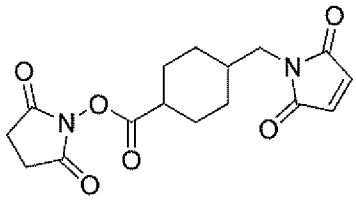
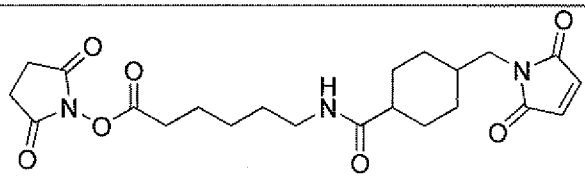
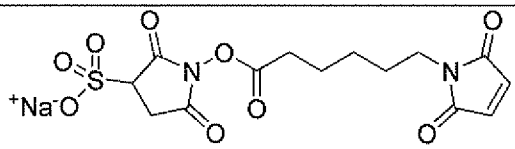
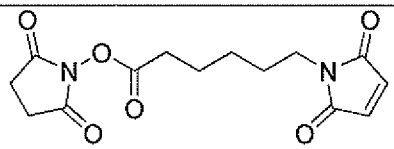
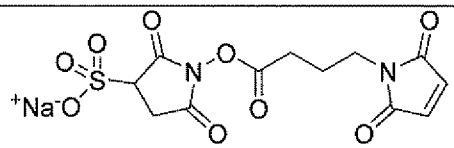
Reactive linker

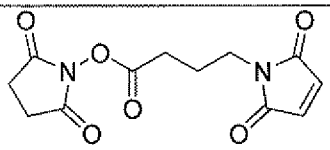
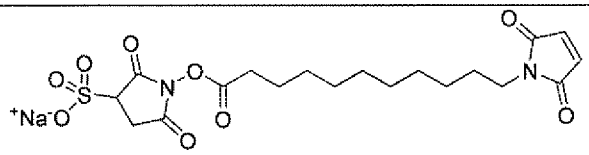
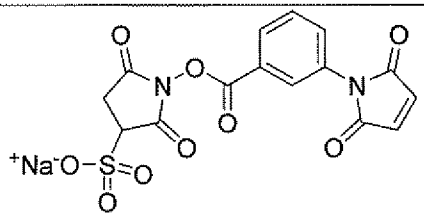
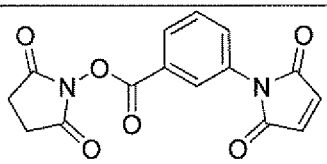
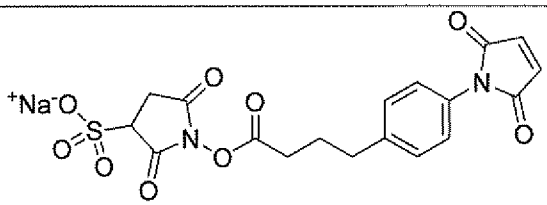
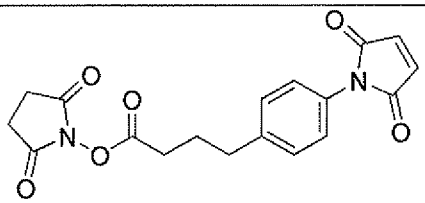
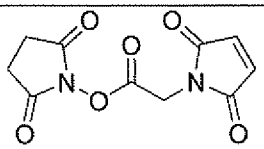
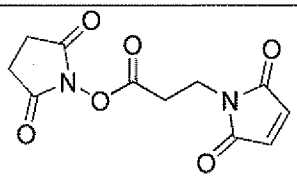
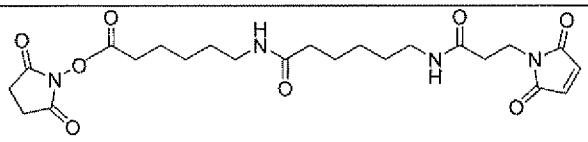
Affinity ligands are covalently linked to a cysteine residues of the lectin via a reactive linker. Typically, this will involve allowing the recognition element of the ligand to associate with the lectin binding site and then causing the reactive linker to react with the cysteine lectin. In certain embodiments, the reactive linker may be attached to the recognition element at a position that does not substantially interfere with the binding properties of the recognition element. For example, when the recognition element is a saccharide or polysaccharide the linker may be attached to the C1, C2 or C6 position of a terminal saccharide. In certain embodiments, the linker may be attached to the C1 position. The C1 position is also referred to as the anomeric carbon and may be connected to the linker in the alpha or beta conformation. In certain embodiments, the linker is attached to the C1 position as the alpha anomer.

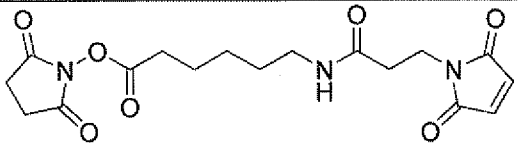
In certain embodiments the reactive linker includes a terminal maleimide group. The maleimide group reacts with the free thiol on the cysteine residue to form a covalent bond.

Exemplary precursors that can be used to prepare reactive linkers with a terminal maleimide group are shown in the table below. In certain embodiments, the NHS groups of a precursor shown below is allowed to react with an amine-containing recognition element (e.g., an amino-terminal saccharide such as AEM) to form a reactive linker.

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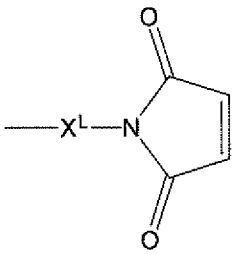
Precursor	Chemical Name	Chemical Structure
Sulfo-SMCC	Sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate	
SM(PEG)$_n$	Succinimidyl-[*N*-maleimidopropionamido]-#-ethyleneglycol) ester	 SM(PEG)$_2$  n = 2, 4, 6, 8, 12, 24
SMCC	Succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate	
LC-SMCC	Succinimidyl-4-[N-maleimidomethyl]cyclohexane-1-carboxy-[6-amidocaproate]	
Sulfo-EMCS	[N-ε-maleimidocaproyloxy] sulfosuccinimide ester	
EMCS	[N-ε-maleimidocaproyloxy] succinimide ester	
Sulfo-GMBS	N-[γ-maleimidobutyryloxy] sulfosuccinimide ester	

Precursor	Chemical Name	Chemical Structure
GMBS	N-[γ-maleimidobutyryloxy] succinimide ester	
Sulfo-KMUS	*N*-[κ-maleimidoundecanoyloxy] sulfosuccinimide ester	
Sulfo-MBS	*m*-maleimidobenzoyl-N-hydroxysulfosuccinimide ester	
MBS	*m*-maleimidobenzoyl-N-hydroxysuccinimide ester	
Sulfo-SMPB	Sulfosuccinimidyl 4-[*p*-maleimidophenyl]butyrate	
SMPB	Succinimidyl 4-[*p*-maleimidophenyl] butyrate	
AMAS	N-[α-maleimidoacetoxy] succinimide ester	
BMPS	*N*-[ß-maleimidopropyloxy] succinimide ester	
LC-SMPH	Succinimidyl-1-[β-maleimidopropionamido]-	

Precursor	Chemical Name	Chemical Structure
	hexanamido-6-hexanoate	
SMPH	Succinimidyl-6-[ß-maleimidopropionamido] hexanoate	

For example, in various embodiments the affinity ligand may be of the general formula (I): R_e–L^1 where R_e is a recognition element and –L^1 is a reactive linker. In certain embodiments R_e is a saccharide moiety. In certain embodiments R_e is a glucose or mannose moiety which is covalently bonded to the linker at the C1 position.

In certain embodiments –L^1 may be of the general formula (II):



(II)

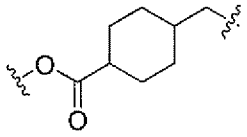
wherein:

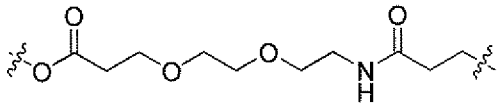
X^L is a covalent bond or a bivalent, straight or branched, saturated or unsaturated, optionally substituted C_{1-100} hydrocarbon chain wherein one or more methylene units of X^L are optionally and independently replaced by -O-, -S-, -N(R')-, -C(O)-, -C(O)O-, -OC(O)-, -N(R')C(O)-, -C(O)N(R')-, -S(O)-, -S(O)$_2$-, -N(R')SO$_2$-, -SO$_2$N(R')-, a carbocyclic group, a heterocyclic group, an aryl group, or a heteroaryl group; and

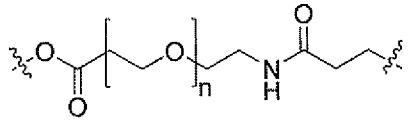
each occurrence of R' is independently hydrogen, a suitable protecting group, or an acyl moiety, arylalkyl moiety, aliphatic moiety, aryl moiety, heteroaryl moiety, or heteroaliphatic moiety.

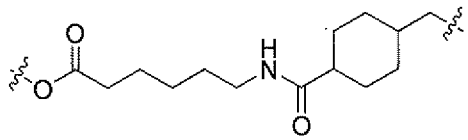
In certain embodiments, one, two, three, four, or five methylene units of X^L are optionally and independently replaced. In certain embodiments, X^L is constructed from a C_{1-20}, C_{1-10}, C_{1-8}, C_{1-6}, C_{1-4}, C_{2-12}, C_{4-12}, C_{6-12}, C_{8-12}, or C_{10-12} hydrocarbon chain wherein one or more

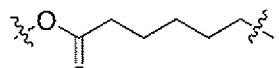
methylene units of X^L are optionally and independently replaced by -O-, -S-, -N(R')-, -C(O)-, -C(O)O-, -OC(O)-, -N(R')C(O)-, -C(O)N(R')-, -S(O)-, -$S(O)_2$-, -N(R')SO_2-, -SO_2N(R')-, a carbocyclic group, a heterocyclic group, an aryl group, or a heteroaryl group. In some embodiments, one or more methylene units of X^L is replaced by a carbocyclic group. In some embodiments, one or more methylene units of X^L is replaced by a cyclohexyl moiety. In some embodiments, one or more methylene units of X^L is replaced by a benzene ring. In certain embodiments, one or more methylene units of X^L is replaced by -C(O)-. In certain embodiments, one or more methylene units of X^L is replaced by -C(O)N(R')-. In certain embodiments, one or more methylene units of X^L is replaced by -O-. In certain embodiments, X^L contains a PEG moiety.

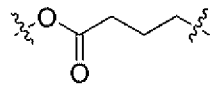
In some embodiments, X^L is .

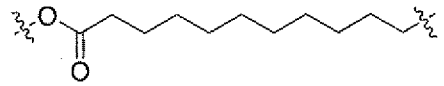
In some embodiments, X^L is .

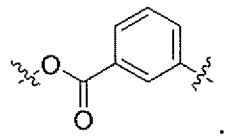
In some embodiments, X^L is , wherein n is 2, 4, 6, 8, 12, or 24.

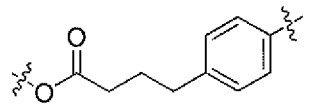
In some embodiments, X^L is .

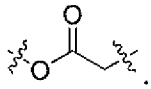
In some embodiments, X^L is .

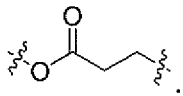
In some embodiments, X^L is .

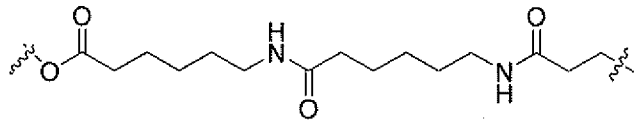
In some embodiments, X^L is .

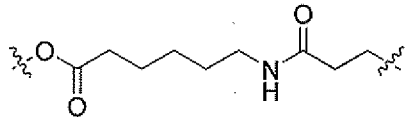
In some embodiments, X^L is .

In some embodiments, X^L is .

In some embodiments, X^L is .

In some embodiments, X^L is .

In some embodiments, X^L is 

In some embodiments, X^L is .

In various embodiments, the reactive linker for a particular lectin / target molecule combination may be selected empirically. According to such embodiments several affinity ligands with the same recognition element and different linkers (e.g., linkers of different lengths, linkers with different reactive groups, linkers with different hydrophobicity, etc.) are screened based on their effect on relevant lectin properties (e.g., MAC value and/or ability to precipitate glycogen, etc.).

Purification of modified lectins

In various embodiments, modified lectins (i.e., recombinant lectins that have been covalently attached to an affinity ligand) can be further processed in order to improve their properties. Thus, in certain embodiments, compositions comprising multivalent lectins can be purified in order to remove protein fragments, unmodified components, etc. In general, these separations can be achieved on the basis of physical properties (e.g., electrical charge; molecular weight; and/or size) and/or chemical properties (e.g., binding affinity for glucose or mannose). In certain embodiments optimal removal may be achieved by combining two or more methods that rely on these differential properties. In one embodiment, these separations are performed under denaturing conditions. For example, unmodified or partially modified lectins can be removed on the basis of their net charge by ion-exchange chromatography. Gel-filtration chromatography may be used to discriminate between differentially modified lectins on the basis of size. Affinity chromatography is another method that may be used to remove unmodified or partially modified lectins. This approach takes advantage of the differential binding affinity of modified, partially modified and unmodified lectins for a specific target molecule (e.g., glucose or mannose).

Characterization of modified lectins

In various embodiments, modified lectins can be screened or further tested in order to confirm or characterize their properties. Representative assays include: affinity assays, agglutination assays, T-cell mitogenicity assays, T-cell viability assays, antigenicity assays, etc.

Affinity assays may involve passing the modified lectins over an affinity column (e.g., a resin with the target molecule) and determining the elution conditions required to remove the lectin from the column. Equilibrium dialysis can also be used as is known in the art. Set point assays in which the modified lectins are combined with one or more conjugates of the present disclosure and then contacted with varying concentrations of the glucose may also be used. Preferably the binding affinity of the modified lectins is at least 75% that of the unmodified lectins. More preferably the binding affinity is at least 85% and yet more preferably at least 95% that of the unmodified lectins.

In certain embodiments, an agglutination assay may be used to determine the minimum agglutinating concentration (MAC) of a modified lectin. For example, in certain embodiments the MAC may be determined using rabbit erythrocytes as described in U.S. Patent Application Publication No. 2007-0110811. In certain embodiments a modified lectin may have a MAC that is higher than the unmodified lectin. Preferably the MAC is 25 times that of the unmodified lectin. More preferably the MAC is 50 times and yet more preferably more than 100 times that of the unmodified lectin. In certain embodiments, the modified lectin exhibits a MAC with a 2% v/v suspension of formaldehyde-stabilized rabbit erythrocytes that is greater than 4 ug/ml. Preferably the MAC is greater than 6 ug/ml, more preferably greater than 10 ug/ml, even more preferably greater than 25 ug/ml.

Mitogenicity assays will typically involve contacting the compositions of interest with a T-cell culture (e.g., PBMC cells) for a period of time and then measuring the level of T-cell proliferation. Various methods for measuring cell proliferation are known. In one embodiment the cell density may be measured spectrophotometrically at 450 nm. In another embodiment an indirect measure can obtained by detecting the reduction of MTT at 570 nm (e.g., see Ohno et al., *J. Immunol. Methods* 145:199-203, 1991). In preferred embodiments, the level of cell proliferation is determined using a tritiated thymidine uptake assay. Those skilled in the art will recognize that other suitable methods may be used and that the invention is in no way limited to a specific proliferation assay. In certain embodiments, the T-cell mitogenicity of a modified

lectin is less than 50% the T-cell mitogenicity of the unmodified lectin. The reduction in T-cell mitogenicity may be assessed by performing a comparative thymidine uptake assay across a range cross-linking agent concentrations, e.g., 0.01, 0.1, 1, 10, 100 and 1000 ug/ml. In preferred embodiments, the thymidine uptake assay is performed with samples that include approximately 500,000 PBMCs. The mitogenicity of the test composition (e.g., a modified composition) is then expressed as the % maximal unmodified mitogenicity. The % maximal unmodified mitogenicity is obtained by dividing the maximal CPM (counts per minute) value for the test composition over all measured concentrations by the maximal CPM value of the unmodified composition over all measured concentrations. Preferably, the test composition with reduced mitogenicity induces a level of T-cell proliferation that is at least 50% lower than the unmodified composition. More preferably, the level is at least 75% lower, even more preferably at least 90%, 95% or 99% lower.

T-cell viability can be measured using a similar experiment by adding Trypan Blue to the T-cell culture and counting a representative sample of the cells (noting those that either take up the trypan or still exclude the trypan, i.e., those that become blue vs. those that do not). The % viability is then calculated by dividing the number of cells that exclude the trypan (alive, "not blue") by the total number of cells counted (dead, "blue," plus live, "not blue"). Those skilled in the art will recognize that other suitable methods may be used and that the invention is in no way limited to a specific viability assay. In certain embodiments, a modified lectin exhibits a percentage cell viability at 100 ug/ml that is greater than 10% when assayed using PBMCs at a concentration of 500,000 cells/ml. Preferably the percentage cell viability is greater than 25%, more preferably greater than 50%, even more preferably greater than 90%.

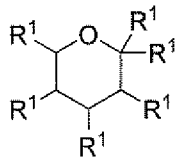
Conjugates

The conjugates include two or more separate affinity ligands bound to a conjugate framework. The two or more separate affinity ligands compete with the target molecule for binding with the modified lectin. Depending on the end application, the conjugates may also include a drug and/or a detectable label. The affinity ligands, drug, and/or detectable label may be covalently or non-covalently bound to the conjugate framework.

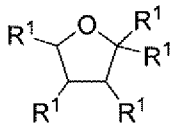
Affinity ligands

The two or more separate affinity ligands may have the same or different chemical structures. The two or more separate affinity ligands may have the same chemical structure as the target molecule itself or may be a chemically related species of the target molecule. The only requirement is that they compete with the target molecule for binding with the modified lectin. In certain embodiments, the relative affinity of the conjugate and target molecule for the modified lectin is in the range of 1:1 to 100:1 (where a relative affinity of 100:1 means that, in an equilibrium mixture of conjugate, target molecule and modified lectin (in pH 7 HEPES buffered saline at 37 C), the modified lectin will bind about equal molar amounts of conjugate and target molecule if the concentration of target molecule is 100 x the concentration of conjugate). In certain embodiments, the relative affinity is in the range of 1:1 to 50:1, 1:1 to 10:1, 1:1 to 5:1 or 1:1 to 2:1. In various embodiments it may be advantageous for the affinity ligands to have a different chemical structure from the target molecule, e.g., in order to fine tune the relative affinity of the modified lectin for the conjugates and the target molecule. For example, when the target molecule is glucose one might use a saccharide or a polysaccharide as one or more of affinity ligands. For example, when the target molecule is glucose the affinity ligands may include a saccharide. Thus, in certain embodiments, the affinity ligands are capable of competing with glucose for binding to a multivalent glucose binding molecule (e.g., without limitation Con A, mannan-binding lectin or MBL, etc.).

In certain embodiments, the affinity ligand is of formula (IIIa) or (IIIb):



IIIa



IIIb

wherein:

each R^1 is independently hydrogen, $-OR^y$, $-N(R^y)_2$, $-SR^y$, -O-Y, -G-Z, or $-CH_2R^x$;

each R^x is independently hydrogen, $-OR^y$, $-N(R^y)_2$, $-SR^y$, or -O-Y;

each R^y is independently $-R^2$, $-SO_2R^2$, $-S(O)R^2$, $-P(O)(OR^2)_2$, $-C(O)R^2$, $-CO_2R^2$, or $-C(O)N(R^2)_2$;

each Y is independently a monosaccharide, disaccharide, or trisaccharide;

each G is independently a covalent bond or an optionally substituted C_{1-9} alkylene, wherein one or more methylene units of G is optionally replaced by –O–, –S–, $-N(R^2)-$, $-C(O)-$, $-OC(O)-$, $-C(O)O-$, $-C(O)N(R^2)-$, $-N(R^2)C(O)-$, $-N(R^2)C(O)N(R^2)-$, $-SO_2-$, $-SO_2N(R^2)-$, $-N(R^2)SO_2-$, or $-N(R^2)SO_2N(R^2)-$;

each Z is independently halogen, $-N(R^2)_2$, $-OR^2$, $-SR^2$, $-N_3$, $-C\equiv CR^2$, $-CO_2R^2$, $-C(O)R^2$, or $-OSO_2R^2$; and

each R^2 is independently hydrogen or an optionally substituted group selected from C_{1-6} aliphatic, phenyl, a 4-7 membered heterocyclic ring having 1-2 heteroatoms selected from nitrogen, oxygen, or sulfur, or a 5-6 membered monocyclic heteroaryl ring having 1-4 heteroatoms selected from nitrogen, oxygen, or sulfur.

In certain embodiments, the affinity ligand of formula (IIIa) or (IIIb) is a monosaccharide. In certain embodiments, the affinity ligand is a disaccharide. In certain embodiments, the affinity ligand is a trisaccharide. In certain embodiments, the affinity ligand is a tetrasaccharide. In certain embodiments, the affinity ligand comprises no more than four saccharide moieties.

As defined generally above, each R^1 is independently hydrogen, $-OR^y$, $-N(R^y)_2$, $-SR^y$, -O-Y, -G-Z, or $-CH_2R^x$. In certain embodiments, R^1 is hydrogen. In certain embodiments, R^1 is –OH. In other embodiments, R^1 is $-NHC(O)CH_3$. In certain embodiments, R^1 is –O-Y. In certain other embodiments, R^1 is –G-Z. In some embodiments, R^1 is $-CH_2OH$. In other embodiments, R^1 is $-CH_2$-O-Y. In yet other embodiments, R^1 is $-NH_2$. One of ordinary skill in the art will appreciate that each R^1 substituent in formula (IIIa) or (IIIb) may be of (*R*) or (*S*) stereochemistry.

As defined generally above, each R^x is independently hydrogen, $-OR^y$, $-N(R^y)_2$, $-SR^y$, or –O-Y. In some embodiments, R^x is hydrogen. In certain embodiments, R^x is –OH. In other embodiments, R^x is –O-Y.

As defined generally above, each R^y is independently $-R^2$, $-SO_2R^2$, $-S(O)R^2$, $-P(O)(OR^2)_2$, $-C(O)R^2$, $-CO_2R^2$, or $-C(O)N(R^2)_2$. In some embodiments, R^y is hydrogen. In other embodiments, R^y is $-R^2$. In some embodiments, R^y is $-C(O)R^2$. In certain embodiments, R^y is acetyl. In other embodiments, R^y is $-SO_2R^2$, $-S(O)R^2$, $-P(O)(OR^2)_2$, $-CO_2R^2$, or $-C(O)N(R^2)_2$.

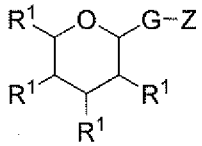
As defined generally above, Y is a monosaccharide, disaccharide, or trisaccharide. In certain embodiments, Y is a monosaccharide. In some embodiments, Y is a disaccharide. In other embodiments, Y is a trisaccharide. In some embodiments, Y is mannose, glucose, fructose, galactose, rhamnose, or xylopyranose. In some embodiments, Y is sucrose, maltose, turanose, trehalose, cellobiose, or lactose. In certain embodiments, Y is mannose. In certain embodiments, Y is D-mannose. One of ordinary skill in the art will appreciate that the

saccharide Y is attached to the oxygen group of –O-Y through anomeric carbon to form a glycosidic bond. The glycosidic bond may be of an alpha or beta configuration.

As defined generally above, each G is independently a covalent bond or an optionally substituted C_{1-9} alkylene, wherein one or more methylene units of G is optionally replaced by –O–, –S–, $-N(R^2)-$, –C(O) –, –OC(O) –, –C(O)O–, $-C(O)N(R^2)-$, $-N(R^2)C(O)-$, $-N(R^2)C(O)N(R^2)-$, $-SO_2-$, $-SO_2N(R^2)-$, $-N(R^2)SO_2-$, or $-N(R^2)SO_2N(R^2)-$. In some embodiments, G is a covalent bond. In certain embodiments, G is –O-C_{1-8} alkylene. In certain embodiments, G is $-OCH_2CH_2-$.

As defined generally above, each Z is independently halogen, $-N(R^2)_2$, $-OR^2$, $-SR^2$, $-N_3$, $-C\equiv CR^2$, $-CO_2R^2$, –C(O)R^2, or $-OSO_2R^2$. In some embodiments, Z is a halogen or $-OSO_2R^2$. In other embodiments, Z is $-N_3$ or -C≡CR^2. In certain embodiments, Z is $-N(R^2)_2$, -OR^2, or –SR^2. In certain embodiments, Z is –SH. In certain embodiments, Z is $-NH_2$. In certain embodiments, -G-Z is $-OCH_2CH_2NH_2$.

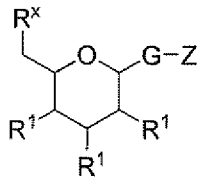
In some embodiments, the R^1 substituent on the C1 carbon of formula (IIIa) is –G-Z to give a compound of formula (IIIa-*i*):



IIIa-*i*

wherein R^1, G, and Z are as defined and described herein.

In some embodiments, the ligand is of formula (IIIa-*ii*):

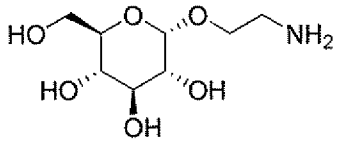


IIIa-*ii*

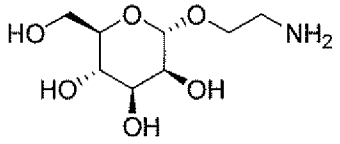
wherein R^1, R^x, G, and Z are as defined and described herein.

In certain embodiments where the target molecule is glucose, it may be advantageous for the affinity ligands to have a different chemical structure from glucose, e.g., in order to fine tune the response of a glucose-responsive material. For example, in certain embodiments, one might use an affinity ligand that includes one or more of the following: glucose, sucrose, maltose, mannose, derivatives of these (e.g., glucosamine, mannosamine, methylglucose, methylmannose, ethylglucose, ethylmannose, etc.) and/or higher order combinations of these (e.g., a bimannose, a

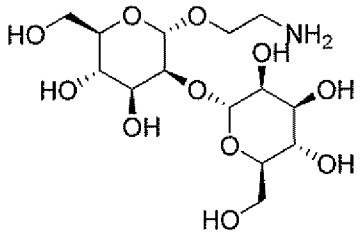
linear and/or branched trimannose, etc.). In certain embodiments, the affinity ligand includes a monosaccharide. In certain embodiments, the affinity ligand includes a disaccharide. In certain embodiments, the affinity ligand includes a trisaccharide. In certain embodiments, the affinity ligand includes a polysaccharide. In some embodiments, the affinity ligand includes a saccharide and one or more amine groups. In some embodiments, the affinity ligand is aminoethylglucose (AEG). In some embodiments, the affinity ligand is aminoethylmannose (AEM). In some embodiments, the affinity ligand is aminoethylbimannose (AEBM). In some embodiments, the affinity ligand is aminoethyltrimannose (AETM). In some embodiments, the affinity ligand is β-aminoethyl-N-acetylglucosamine (AEGA). In some embodiments, the affinity ligand is aminoethylfucose (AEF). In other embodiments, the affinity ligand is D-glucosamine (GA). In certain embodiments, a saccharide ligand is of the "D" configuration. In other embodiments, a saccharide ligand is of the "L" configuration. Below we show the structures of these exemplary affinity ligands. Other exemplary affinity ligands will be recognized by those skilled in the art.



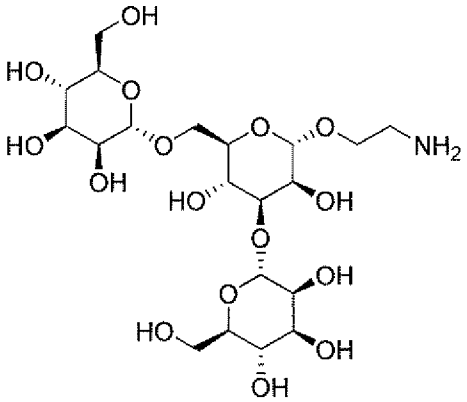
AEG



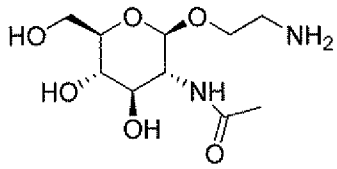
AEM



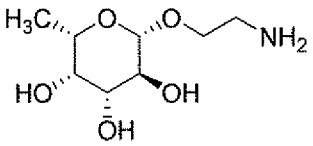
AEBM



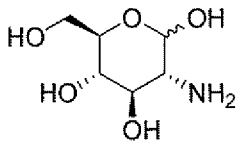
AETM



AEGA



AEF



GA

In various embodiments, the affinity ligand is a polysaccharide, glycopeptide or glycolipid. In certain embodiments, the affinity ligand includes from 2-10 saccharide moieties, e.g., 2, 3, 4, 5, 6, 7, 8, 9 or 10 moieties. The terminal and/or internal residues of the

polysaccharide, glycopeptide or glycolipid may be selected based on the saccharide specificity of the lectin in question (e.g., see Goldstein et al., *Biochem. Biophys. Acta* 317:500-504, 1973 and Lis et al., *Ann. Rev. Biochem.* 55:35-67, 1986).

In various embodiments, the affinity ligands for a particular conjugate / modified lectin combination may be selected empirically. According to such embodiments one or more affinity ligands are screened based on their relative binding affinities for the modified lectin as compared to glucose. In certain embodiments a library of saccharides and/or polysaccharides are screened in this manner. A suitable affinity ligand will exhibit a detectable level of competition with glucose but will not compete so strongly that it prevents all binding between the modified lectin and glucose.

Other exemplary target molecule / affinity ligand combinations will be recognized by those skilled in the art. In general, an affinity ligand can be generated for any target molecule using the target molecule itself and/or by generating derivatives of the target molecule (e.g., by making chemical and/or stereochemical modifications to the target molecule and then screening the resulting derivative for its relative affinity to the modified lectin in question).

As discussed in more detail below, the affinity ligands may be naturally present within the framework of the conjugate (e.g., as part of a polymer backbone or as a side group of a monomer). Alternatively (or additionally) affinity ligands may be artificially incorporated into the conjugate framework (e.g., in the form of a chemical group that is synthetically added to a conjugate framework). In certain embodiments, a conjugate may include a framework which comprises 5 or more, 10 or more, 20 or more, 25 or more, 50 or more, or 100 or more affinity ligands. In certain embodiments, a conjugate may include a framework which comprises 2-5, 2-10, 2-20, 2-25, 2-50 or 2-100 affinity ligands. In certain embodiments, a conjugate may include a framework which comprises as few as 2, 3 or 4 separate affinity ligands.

Methods for conjugating affinity ligands to a conjugate framework are discussed in more detail below. In certain embodiments, when the affinity ligands include a saccharide, the conjugation (whether direct or indirect) involves the C1, C2 or C6 position of the saccharide. In certain embodiments, the conjugation involves the C1 position. The C1 position is also referred to as the anomeric carbon and may be connected to the conjugate framework in the alpha or beta conformation. In certain embodiments, the C1 position is configured as the alpha anomer. In other embodiments, the C1 position is configured as the beta anomer.

Drug

As noted above, in various embodiments, a conjugate may comprise a drug. For example, a drug may be included when the material is to be used for therapeutic purposes, e.g., to controllably deliver a drug in a patient. It is to be understood that a conjugate can comprise any drug. A conjugate can comprise more than one copy of the same drug and/or can comprise more than one type of drug. The conjugates are not limited to any particular drug and may include small molecule drugs or biomolecular drugs. In general, the drug(s) used will depend on the disease or disorder to be treated.

For example, without limitation, in various embodiments a conjugate can comprise any one of the following drugs: diclofenac, nifedipine, rivastigmine, methylphenidate, fluoroxetine, rosiglitazone, prednison, prednisolone, codeine, ethylmorphine, dextromethorphan, noscapine, pentoxiverine, acetylcysteine, bromhexine, epinephrine, isoprenaline, orciprenaline, ephedrine, fenoterol, rimiterol, ipratropium, cholinetheophyllinate, proxiphylline, bechlomethasone, budesonide, deslanoside, digoxine, digitoxin, disopyramide, proscillaridin, chinidine, procainamide, mexiletin, flecainide, alprenolol, proproanolol, nadolol, pindolol, oxprenolol, labetalol, tirnolol, atenolol, pentaeritrityltetranitrate, isosorbiddinitrate, isosorbidmononitrate, niphedipin, phenylamine, verapamil, diltiazem, cyclandelar, nicotinylalcholhol, inositolnicotinate, alprostatdil, etilephrine, prenalterol, dobutamine, dopamine, dihydroergotamine, guanetidine, betanidine, methyldopa, reserpine, guanfacine, trimethaphan, hydralazine, dihydralazine, prazosine, diazoxid, captopril, nifedipine, enalapril, nitroprusside, bendroflumethiazide, hydrochlorthiazide, metychlothiazide, polythiazide, chlorthalidon, cinetazon, clopamide, mefruside, metholazone, bumetanide, ethacrynacide, spironolactone, amiloride, chlofibrate, nicotinic acid, nicheritrol, brompheniramine, cinnarizine, dexchlorpheniramine, clemastine, antazoline, cyproheptadine, proethazine, cimetidine, ranitidine, sucralfat, papaverine, moxaverine, atropin, butylscopolamin, emepron, glucopyrron, hyoscyamine, mepensolar, methylscopolamine, oxiphencyclimine, probanteline, terodilin, sennaglycosides, sagradaextract, dantron, bisachodyl, sodiumpicosulfat, etulos, diphenolxylate, loperamide, salazosulfapyridine, pyrvin, mebendazol, dimeticon, ferrofumarate, ferrosuccinate, ferritetrasemisodium, cyanochobalamine, folid acid heparin, heparin co-factor, diculmarole, warfarin, streptokinase, urokinase, factor VIII, factor IX, vitamin K, thiopeta, busulfan, chlorambucil, cyclophosphamid, melfalan, carmustin, mercatopurin, thioguanin, azathioprin, cytarabin, vinblastin, vinchristin, vindesin, procarbazine, dacarbazine, lomustin, estramustin,

teniposide, etoposide, cisplatin, amsachrin, aminogluthetimid, phosphestrol, medroxiprogresterone, hydroxiprogesterone, megesterol, noretisteron, tamoxiphen, ciclosporin, sulfosomidine, bensylpenicillin, phenoxymethylpenicillin, dicloxacillin, cloxacillin, flucoxacillin, ampicillin, amoxicillin, pivampicillin, bacampicillin, piperacillin, meziocillin, mecillinam, pivmecillinam, cephalotin, cephalexin, cephradin, cephadroxil, cephaclor, cefuroxim, cefotaxim, ceftazidim, cefoxitin, aztreonam, imipenem, cilastatin, tetracycline, lymecycline, demeclocycline, metacycline, oxitetracycline, doxycycline, chloramphenicol, spiramycin, fusidic acid, lincomycin, clindamycin, spectinomycin, rifampicin, amphotericin B, griseofulvin, nystatin, vancomycin, metronidazole, tinidazole, trimethoprim, norfloxacin, salazosulfapyridin, aminosalyl, isoniazid, etambutol, nitrofurantoin, nalidixic acid, metanamine, chloroquin, hydroxichloroquin, tinidazol, ketokonazol, acyclovir, interferon idoxuridin, retinal, tiamin, dexpantenol, pyridoxin, folic acid, ascorbic acid, tokoferol, phytominadion, phenfluramin, corticotropin, tetracosactid, tyrotropin, somatotoprin, somatrem, vasopressin, lypressin, desmopressin, oxytocin, chloriongonadotropin, cortison, hydrocortisone, fluodrocortison, prednison, prednisolon, fluoximesteron, mesterolon, nandrolon, stanozolol, oximetolon, cyproteron, levotyroxin, liotyronin, propylthiouracil, carbimazol, tiamazol, dihydrotachysterol, alfacalcidol, calcitirol, insulin, tolbutamid, chlorpropamid, tolazamid, glipizid, glibenclamid, phenobarbital, methyprylon, pyrityidion, meprobamat, chlordiazepoxid, diazepam, nitrazepam, baclofen, oxazepam, dikaliumclorazepat, lorazepam, flunitrazepam, alprazolam, midazolam, hydroxizin, dantrolene, chlometiazol, propionmazine, alimemazine, chlorpromazine, levomepromazine, acetophenazine, fluphenazine, perphenazine, prochlorperazine, trifluoperazine, dixyrazine, thiodirazine, periciazin, chloprothixene, tizanidine, zaleplon, zuclopentizol, flupentizol, thithixen, haloperidol, trimipramin, opipramol, chlomipramin, desipramin, lofepramin, amitriptylin, nortriptylin, protriptylin, maptrotilin, caffeine, cinnarizine, cyclizine, dimenhydinate, meclozine, prometazine, thiethylperazine, metoclopramide, scopolamine, phenobarbital, phenytoine, ethosuximide, primidone, carbamazepine, chlonazepam, orphenadrine, atropine, bensatropine, biperiden, metixene, procylidine, levodopa, bromocriptin, amantadine, ambenon, pyridostigmine, synstigmine, disulfiram, morphine, codeine, pentazocine, buprenorphine, pethidine, phenoperidine, phentanyl, methadone, piritramide, dextropropoxyphene, ketobemidone, acetylsalicylic acid, celecoxib, phenazone, phenylbutazone, azapropazone, piroxicam, ergotamine, dihydroergotamine, cyproheptadine, pizitifen, flumedroxon, allopurinol, probenecid, sodiummaurothiomalate

auronofin, penicillamine, estradiol, estradiolvalerianate, estriol, ethinylestradiol, dihydrogesteron, lynestrenol, medroxiprogresterone, noretisterone, cyclophenile, clomiphene, levonorgestrel, mestranol, ornidazol, tinidazol, ekonazol, chlotrimazol, natamycine, miconazole, sulbentin, methylergotamine, dinoprost, dinoproston, gemeprost, bromocriptine, phenylpropanolamine, sodiumchromoglicate, azetasolamide, dichlophenamide, betacarotene, naloxone, calciumfolinate, in particular clonidine, thephylline, dipyradamol, hydrochlothiazade, scopolamine, indomethacine, furosemide, potassium chloride, morphine, ibuprofen, salbutamol, terbutalin, calcitonin, etc. It is to be undersrtood that this list is intended to be exemplary and that any drug, whether known or later discovered, may be used in a conjugate of the present disclosure.

In various embodiments, a conjugate may include a hormonal drug which may be peptidic or non-peptidic, e.g., adrenaline, noradrenaline, angiotensin, atriopeptin, aldosterone, dehydroepiandrosterone, androstenedione, testosterone, dihydrotestosterone, calcitonin, calcitriol, calcidiol, corticotropin, cortisol, dopamine, estradiol, estrone, estriol, erythropoietin, follicle-stimulating hormone, gastrin, ghrelin, glucagon, gonadotropin-releasing hormone, growth hormone, growth hormone-releasing hormone, human chorionic gonadotropin, histamine, human placental lactogen, insulin, insulin-like growth factor, inhibin, leptin, a leukotriene, lipotropin, melatonin, orexin, oxytocin, parathyroid hormone, progesterone, prolactin, prolactin-releasing hormone, a prostglandin, renin, serotonin, secretin, somatostatin, thrombopoietin, thyroid-stimulating hormone, thyrotropin-releasing hormone (or thyrotropin), thyrotropin-releasing hormone, thyroxine, triiodothyronine, vasopressin, etc. In certain embodiments, the hormone may be selected from glucagon, insulin, insulin-like growth factor, leptin, thyroid-stimulating hormone, thyrotropin-releasing hormone (or thyrotropin), thyrotropin-releasing hormone, thyroxine, and triiodothyronine. It is to be understood that this list is intended to be exemplary and that any hormonal drug, whether known or later discovered, may be used in a conjugate of the present disclosure.

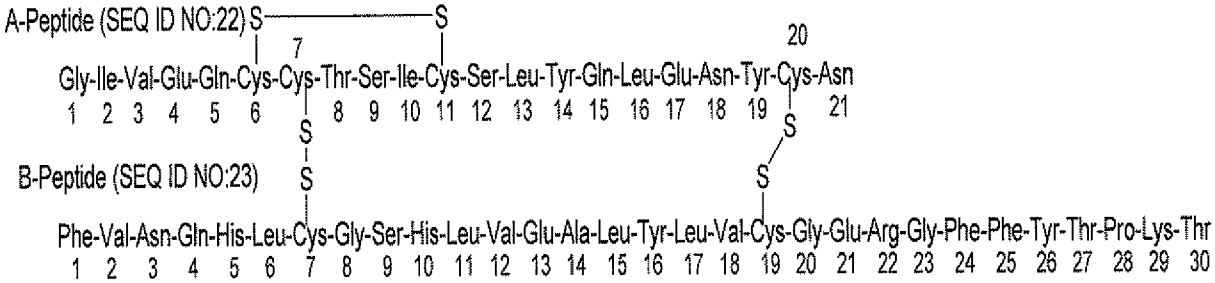
In various embodiments, a conjugate may include a thyroid hormone.

In various embodiments, a conjugate may include an anti-diabetic drug (i.e., a drug which has a beneficial effect on patients suffering from diabetes).

In various embodiments, a conjugate may include an insulin molecule. As used herein, the term "insulin" or "insulin molecule" encompasses all salt and non-salt forms of the insulin molecule. It will be appreciated that the salt form may be anionic or cationic depending on the

insulin molecule. By "insulin" or "an insulin molecule" we intend to encompass both wild-type and modified forms of insulin as long as they are bioactive (i.e., capable of causing a detectable reduction in glucose when administered *in vivo*). Wild-type insulin includes insulin from any species whether in purified, synthetic or recombinant form (e.g., human insulin, porcine insulin, bovine insulin, rabbit insulin, sheep insulin, etc.). A number of these are available commercially, e.g., from Sigma-Aldrich (St. Louis, MO).

The wild-type sequence of human insulin is shown below and in Figure 25:



The present disclosure is not limited to human insulin molecules (i.e., human proinsulin or bioactive human insulin molecules). In general, the present disclosure encompasses any human or non-human insulin that retains insulin-like bioactivity (i.e., is capable of causing a detectable reduction in glucose when administered to a suitable species at an appropriate dose *in vivo*). For example, as discussed below, the present disclosure also encompasses modified porcine insulin, bovine insulin, rabbit insulin, sheep insulin, etc.

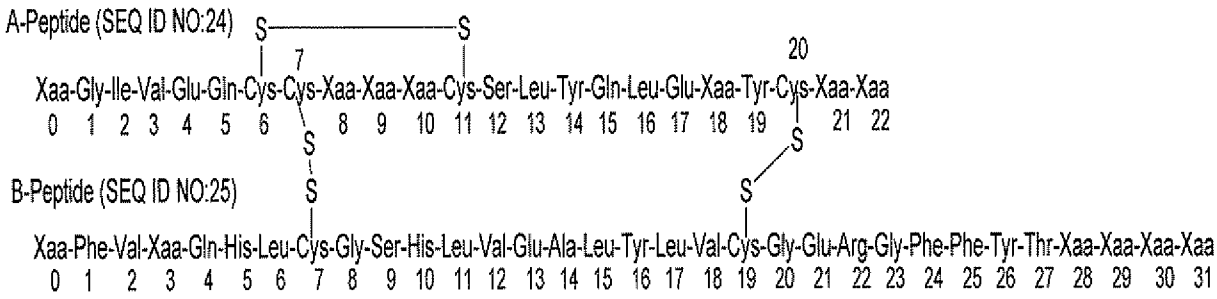
It is to be understood that an insulin molecule of the present disclosure may include chemical modifications and/or mutations that are not present in a wild-type insulin. A variety of modified insulins are known in the art (e.g., see Crotty and Reynolds, *Pediatr. Emerg. Care.* 23:903-905, 2007 and Gerich, *Am. J. Med.* 113:308-16, 2002 and references cited therein). Modified forms of insulin may be chemically modified (e.g., by addition of a chemical moiety such as a PEG group or a fatty acyl chain as described below) and/or mutated (i.e., by addition, deletion or substitution of amino acids).

In certain embodiments, an insulin molecule of the present disclosure will differ from a wild-type insulin by 1-10 (e.g., 1-9, 1-8, 1-7, 1-6, 1-5, 1-4, 1-3, 1-2, 2-9, 2-8, 2-7, 2-6, 2-5, 2-4, 2-3, 3-9, 3-8, 3-7, 3-6, 3-5, 3-4, 4-9, 4-8, 4-7, 4-6, 4-5, 5-9, 5-8, 5-7, 5-6, 6-9, 6-8, 6-7, 7-9, 7-8, 8-9, 9, 8, 7, 6, 5, 4, 3, 2 or 1) amino acid substitutions, additions and/or deletions. In certain embodiments, an insulin molecule of the present disclosure will differ from a wild-type insulin

by amino acid substitutions only. In certain embodiments, an insulin molecule of the present disclosure will differ from a wild-type insulin by amino acid additions only. In certain embodiments, an insulin molecule of the present disclosure will differ from a wild-type insulin by both amino acid substitutions and additions. In certain embodiments, an insulin molecule of the present disclosure will differ from a wild-type insulin by both amino acid substitutions and deletions.

In certain embodiments, amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. In certain embodiments, a substitution may be conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and tyrosine, phenylalanine. In certain embodiments, the hydrophobic index of amino acids may be considered in choosing suitable mutations. The importance of the hydrophobic amino acid index in conferring interactive biological function on a polypeptide is generally understood in the art. Alternatively, the substitution of like amino acids can be made effectively on the basis of hydrophilicity. The importance of hydrophilicity in conferring interactive biological function of a polypeptide is generally understood in the art. The use of the hydrophobic index or hydrophilicity in designing polypeptides is further discussed in U.S. Patent No. 5,691,198.

In certain embodiments, an insulin molecule of the present disclosure comprises an amino acid sequence of SEQ ID NO:24 (A-peptide) and an amino acid sequence of SEQ ID NO:25 (B-peptide) and three disulfide bridges as shown in formula (X^I):



(X^I)

where Xaa at each of positions A0, A22, B0 and B31 is independently a codable amino acid, a sequence of codable amino acids (e.g., 1-2, 1-3, 1-4, etc. codable amino acids), or missing; Xaa at each of positions A8, A9, A10, A18, and A21 is independently a codable amino acid; and Xaa at each of positions B3, B28, B29, and B30 is independently a codable amino acid or missing.

In some embodiments, Xaa at each of positions A0, A22, B0 and B31 is missing.

In certain embodiments, Xaa at one or more of the positions of the A- and B-peptides in formula (X^I) is selected from the choices that are set forth in Table 1 and 2 below.

Table 1 – A-peptide

Position	Amino Acid Identity
A0	Any codable amino acid, sequence of codable amino acids, or missing
A8	Thr or Ala
A9	Ser or Gly
A10	Ile or Val
A18	Asn, Asp or Glu
A21	Asn, Asp, Glu, Gly or Ala
A22	Any codable amino acid, sequence of codable amino acids, or missing

Table 2 – B-peptide

Position	Amino Acid Identity
B0	Any codable amino acid, sequence of codable amino acids, or missing
B3	Asn, Lys, Asp or Glu, or missing
B28	Pro, Ala, Lys, Leu, Val, or Asp, or missing
B29	Lys, Pro, or Glu, or missing
B30	Thr, Ala, Lys, Glu, Ser or Arg, or missing
B31	Any codable amino acid, sequence of codable amino acids, Arg-Arg, or missing

In some embodiments, an insulin molecule of formula (X^I) comprises amino acids at positions A8, A9, A10, and B30 selected from those shown in Table 3 below. In some embodiments, an insulin molecule of formula (X^I) comprises amino acids at positions A8, A9, A10, and B30 selected from those shown in Table 3 below for a single species (e.g., from the human sequence or Thr at A8, Ser at A9, Ile at A10 and Thr at B30).

Table 3

Species	Amino Acid Position			
	A8	A9	A10	B30
Human	Thr	Ser	Ile	Thr
Rabbit	Thr	Ser	Ile	Ser
Porcine	Thr	Ser	Ile	Ala
Bovine	Ala	Ser	Val	Ala
Sheep	Ala	Gly	Val	Ala

In various embodiments, an insulin molecule of the present disclosure is mutated at the B28 and/or B29 positions of the B-peptide sequence. For example, insulin lispro (HUMALOG®) is a rapid acting insulin mutant in which the penultimate lysine and proline residues on the C-terminal end of the B-peptide have been reversed ($Lys^{B28}Pro^{B29}$-human insulin). This modification blocks the formation of insulin multimers. Insulin aspart (NOVOLOG®) is another rapid acting insulin mutant in which proline at position B28 has been substituted with aspartic acid (Asp^{B28}-human insulin). This mutant also prevents the formation of multimers. In some embodiments, mutation at positions B28 and/or B29 is accompanied by one or more mutations elsewhere in the insulin molecule. For example, insulin glulisine (APIDRA®) is yet another rapid acting insulin mutant in which aspartic acid at position B3 has been replaced by a lysine residue and lysine at position B29 has been replaced with a glutamic acid residue ($Lys^{B3}Glu^{B29}$-human insulin).

In various embodiments, an insulin molecule of the present disclosure has an isoelectric point that is shifted relative to human insulin. In some embodiments, the shift in isoelectric point is achieved by adding one or more arginine residues to the N-terminus of the insulin A-peptide and/or the C-terminus of the insulin B- peptide. Examples of such insulin molecules include Arg^{A0}-human insulin, $Arg^{B31}Arg^{B32}$-human insulin, $Gly^{A21}Arg^{B31}Arg^{B32}$-human insulin, $Arg^{A0}Arg^{B31}Arg^{B32}$-human insulin, and $Arg^{A0}Gly^{A21}Arg^{B31}Arg^{B32}$-human insulin. By way of further example, insulin glargine (LANTUS®) is an exemplary long acting insulin mutant in which Asp^{A21} has been replaced by glycine, and two arginine residues have been added to the C-terminus of the B-peptide. The effect of these changes is to shift the isoelectric point, producing

a solution that is completely soluble at pH 4. Thus, in some embodiments, an insulin molecule of the present disclosure comprises an A-peptide sequence wherein A21 is Gly and B-peptide sequence wherein B31 is Arg-Arg. It is to be understood that the present disclosure encompasses all single and multiple combinations of these mutations and any other mutations that are described herein (e.g., Gly^{A21}-human insulin, $Gly^{A21}Arg^{B31}$-human insulin, $Arg^{B31}Arg^{B32}$-human insulin, Arg^{B31}-human insulin).

In various embodiments, an insulin molecule of the present disclosure may be truncated. For example, the B-peptide sequence may be missing residues B(1-2), B(1-3), B30, B(29-30) or B(28-30). In some embodiments, these deletions and/or truncations apply to any of the aforementioned insulin molecules (e.g., without limitation to produce des(B30) insulin lispro, des(B30) insulin aspart, des(B30) insulin glulisine, des(B30) insulin glargine, etc.).

In some embodiments, an insulin molecule contains additional amino acid residues on the N- or C-terminus of the A or B-peptide sequences. In some embodiments, one or more amino acid residues are located at positions A0, A22, B0, and/or B31. In some embodiments, one or more amino acid residues are located at position A0. In some embodiments, one or more amino acid residues are located at position A22. In some embodiments, one or more amino acid residues are located at position B0. In some embodiments, one or more amino acid residues are located at position B31. In certain embodiments, an insulin molecule does not include any additional amino acid residues at positions A0, A22, B0, or B31.

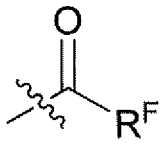
In certain embodiments, an insulin molecule of the present disclosure may have mutations wherein one or more amidated amino acids are replaced with acidic forms. For example, asparagine may be replaced with aspartic acid or glutamic acid. Likewise, glutamine may be replaced with aspartic acid or glutamic acid. In particular, Asn^{A18}, Asn^{A21}, or Asn^{B3}, or any combination of those residues, may be replaced by aspartic acid or glutamic acid. Gln^{A15} or Gln^{B4}, or both, may be replaced by aspartic acid or glutamic acid. In certain embodiments, an insulin molecule has aspartic acid at position A21 or aspartic acid at position B3, or both.

One skilled in the art will recognize that it is possible to mutate yet other amino acids in the insulin molecule while retaining biological activity. For example, without limitation, the following modifications are also widely accepted in the art: replacement of the histidine residue of position B10 with aspartic acid ($His^{B10} \rightarrow Asp^{B10}$); replacement of the phenylalanine residue at position B1 with aspartic acid ($Phe^{B1} \rightarrow Asp^{B1}$); replacement of the threonine residue at position B30 with alanine ($Thr^{B30} \rightarrow Ala^{B30}$); replacement of the tyrosine residue at position B26 with

alanine ($Tyr^{B26} \rightarrow Ala^{B26}$); and replacement of the serine residue at position B9 with aspartic acid ($Ser^{B9} \rightarrow Asp^{B9}$).

In various embodiments, an insulin molecule of the present disclosure has a protracted profile of action. Thus, in certain embodiments, an insulin molecule of the present disclosure may be acylated with a fatty acid. That is, an amide bond is formed between an amino group on the insulin molecule and the carboxylic acid group of the fatty acid. The amino group may be the alpha-amino group of an N-terminal amino acid of the insulin molecule, or may be the epsilon-amino group of a lysine residue of the insulin molecule. An insulin molecule of the present disclosure may be acylated at one or more of the three amino groups that are present in wild-type insulin or may be acylated on lysine residue that has been introduced into the wild-type sequence. In certain embodiments, an insulin molecule may be acylated at position B1. In certain embodiments, an insulin molecule may be acylated at position B29. In certain embodiments, the fatty acid is selected from myristic acid (C14), pentadecylic acid (C15), palmitic acid (C16), heptadecylic acid (C17) and stearic acid (C18). For example, insulin detemir (LEVEMIR®) is a long acting insulin mutant in which Thr^{B30} has been deleted, and a C14 fatty acid chain (myristic acid) has been attached to Lys^{B29}.

In some embodiments, the N-terminus of the A-peptide, the N-terminus of the B-peptide, the epsilon-amino group of Lys at position B29 or any other available amino group in an insulin molecule of the present disclosure is covalently linked to a fatty acid moiety of general formula:



wherein R^F is hydrogen or a C_{1-30} alkyl group. In some embodiments, R^F is a C_{1-20} alkyl group, a C_{3-19} alkyl group, a C_{5-18} alkyl group, a C_{6-17} alkyl group, a C_{8-16} alkyl group, a C_{10-15} alkyl group, or a C_{12-14} alkyl group. In certain embodiments, the insulin polypeptide is conjugated to the moiety at the A1 position. In certain embodiments, the insulin polypeptide is conjugated to the moiety at the B1 position. In certain embodiments, the insulin polypeptide is conjugated to the moiety at the epsilon-amino group of Lys at position B29. In certain embodiments, position B28 of the insulin molecule is Lys and the epsilon-amino group of Lys^{B28} is conjugated to the fatty acid moiety. In certain embodiments, position B3 of the insulin molecule is Lys and the epsilon-amino group of Lys^{B3} is conjugated to the fatty acid moiety. In some embodiments, the fatty acid chain is 8-20 carbons long. In some embodiments, the fatty acid is octanoic acid (C8),

nonanoic acid (C9), decanoic acid (C10), undecanoic acid (C11), dodecanoic acid (C12), or tridecanoic acid (C13). In certain embodiments, the fatty acid is myristic acid (C14), pentadecanoic acid (C15), palmitic acid (C16), heptadecanoic acid (C17), stearic acid (C18), nonadecanoic acid (C19), or arachidic acid (C20).

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $Lys^{B28}Pro^{B29}$-human insulin (insulin lispro), Asp^{B28}-human insulin (insulin aspart), $Lys^{B3}Glu^{B29}$-human insulin (insulin glulisine), $Arg^{B31}Arg^{B32}$-human insulin (insulin glargine), $N^{\varepsilon B29}$-myristoyl-des(B30)-human insulin (insulin detemir), Ala^{B26}-human insulin, Asp^{B1}-human insulin, Arg^{A0}-human insulin, $Asp^{B1}Glu^{B13}$-human insulin, Gly^{A21}-human insulin, $Gly^{A21}Arg^{B31}Arg^{B32}$-human insulin, $Arg^{A0}Arg^{B31}Arg^{B32}$-human insulin, $Arg^{A0}Gly^{A21}Arg^{B31}Arg^{B32}$-human insulin, des(B30)-human insulin, des(B27)-human insulin, des(B28-B30)-human insulin, des(B1)-human insulin, des(B1-B3)-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-palmitoyl-human insulin, $N^{\varepsilon B29}$-myrisotyl-human insulin, $N^{\varepsilon B28}$-palmitoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-myristoyl-$Lys^{B28}Pro^{B29}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-palmitoyl-des(B30)-human insulin, $N^{\varepsilon B30}$-myristoyl-$Thr^{B29}Lys^{B30}$-human insulin, $N^{\varepsilon B30}$-palmitoyl-$Thr^{B29}Lys^{B30}$-human insulin, $N^{\varepsilon B29}$-(N-palmitoyl-γ-glutamyl)-des(B30)-human insulin, $N^{\varepsilon B29}$-(N-lithocolyl-γ-glutamyl)-des(B30)-human insulin, $N^{\varepsilon B29}$-(ω-carboxyheptadecanoyl)-des(B30)-human insulin, $N^{\varepsilon B29}$-(ω-carboxyheptadecanoyl)- human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-octanoyl-human insulin, $N^{\varepsilon B29}$-myristoyl-$Gly^{A21}Arg^{B31}Arg^{B31}$-human insulin, $N^{\varepsilon B29}$-myristoyl-$Gly^{A21}Gln^{B3}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B29}$-myristoyl-$Arg^{A0}Gly^{A21}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B29}$-$Arg^{A0}Gly^{A21}Gln^{B3}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B29}$-myristoyl-$Arg^{A0}Gly^{A21}Asp^{B3}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B29}$-myristoyl-$Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B29}$-myristoyl-$Arg^{A0}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B29}$-octanoyl-$Gly^{A21}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B29}$-octanoyl-$Gly^{A21}Gln^{B3}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B29}$-octanoyl-$Arg^{A0}Gly^{A21}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B29}$-octanoyl-$Arg^{A0}Gly^{A21}Gln^{B3}Arg^{B31}Arg^{B32}$-human

insulin, $N^{\varepsilon B29}$-octanoyl-$Arg^{B0}Gly^{A21}Asp^{B3}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B29}$-octanoyl-$Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B29}$-octanoyl-$Arg^{A0}Arg^{B31}Arg^{B32}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B28}$-myristoyl-$Gly^{A21}Lys^{B28}Pro^{B29}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B28}$-myristoyl-$Gly^{A21}Gln^{B3}Lys^{B28}Pro^{B30}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B28}$-myristoyl-$Arg^{A0}Gly^{A21}Lys^{B28}Pro^{B29}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B28}$-myristoyl-$Arg^{A0}Gly^{A21}Gln^{B3}Lys^{B28}Pro^{B29}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B28}$-myristoyl-$Arg^{A0}Gly^{A21}Asp^{B3}Lys^{B28}Pro^{B29}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B28}$-myristoyl-$Lys^{B28}Pro^{B29}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B28}$-myristoyl-$arg^{A0}Lys^{B28}Pro^{B29}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B28}$-octanoyl-$Gly^{A21}Lys^{B28}Pro^{B29}Arg^{B31}Arg^{B32}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B28}$-octanoyl-$Gly^{A21}Gln^{B3}Lys^{B28}Pro^{B29}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B28}$-octanoyl-$Arg^{A0}Gly^{A21}Lys^{B28}Pro^{B29}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B28}$-octanoyl-$Arg^{A0}Gly^{A21}Gln^{B3}Lys^{B28}Pro^{B29}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B28}$-octanoyl-$Arg^{A0}Gly^{A21}Asp^{B3}Lys^{B28}Pro^{B29}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B28}$-octanoyl-$Lys^{B28}Pro^{B29}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B28}$-octanoyl-$Arg^{A0}Lys^{B28}Pro^{B29}Arg^{B31}Arg^{B32}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-tridecanoyl-des(B30)-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-des(B30)-human insulin, $N^{\varepsilon B29}$-decanoyl-des(B30)-human insulin, $N^{\varepsilon B29}$-dodecanoyl-des(B30)-human insulin, $N^{\varepsilon B29}$-tridecanoyl-Gly^{A21}-des(B30)-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-Gly^{A21}-des(B30)-human insulin, $N^{\varepsilon B29}$-decanoyl-Gly^{A21}-des(B30)-human insulin, $N^{\varepsilon B29}$-dodecanoyl-Gly^{A21}-des(B30)-human insulin, $N^{\varepsilon B29}$-tridecanoyl-$Gly^{A21}Gln^{B3}$-des(B30)-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-$Gly^{A21}Gln^{B3}$-des(B30)-human insulin, $N^{\varepsilon B29}$-decanoyl-Gly^{A21}-Gln^{B3}-des(B30)-human insulin, $N^{\varepsilon B29}$-dodecanoyl-Gly^{A21}-Gln^{B3}-des(B30)-human insulin, $N^{\varepsilon B29}$-tridecanoyl-Ala^{A21}-des(B30)-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-Ala^{A21}-des(B30)-human insulin, $N^{\varepsilon B29}$-decanoyl-Ala^{A21}-des(B30)-human insulin, $N^{\varepsilon B29}$-dodecanoyl-Ala^{A21}-des(B30)-human insulin, $N^{\varepsilon B29}$-tridecanoyl-Ala^{A21}-Gln^{B3}-des(B30)-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-$Ala^{A21}Gln^{B3}$-des(B30)-human insulin, $N^{\varepsilon B29}$-decanoyl-$Ala^{A21}Gln^{B3}$-des(B30)-human insulin, $N^{\varepsilon B29}$-dodecanoyl-$Ala^{A21}Gln^{B3}$-des(B30)-human

insulin, $N^{\varepsilon B29}$-tridecanoyl-Gln^{B3}-des(B30)-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-Gln^{B3}-des(B30)-human insulin, $N^{\varepsilon B29}$-decanoyl-Gln^{B3}-des(B30)-human insulin, $N^{\varepsilon B29}$-dodecanoyl-Gln^{B3}-des(B30)-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-tridecanoyl-Gly^{A21}-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-Gly^{A21}-human insulin, $N^{\varepsilon B29}$-decanoyl-Gly^{A21}-human insulin, $N^{\varepsilon B29}$-dodecanoyl-Gly^{A21}-human insulin, $N^{\varepsilon B29}$-tridecanoyl-Ala^{A21}-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-Ala^{A21}-human insulin, $N^{\varepsilon B29}$-decanoyl-Ala^{A21}-human insulin, $N^{\varepsilon B29}$-dodecanoyl-Ala^{A21}-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-tridecanoyl-$Gly^{A21}Gln^{B3}$-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-$Gly^{A21}Gln^{B3}$-human insulin, $N^{\varepsilon B29}$-decanoyl-$Gly^{A21}Gln^{B3}$-human insulin, $N^{\varepsilon B29}$-dodecanoyl-$Gly^{A21}Gln^{B3}$-human insulin, $N^{\varepsilon B29}$-tridecanoyl-$Ala^{A21}Gln^{B3}$-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-$Ala^{A21}Gln^{B3}$-human insulin, $N^{\varepsilon B29}$-decanoyl-$Ala^{A21}Gln^{B3}$-human insulin, $N^{\varepsilon B29}$-dodecanoyl-$Ala^{A21}Gln^{B3}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-tridecanoyl-Gln^{B3}-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-Gln^{B3}-human insulin, $N^{\varepsilon B29}$-decanoyl-Gln^{B3}-human insulin, $N^{\varepsilon B29}$-dodecanoyl-Gln^{B3}-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-tridecanoyl-Glu^{B30}-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-Glu^{B30}-human insulin, $N^{\varepsilon B29}$-decanoyl-Glu^{B30}-human insulin, $N^{\varepsilon B29}$-dodecanoyl-Glu^{B30}-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-tridecanoyl-$Gly^{A21}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-$Gly^{A21}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-decanoyl-$Gly^{A21}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-dodecanoyl-$Gly^{A21}Glu^{B30}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-tridecanoyl-$Gly^{A21}Gln^{B3}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-$Gly^{A21}Gln^{B3}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-decanoyl-$Gly^{A21}Gln^{B3}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-dodecanoyl-$Gly^{A21}Gln^{B3}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-tridecanoyl-$Ala^{A21}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-$Ala^{A21}Glu^{B30}$-

human insulin, $N^{\varepsilon B29}$-decanoyl-$Ala^{A21}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-dodecanoyl-$Ala^{A21}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-tridecanoyl-$Ala^{A21}Gln^{B3}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-$Ala^{A21}Gln^{B3}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-decanoyl-$Ala^{A21}Gln^{B3}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-dodecanoyl-$Ala^{A21}Gln^{B3}Glu^{B30}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-tridecanoyl-$Gln^{B3}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-tetradecanoyl-$Gln^{B3}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-decanoyl-$Gln^{B3}Glu^{B30}$-human insulin, $N^{\varepsilon B29}$-dodecanoyl-$Gln^{B3}Glu^{B30}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-formyl-human insulin, $N^{\alpha B1}$-formyl-human insulin, $N^{\alpha A1}$-formyl-human insulin, $N^{\varepsilon B29}$-formyl-$N^{\alpha B1}$-formyl-human insulin, $N^{\varepsilon B29}$-formyl-$N^{\alpha A1}$-formyl-human insulin, $N^{\alpha A1}$-formyl-$N^{\alpha B1}$-formyl-human insulin, $N^{\varepsilon B29}$-formyl-$N^{\alpha A1}$-formyl-$N^{\alpha B1}$-formyl-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-acetyl-human insulin, $N^{\alpha B1}$-acetyl-human insulin, $N^{\alpha A1}$-acetyl-human insulin, $N^{\varepsilon B29}$-acetyl- $N^{\alpha B1}$-acetyl-human insulin, $N^{\varepsilon B29}$-acetyl-$N^{\alpha A1}$-acetyl-human insulin, $N^{\alpha A1}$-acetyl-$N^{\alpha B1}$-acetyl-human insulin, $N^{\varepsilon B29}$-acetyl-$N^{\alpha A1}$-acetyl- $N^{\alpha B1}$-acetyl-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-propionyl-human insulin, $N^{\alpha B1}$-propionyl-human insulin, $N^{\alpha A1}$-propionyl-human insulin, $N^{\varepsilon B29}$-acetyl- $N^{\alpha B1}$-propionyl-human insulin, $N^{\varepsilon B29}$-propionyl- $N^{\alpha A1}$-propionyl-human insulin, $N^{\alpha A1}$-propionyl- $N^{\alpha B1}$-propionyl-human insulin, $N^{\varepsilon B29}$-propionyl-$N^{\alpha A1}$-propionyl-$N^{\alpha B1}$-propionyl-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-butyryl-human insulin, $N^{\alpha B1}$-butyryl-human insulin, $N^{\alpha A1}$-butyryl-human insulin, $N^{\varepsilon B29}$-butyryl-$N^{\alpha B1}$-butyryl-human insulin, $N^{\varepsilon B29}$-butyryl-$N^{\alpha A1}$-butyryl-human insulin, $N^{\alpha A1}$-butyryl-$N^{\alpha B1}$-butyryl-human insulin, $N^{\varepsilon B29}$-butyryl-$N^{\alpha A1}$-butyryl-$N^{\alpha B1}$-butyryl-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-pentanoyl-human insulin, $N^{\alpha B1}$-pentanoyl-human insulin, $N^{\alpha A1}$-pentanoyl-human insulin, $N^{\varepsilon B29}$-

pentanoyl-$N^{\alpha B1}$-pentanoyl-human insulin, $N^{\varepsilon B29}$-pentanoyl-$N^{\alpha A1}$-pentanoyl-human insulin, $N^{\alpha A1}$-pentanoyl-$N^{\alpha B1}$-pentanoyl-human insulin, $N^{\varepsilon B29}$-pentanoyl-$N^{\alpha A1}$-pentanoyl-$N^{\alpha B1}$-pentanoyl-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-hexanoyl-human insulin, $N^{\alpha B1}$-hexanoyl-human insulin, $N^{\alpha A1}$-hexanoyl-human insulin, $N^{\varepsilon B29}$-hexanoyl-$N^{\alpha B1}$-hexanoyl-human insulin, $N^{\varepsilon B29}$-hexanoyl-$N^{\alpha A1}$-hexanoyl-human insulin, $N^{\alpha A1}$-hexanoyl-$N^{\alpha B1}$-hexanoyl-human insulin, $N^{\varepsilon B29}$-hexanoyl-$N^{\alpha A1}$-hexanoyl-$N^{\alpha B1}$-hexanoyl-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-heptanoyl-human insulin, $N^{\alpha B1}$-heptanoyl-human insulin, $N^{\alpha A1}$-heptanoyl-human insulin, $N^{\varepsilon B29}$-heptanoyl-$N^{\alpha B1}$-heptanoyl-human insulin, $N^{\varepsilon B29}$-heptanoyl-$N^{\alpha A1}$-heptanoyl-human insulin, $N^{\alpha A1}$-heptanoyl-$N^{\alpha B1}$-heptanoyl-human insulin, $N^{\varepsilon B29}$-heptanoyl-$N^{\alpha A1}$-heptanoyl-$N^{\alpha B1}$-heptanoyl-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\alpha B1}$-octanoyl-human insulin, $N^{\alpha A1}$-octanoyl-human insulin, $N^{\varepsilon B29}$-octanoyl-$N^{\alpha B1}$-octanoyl-human insulin, $N^{\varepsilon B29}$-octanoyl-$N^{\alpha A1}$-octanoyl-human insulin, $N^{\alpha A1}$-octanoyl-$N^{\alpha B1}$-octanoyl-human insulin, $N^{\varepsilon B29}$-octanoyl-$N^{\alpha A1}$-octanoyl-$N^{\alpha B1}$-octanoyl-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-nonanoyl-human insulin, $N^{\alpha B1}$-nonanoyl-human insulin, $N^{\alpha A1}$-nonanoyl-human insulin, $N^{\varepsilon B29}$-nonanoyl-$N^{\alpha B1}$-nonanoyl-human insulin, $N^{\varepsilon B29}$-nonanoyl-$N^{\alpha A1}$-nonanoyl-human insulin, $N^{\alpha A1}$-nonanoyl-$N^{\alpha B1}$-nonanoyl-human insulin, $N^{\varepsilon B29}$-nonanoyl-$N^{\alpha A1}$-nonanoyl-$N^{\alpha B1}$-nonanoyl-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-decanoyl-human insulin, $N^{\alpha B1}$-decanoyl-human insulin, $N^{\alpha A1}$-decanoyl-human insulin, $N^{\varepsilon B29}$-decanoyl-$N^{\alpha B1}$-decanoyl-human insulin, $N^{\varepsilon B29}$-decanoyl-$N^{\alpha A1}$-decanoyl-human insulin, $N^{\alpha A1}$-decanoyl-$N^{\alpha B1}$-decanoyl-human insulin, $N^{\varepsilon B29}$-decanoyl-$N^{\alpha A1}$-decanoyl-$N^{\alpha B1}$-decanoyl-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B28}$-formyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha B1}$-formyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-formyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-formyl-$N^{\alpha B1}$-formyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-formyl-$N^{\alpha A1}$-formyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-formyl-$N^{\alpha B1}$-formyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-formyl-$N^{\alpha A1}$-formyl-$N^{\alpha B1}$-formyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B29}$-acetyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha B1}$-acetyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-acetyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-acetyl-$N^{\alpha B1}$-acetyl-$Lys^{B28}Pro^{B29}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B28}$-acetyl-$N^{\alpha A1}$-acetyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-acetyl-$N^{\alpha B1}$-acetyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-acetyl-$N^{\alpha A1}$-acetyl-$N^{\alpha B1}$-acetyl-$Lys^{B28}Pro^{B29}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B28}$-propionyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha B1}$-propionyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-propionyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-propionyl-$N^{\alpha B1}$-propionyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-propionyl-$N^{\alpha A1}$-propionyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-propionyl-$N^{\alpha B1}$-propionyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-propionyl-$N^{\alpha A1}$-propionyl-$N^{\alpha B1}$-propionyl-$Lys^{B28}Pro^{B29}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B28}$-butyryl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha B1}$-butyryl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-butyryl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-butyryl-$N^{\alpha B1}$-butyryl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-butyryl-$N^{\alpha A1}$-butyryl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-butyryl-$N^{\alpha B1}$-butyryl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-butyryl-$N^{\alpha A1}$-butyryl-$N^{\alpha B1}$-butyryl-$Lys^{B28}Pro^{B29}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B28}$-pentanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha B1}$-pentanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-pentanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-pentanoyl-$N^{\alpha B1}$-pentanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-pentanoyl-$N^{\alpha A1}$-pentanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-pentanoyl-$N^{\alpha B1}$-pentanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-pentanoyl-$N^{\alpha A1}$-pentanoyl-$N^{\alpha B1}$-pentanoyl-$Lys^{B28}Pro^{B29}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B28}$-hexanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha B1}$-hexanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-hexanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-hexanoyl-$N^{\alpha B1}$-hexanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-hexanoyl-$N^{\alpha A1}$-hexanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-hexanoyl-$N^{\alpha B1}$-hexanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-hexanoyl-$N^{\alpha A1}$-hexanoyl-$N^{\alpha B1}$-hexanoyl-$Lys^{B28}Pro^{B29}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B28}$-heptanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha B1}$-heptanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-heptanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-heptanoyl-$N^{\alpha B1}$-heptanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-heptanoyl-$N^{\alpha A1}$-heptanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-heptanoyl-$N^{\alpha B1}$-heptanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-heptanoyl-$N^{\alpha A1}$-heptanoyl-$N^{\alpha B1}$-heptanoyl-$Lys^{B28}Pro^{B29}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B28}$-octanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha B1}$-octanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-octanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-octanoyl-$N^{\alpha B1}$-octanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-octanoyl-$N^{\alpha A1}$-octanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-octanoyl-$N^{\alpha B1}$-octanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-octanoyl-$N^{\alpha A1}$-octanoyl-$N^{\alpha B1}$-octanoyl-$Lys^{B28}Pro^{B29}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B28}$-nonanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha B1}$-nonanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-nonanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-nonanoyl-$N^{\alpha B1}$-nonanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-nonanoyl-$N^{\alpha A1}$-nonanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-nonanoyl-$N^{\alpha B1}$-nonanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-nonanoyl-$N^{\alpha A1}$-nonanoyl-$N^{\alpha B1}$-nonanoyl-$Lys^{B28}Pro^{B29}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B28}$-decanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha B1}$-decanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-decanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-decanoyl-$N^{\alpha B1}$-decanoyl-$Lys^{B28}Pro^{B29}$-human

insulin, $N^{\varepsilon B28}$-decanoyl-$N^{\alpha A1}$-decanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\alpha A1}$-decanoyl-$N^{\alpha B1}$-decanoyl-$Lys^{B28}Pro^{B29}$-human insulin, $N^{\varepsilon B28}$-decanoyl-$N^{\alpha A1}$-decanoyl-$N^{\alpha B1}$-decanoyl-$Lys^{B28}Pro^{B29}$-human insulin.

In certain embodiments, an insulin molecule of the present disclosure comprises the mutations and/or chemical modifications of one of the following insulin molecules: $N^{\varepsilon B29}$-pentanoyl-$Gly^{A21}Arg^{B31}Arg^{B32}$-human insulin, $N^{\alpha B1}$-hexanoyl-$Gly^{A21}Arg^{B31}Arg^{B32}$-human insulin, $N^{\alpha A1}$-heptanoyl-$Gly^{A21}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B29}$-octanoyl- $N^{\alpha B1}$-octanoyl-$Gly^{A21}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B29}$-propionyl- $N^{\alpha A1}$-propionyl-$Gly^{A21}Arg^{B31}Arg^{B32}$-human insulin, $N^{\alpha A1}$-acetyl- $N^{\alpha B1}$-acetyl-$Gly^{A21}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B29}$-formyl- $N^{\alpha A1}$-formyl-$N^{\alpha B1}$-formyl-$Gly^{A21}Arg^{B31}Arg^{B32}$-human insulin, $N^{\varepsilon B29}$-formyl-des(B26)-human insulin, $N^{\alpha B1}$-acetyl-Asp^{B28}-human insulin, $N^{\varepsilon B29}$-propionyl- $N^{\alpha A1}$-propionyl- $N^{\alpha B1}$-propionyl-$Asp^{B1}Asp^{B3}AspB^{21}$-human insulin, $N^{\varepsilon B29}$-pentanoyl-Gly^{A21}-human insulin, $N^{\alpha B1}$-hexanoyl-Gly^{A21}-human insulin, $N^{\alpha A1}$-heptanoyl-Gly^{A21}-human insulin, $N^{\varepsilon B29}$-octanoyl- $N^{\alpha B1}$-octanoyl-Gly^{A21}-human insulin, $N^{\varepsilon B29}$-propionyl- $N^{\alpha A1}$-propionyl-Gly^{A21}-human insulin, $N^{\alpha A1}$-acetyl-$N^{\alpha B1}$-acetyl-Gly^{A21}-human insulin, $N^{\varepsilon B29}$-formyl- $N^{\alpha A1}$-formyl- $N^{\alpha B1}$-formyl-Gly^{A21}-human insulin, $N^{\varepsilon B29}$-butyryl-des(B30)-human insulin, $N^{\alpha B1}$-butyryl-des(B30)-human insulin, $N^{\alpha A1}$-butyryl-des(B30)-human insulin, $N^{\varepsilon B29}$-butyryl- $N^{\alpha B1}$-butyryl-des(B30)-human insulin, $N^{\varepsilon B29}$-butyryl-$N^{\alpha A1}$-butyryl-des(B30)-human insulin, $N^{\alpha A1}$-butyryl- $N^{\alpha B1}$-butyryl-des(B30)-human insulin, $N^{\varepsilon B29}$-butyryl- $N^{\alpha A1}$-butyryl- $N^{\alpha B1}$-butyryl-des(B30)-human insulin.

The present disclosure also encompasses modified forms of non-human insulins (e.g., porcine insulin, bovine insulin, rabbit insulin, sheep insulin, etc.) that comprise any one of the aforementioned mutations and/or chemical modifications.

These and other modified insulin molecules are described in detail in U.S. Patent Nos. 6,906,028; 6,551,992; 6,465,426; 6,444,641; 6,335,316; 6,268,335; 6,051,551; 6,034,054; 5,952,297; 5,922,675; 5,747,642; 5,693,609; 5,650,486; 5,547,929; 5,504,188; 5,474,978; 5,461,031; and 4,421,685; and in U.S. Patent Nos. 7,387,996; 6,869,930; 6,174,856; 6,011,007; 5,866,538; and 5,750,497.

Methods for conjugating drugs including insulin molecules are described below. In certain embodiments, an insulin molecule is conjugated to the conjugate framework via the A1 amino acid residue. In certain embodiments the A1 amino acid residue is glycine. It is to be understood however, that the present disclosure is not limited to N-terminal conjugation and that in certain embodiments an insulin molecule may be conjugated via a non-terminal A-peptide

amino acid residue. In particular, the present disclosure encompasses conjugation via the epsilon-amine group of a lysine residue present at any position in the A-peptide (wild-type or introduced by site-directed mutagenesis). It will be appreciated that different conjugation positions on the A-peptide may lead to different reductions in insulin activity. In certain embodiments, an insulin molecule is conjugated to the conjugate framework via the B1 amino acid residue. In certain embodiments the B1 amino acid residue is phenylalanine. It is to be understood however, that the present disclosure is not limited to N-terminal conjugation and that in certain embodiments an insulin molecule may be conjugated via a non-terminal B-peptide amino acid residue. In particular, the present disclosure encompasses conjugation via the epsilon-amine group of a lysine residue present at any position in the B-peptide (wild-type or introduced by site-directed mutagenesis). For example, in certain embodiments an insulin molecule may be conjugated via the B29 lysine residue. In the case of insulin glulisine, conjugation to the conjugate framework via the B3 lysine residue may be employed. It will be appreciated that different conjugation positions on the B-peptide may lead to different reductions in insulin activity.

In certain embodiments, the ligands are conjugated to more than one conjugation point on a drug such as an insulin molecule. For example, an insulin molecule can be conjugated at both the A1 N-terminus and the B29 lysine. In some embodiments, amide conjugation takes place in carbonate buffer to conjugate at the B29 and A1 positions, but not at the B1 position. In other embodiments, an insulin molecule can be conjugated at the A1 N-terminus, the B1 N-terminus, and the B29 lysine. In yet other embodiments, protecting groups are used such that conjugation takes place at the B1 and B29 or B1 and A1 positions. It will be appreciated that any combination of conjugation points on an insulin molecule may be employed. In some embodiments, at least one of the conjugation points is a mutated lysine residue, e.g., Lys^{A3}.

In various embodiments, a conjugate may include an insulin sensitizer (i.e., a drug which potentiates the action of insulin). Drugs which potentiate the effects of insulin include biguanides (e.g., metformin) and glitazones. The first glitazone drug was troglitazone which turned out to have severe side effects. Second generation glitazones include pioglitazone and rosiglitazone which are better tolerated although rosiglitazone has been associated with adverse cardiovascular events in certain trials.

In various embodiments, a conjugate may include an insulin secretagogue (i.e., a drug which stimulates insulin secretion by beta cells of the pancreas). For example, in various

embodiments, a conjugate may include a sulfonylurea. Sulfonylureas stimulate insulin secretion by beta cells of the pancreas by sensitizing them to the action of glucose. Sulfonylureas can, moreover, inhibit glucagon secretion and sensitize target tissues to the action of insulin. First generation sulfonylureas include tolbutamide, chlorpropamide and carbutamide. Second generation sulfonylureas which are active at lower doses include glipizide, glibenclamide, gliclazide, glibornuride and glimepiride. In various embodiments, a conjugate may include a meglitinide. Suitable meglitinides include nateglinide, mitiglinide and repaglinide. Their hypoglycemic action is faster and shorter than that of sulfonylureas. Other insulin secretagogues include glucagon-like peptide 1 (GLP-1) and GLP-1 analogs (i.e., a peptide with GLP-1 like bioactivity that differs from GLP-1 by 1-10 amino acid substitutions, additions or deletions and/or by a chemical modification). GLP-1 reduces food intake by inhibiting gastric emptying, increasing satiety through central actions and by suppressing glucagon release. GLP-1 lowers plasma glucose levels by increasing pancreas islet cell proliferation and increases insulin production following food consumption. GLP-1 may be chemically modified, e.g., by lipid conjugation as in liraglutide to extend its *in vivo* half-life. Yet other insulin secretagogues include exendin-4 and exendin-4 analogs (i.e., a peptide with exendin-4 like bioactivity that differs from exendin-4 by 1-10 amino acid substitutions, additions or deletions and/or by a chemical modification). Exendin-4, found in the venom of the Gila Monster, exhibits GLP-1 like bioactivity. It has a much longer half-life than GLP-1 and, unlike GLP-1, it can be truncated by 8 amino acid residues at its N-terminus without losing bioactivity. The N-terminal region of GLP-1 and exendin-4 are almost identical, a significant difference being the second amino acid residue, alanine in GLP-1 and glycine in exendin-4, which gives exendin-4 its resistance to *in vivo* digestion. Exendin-4 also has an extra 9 amino acid residues at its C-terminus as compared to GLP-1. Mann et al. *Biochem. Soc. Trans.* 35:713-716, 2007 and Runge et al., *Biochemistry* 46:5830-5840, 2007 describe a variety of GLP-1 and exendin-4 analogs which may be used in a conjugate of the present disclosure. The short half-life of GLP-1 results from enzymatic digestion by dipeptidyl peptidase IV (DPP-IV). In certain embodiments, the effects of endogenous GLP-1 may be enhanced by administration of a DPP-IV inhibitor (e.g., vildagliptin, sitagliptin, saxagliptin, linagliptin or alogliptin).

In various embodiments, a conjugate may include amylin or an amylin analog (i.e., a peptide with amylin like bioactivity that differs from amylin by 1-10 amino acid substitutions, additions or deletions and/or by a chemical modification). Amylin plays an important role in

glucose regulation (e.g., see Edelman and Weyer, *Diabetes Technol. Ther.* 4:175-189, 2002). Amylin is a neuroendocrine hormone that is co-secreted with insulin by the beta cells of the pancreas in response to food intake. While insulin works to regulate glucose disappearance from the bloodstream, amylin works to help regulate glucose appearance in the bloodstream from the stomach and liver. Pramlintide acetate (SYMLIN®) is an exemplary amylin analog. Since native human amylin is amyloidogenic, the strategy for designing pramlintide involved substituting certain residues with those from rat amylin, which is not amyloidogenic. In particular, proline residues are known to be structure-breaking residues, so these were directly grafted from the rat sequence into the human sequence. Glu-10 was also substituted with an asparagine.

In various embodiments, a pre-conjugated drug may contain one or more reactive moieties (e.g., carboxyl or reactive ester, amine, hydroxyl, aldehyde, sulfhydryl, maleimidyl, alkynyl, azido, etc. moieties). As discussed below, these reactive moieties may, in certain embodiments, facilitate the conjugation process. Specific examples include peptidic drugs bearing alpha-terminal amine and/or epsilon-amine lysine groups. It will be appreciated that any of these reactive moieties may be artificially added to a known drug if not already present. For example, in the case of peptidic drugs a suitable amino acid (e.g., a lysine) may be added or substituted into the amino acid sequence. In addition, as discussed in more detail below, it will be appreciated that the conjugation process may be controlled by selectively blocking certain reactive moieties prior to conjugation.

As discussed above, the present disclosure is not limited to any particular combination of drug and target molecule.

In various embodiments, a material of the present disclosure may be exploited to manipulate a natural feedback mechanism. For example, there are many natural feedback mechanisms (including most hormonal control mechanisms) in which the level of two endogenous substances are interrelated (e.g., glucose and insulin where the level of insulin increases as the level of glucose increases and the level of glucose decreases as the level of insulin increases). In such embodiments one of the endogenous substances can become the target molecule (e.g., glucose) while the other becomes the drug (e.g., insulin). Alternatively, in various embodiments, the drug can be a molecule that (a) has the same function as the other endogenous substance (e.g., reduces glucose levels), (b) stimulates the production of the other endogenous substance and/or (c) potentiates the effect(s) of the other endogenous substance. For

example, when glucose is the target molecule one could use an insulin secretagogue or an insulin sensitizer instead of insulin as the drug.

Other non-limiting examples of artificial feedback systems, include, a material which releases glucagon conjugates in response to high levels of insulin, a material which releases anticoagulant conjugates (e.g., coumarines such as warfarin, acenocoumarol, phenprocoumon and phenindione, heparin, direct thrombin inhibitors such as argatroban, lepirudin, bivalirudin, and dabigatran, etc.) in response to thrombosis indicators; a material which releases lactate-lowering drug conjugates (e.g., dichloroacetate) in response to increased lactate levels; etc.

In various embodiments, a material can be designed to release conjugates which include a drug with a function that is not directly related to the target molecule. Without limitation, a material which responds to a target molecule which increases in concentration after a meal (e.g., glucose) may be used to provide long-term, mealtime dosing of a drug. Any drug which needs to be dosed periodically and/or with food would benefit from such a delivery system. As is well known in the art, many traditional drugs need to be administered with food or at mealtimes. For example, drugs which inhibit the absorption of fats (e.g., orlistat) are advantageously present during mealtime. Similarly, drugs which lower lipid levels, e.g., lovastatin, attorvastatin, or simvastatin, or triglyceride levels, e.g., gemfibrozil, may also be advantageously released at mealtimes.

Detectable label

As noted above, in various embodiments, a conjugate may comprise a detectable label. For example, a detectable label may be included in order to detect the location of conjugates within an organism, tissue or cell; when the conjugates are used in a sensor; etc. It is to be understood that a conjugate can comprise any detectable label known in the art. A conjugate can comprise more than one copy of the same label and/or can comprise more than one type of label. In general, the label(s) used will depend on the end application and the method used for detection.

The detectable label may be directly detectable or indirectly detectable, e.g., through combined action with one or more additional members of a signal producing system. Examples of directly detectable labels include radioactive, paramagnetic, fluorescent, light scattering, absorptive and colorimetric labels. Fluorescein isothiocyanate, rhodamine, phycoerythrin phycocyanin, allophycocyanin, γ-phthalaldehyde, fluorescamine, etc. are all exemplary

fluorescent labels. Chemiluminescent labels, i.e., labels that are capable of converting a secondary substrate to a chromogenic product are examples of indirectly detectable labels. For example, horseradish peroxidase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenate, α-glycerophosphate dehydrogenase, triose phosphate isomerase, asparaginase, glucose oxidase, β-galactosidase, ribonuclease, urease, catalase, glucoamylase, acetylcholinesterase, luciferin, luciferase, aequorin and the like are all exemplary protein based chemiluminescent labels. Luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt, oxalate ester, etc. are exemplary non-protein based chemiluminescent labels. Another non-limiting and commonly used example of an indirectly detectable label is an affinity ligand, i.e., a label with strong affinity for a secondary binding partner (e.g., an antibody or aptamer) which may itself be directly or indirectly detectable.

In general, a detectable label may be visualized or detected in a variety of ways, with the particular manner of detection being chosen based on the particular detectable label, where representative detection means include, e.g., scintillation counting, autoradiography, measurement of paramagnetism, fluorescence measurement, light absorption measurement, measurement of light scattering and the like.

In various embodiments, a pre-conjugated label may contain one or more reactive moieties (e.g., carboxyl or reactive ester, amine, hydroxyl, aldehyde, sulfhydryl, maleimidyl, alkynyl, azido, etc. moieties). As discussed below, these reactive moieties may, in certain embodiments, facilitate the conjugation process. Specific examples include peptidic labels bearing alpha-terminal amine and/or epsilon-amine lysine groups. It will be appreciated that any of these reactive moieties may be artificially added to a known label if not already present. For example, in the case of peptidic labels a suitable amino acid (e.g., a lysine) may be added or substituted into the amino acid sequence. In addition, as discussed in more detail below, it will be appreciated that the conjugation process may be controlled by selectively blocking certain reactive moieties prior to conjugation.

Conjugate framework

Conjugates can be prepared from frameworks that naturally include affinity ligands (e.g., polysaccharides such as glycogen and dextran naturally include glucose affinity ligands) and/or by artificially incorporating affinity ligands into a natural or synthetic framework. It is to be

understood that the conjugates of the present disclosure are not limited to a particular framework. For example, conjugates may be prepared using frameworks that include polymeric and/or non-polymeric structures. It is also to be understood that the conjugate frameworks may be linear, branched, hyperbranched and/or a combination of these. The following section describes some exemplary conjugate frameworks.

In various embodiments, a conjugate may be prepared from a framework that includes a polymeric structure. For example, a polymer with pendant reactive groups (e.g., carboxyl or reactive ester, amine, hydroxyl, aldehyde, sulfhydryl, maleimidyl, alkynyl, azido, etc.) may be employed. It will be appreciated that different pendant groups may be mixed in a single framework (e.g., by co-polymerizing appropriate monomers in desired ratios to produce a polymeric framework). As discussed below, these reactive groups may be used to attach affinity ligands, drugs and/or detectable labels to the framework. Co-polymers, mixtures, and adducts of different frameworks may also be used. Such combinations may be useful for optimizing the mechanical and chemical properties of a material.

In various embodiments, frameworks having carboxyl (or reactive ester) pendant groups (-COOH bearing frameworks, or CBFs) may be used. Such frameworks may naturally include carboxyl groups or may be modified to include them. Exemplary polymeric CBFs include but are not limited to carboxylated polysaccharides (CPS) such as alginate (Ag), carboxymethylated-D-manno-D-glucan (CMMG, available from Daiichi Pharmaceutical Co.), carboxymethyldextran (CMDex), carboxymethylchitin (CMCh, available from Katakura Chikkalin Co.), N-desulfated N-acetylated heparin (DSH), and hyaluronic acid (HA). DSH and CMDex may be synthesized according to Sugahara, et al., Biol. Pharm. Bull., 24, 535-543 (2001). In general, hydroxylated frameworks may be carboxylated through reaction with chloroacetic acid under basic conditions. In the case of a polymeric framework the degree of carboxyl substitution with respect to monomer may vary between 1 and 100 mol %. Naturally occurring carboxylated polymers include but are not limited to carboxylated poly(amino acids) (CPAA) such as poly-L-glutamate and poly-L-aspartate. The carboxylate content may be varied between 1 and 100% mol COOH/mol AA residue by copolymerizing carboxylated amino acids (e.g., amino acids with a carboxyl group in addition to the carboxyl group which becomes part of the polymer backbone) with non-carboxylated amino acids (e.g., amino acids whose only carboxyl group becomes part of the polymer backbone).

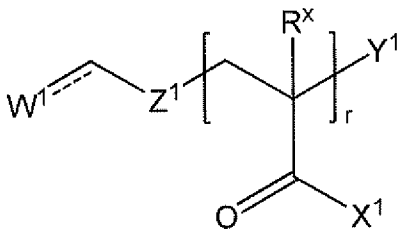
In various embodiments, frameworks having amine pendant groups (-NH_2 bearing frameworks, or NBFs) may be used. Such frameworks may be naturally occurring or may be chemically modified to include a primary amine. The latter include but are not limited to polymeric frameworks, e.g., amine pendant polysaccharides (NPS) such as deacetylated chitosan (Ch) (Sigma Aldrich, Milwaukee, Wis.) and diethylaminoethyl ether dextran (DEAEDex), MW 500,000 g/mol (Polysciences, Warrington, Pa.). In the case of such polymeric frameworks the degree of amine substitution with respect to monomer may vary between 1 and 100 mol %. Other suitable NBFs include, but are not limited to, polynucleotides where one or more of the purine bases has been derivatized with an amine group at the 2' location. Naturally occuring aminated polymers include but are not limited to poly(amino acids) such as poly-L-lysine (PLL) and its enantiomer. The amine content may be varied between 1 and 100% mol NH_2/mol amino acid residue by copolymerizing an aminated amino acid (e.g., an amino acid with an amine in addition to the amine group that eventually becomes part of the polymer backbone) with non-aminated amino acids (e.g., an amino acid whose only amine is that which eventually becomes part of the polymer backbone).

In various embodiments, polymers having hydroxyl pendant groups (-OH bearing frameworks, or OBFs) may be used. Such frameworks may be naturally hydroxylated or may be chemically modified to include a hydroxyl group. In addition to dextran, naturally occurring polymeric OBFs include but are not limited to polysaccharides such as yeast mannan (Mn), pullulan (Pl), amylose (Am), amylopectin (AmP), glycogen (Gl), cellulose (Cl), hyaluronate (Hy), chondroitin (ChD), and dextrin (Dx), all of which may be obtained commercially from Sigma Aldrich. In addition, poly(amino acids) such as poly(serine), poly(threonine), poly(tyrosine), and poly(4-hydroxyproline) may also be employed as hydroxylated polymers. The hydroxyl content of the poly(amino acids) may be varied between 1 and 100% mol -OH/mol amino acid residue by co-polymerizing hydroxylated amino acids with non-hydroxylated amino acids. Of course, carboxyl (or reactive ester), amino, and hydroxyl pendant groups may be mixed in a single polymer by co-polymerizing the appropriate amino acids in desired ratios.

In various embodiments, frameworks having sulfhydryl pendant groups (-SH bearing frameworks, or SBFs) may be used. SBFs may be naturally sulfhydrylated or may be chemically modified using standard organic chemistry techniques to include a sulfhydryl group. In other embodiments, frameworks having aldehyde, maleimidyl, alkynyl, azido, etc. pendant groups may be used.

In addition to the aforementioned classes of frameworks, some exemplary polymers that may be used include poly(lactic acid) (PLA), poly(glycolic acid) (PGA), PLA-PGA co-polymers (PLGA), poly(anhydrides), poly(hydroxy acids), poly(ortho esters), poly(propylfumerates), poly(caprolactones), polyamides, polyacetals, biodegradable polycyanoacrylates and biodegradable polyurethanes.

In various embodiments, conjugates of the following general formula (IV) may be employed:



IV

Various embodiments of the conjugates of formula (IV) are described in more detail in Example 57; however, in general it is to be understood that:

R^x is hydrogen or optionally substituted C_{1-6} alkyl;

Z^1 is an optionally substituted bivalent C_{1-10} hydrocarbon chain, wherein 1, 2, 3, 4 or 5 methylene units of Z^1 are optionally and independently replaced with one or more groups selected from –S–, –O–, $–NR^a–$, $–(C=NR^a)–$, –(C=O)–, –(S=O)–, $–S(=O)_2–$, $–(CR^b=CR^b)–$, –(N=N)–, an optionally substituted arylene moiety or an optionally substituted heteroarylene moiety, wherein R^a is hydrogen, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, optionally substituted heteroaryl, or a suitable amino protecting group; and R^b is hydrogen, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, or optionally substituted heteroaryl;

each occurrence of X^1 is independently $–OR^c$ or $–N(R^d)_2$, wherein R^c is hydrogen, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, optionally substituted heteroaryl, a suitable hydroxyl protecting group, a cation group, or an affinity ligand, and each R^d is, independently, hydrogen, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, optionally substituted heteroaryl, a suitable amino protecting group,

or an affinity ligand, with the proviso that at least two occurrences of X^1 include an affinity ligand;

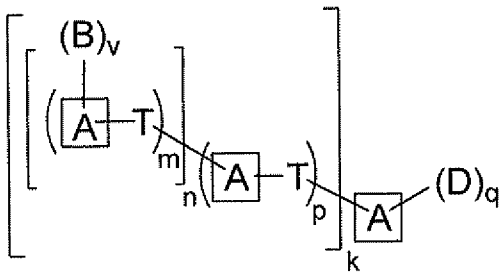
Y^1 is hydrogen, halogen, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, optionally substituted heteroaryl, $-OR^e$ or $-SR^e$ wherein R^e is hydrogen, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, or optionally substituted heteroaryl;

r is an integer between 5–25, inclusive;

W^1 is a drug or detectable label; and

====== corresponds to a single or double covalent bond.

In various embodiments, conjugates of the following general formula (V) may be employed:



V

wherein:

each occurrence of $\left[\left(\boxed{A}-T\right)\right]$ represents a potential branch within the conjugate;

each occurrence of $\left(\boxed{A}-T\right)$ represents a potential repeat within a branch of the conjugate;

each occurrence of $\boxed{A}$ is independently a covalent bond, a carbon atom, a heteroatom, or an optionally substituted group selected from the group consisting of acyl, aliphatic, heteroaliphatic, aryl, heteroaryl, and heterocyclic;

each occurrence of T is independently a covalent bond or a bivalent, straight or branched, saturated or unsaturated, optionally substituted C_{1-30} hydrocarbon chain wherein one or more methylene units of T are optionally and independently replaced by -O-, -S-, -N(R)-, -C(O)-, -C(O)O-, -OC(O)-, -N(R)C(O)-, -C(O)N(R)-, -S(O)-, $-S(O)_2-$, $-N(R)SO_2-$, $-SO_2N(R)-$, a heterocyclic group, an aryl group, or a heteroaryl group;

each occurrence of R is independently hydrogen, a suitable protecting group, or an acyl moiety, arylalkyl moiety, aliphatic moiety, aryl moiety, heteroaryl moiety, or heteroaliphatic moiety;

–B is –T–L^B–X;

each occurrence of X is independently an affinity ligand;

each occurrence of L^B is independently a covalent bond or a group derived from the covalent conjugation of a T with an X;

–D is –T–L^D–W;

each occurrence of W is independently a drug or a detectable label;

each occurrence of L^D is independently a covalent bond or a group derived from the covalent conjugation of a T with a W;

k is an integer from 2 to 11, inclusive, defining at least two k-branches within the conjugate;

q is an integer from 1 to 4, inclusive;

k + q is an integer from 3 to 12, inclusive;

each occurrence of p is independently an integer from 1 to 5, inclusive; and

each occurrence of n is independently an integer from 0 to 5, inclusive; and

each occurrence of m is independently an integer from 1 to 5, inclusive; and

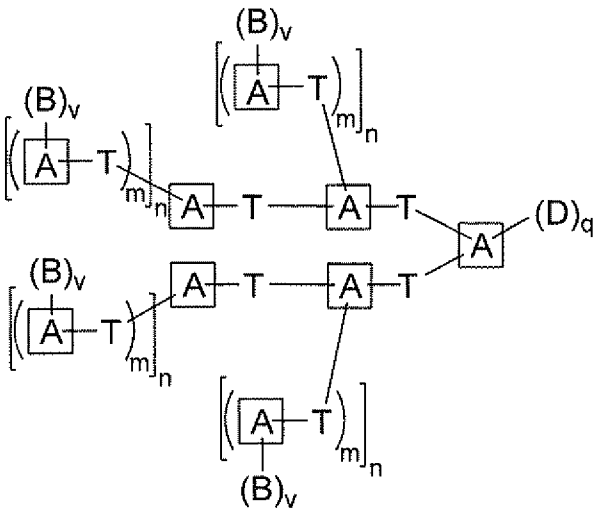
each occurrence of v is independently an integer from 0 to 5, inclusive, with the proviso that within each k-branch at least one occurrence of n is ≥ 1 and at least one occurrence of v is ≥ 1.

It is to be understood that general formula (V) (and other formulas herein) does not expressly list every hydrogen. For example, if the central A is a C_6 aryl group and $k + q < 6$ it will be appreciated that the open position(s) on the C_6 aryl ring include a hydrogen.

In general, it will be appreciated that each occurrence of A represents a potential branching node and that the number of branches at each node are determined by the values of k for the central A and n for non-central occurrences of A. Since $k \geq 2$ the conjugate will always include at least two k-branches. One of ordinary skill will appreciate that because each occurrence of n may be an integer from 0 to 5, the present disclosure contemplates both branched and hyperbranched (e.g., dendrimer-like) embodiments of these conjugates. The proviso which requires that within each k-branch at least one occurrence of n is ≥ 1 and at least one occurrence

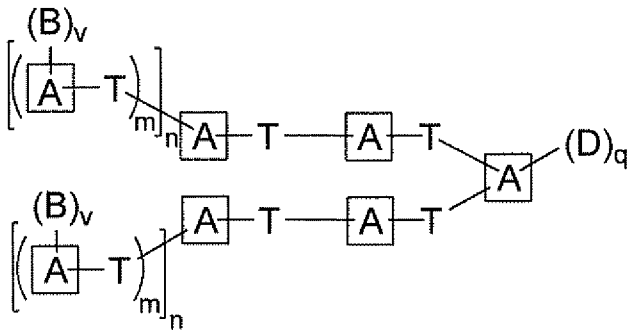
of v is ≥ 1 ensures that every conjugate includes at least two separate k-branches with an occurrence of B (i.e., an affinity ligand).

In certain embodiments, each occurrence of [A] in a p-bracketed moiety is substituted by a number of n-bracketed moieties corresponding to a value of n ≥ 1. For example, when k = 2 and p = 2 in both k-branches, the conjugate may be of the formula (Va):



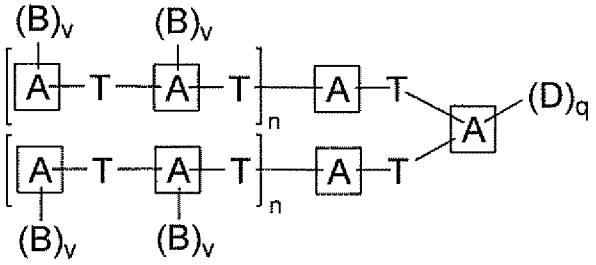
Va

In other embodiments, only terminal occurrences of [A] in a p-bracketed moiety are substituted by a number of n-bracketed moieties corresponding to a value of n ≥ 1. For example, when k = 2 and p = 2 in both k-branches (and n = 0 for the first p-bracketed moiety in both k-branches), the conjugate may be of the formula (Vb):



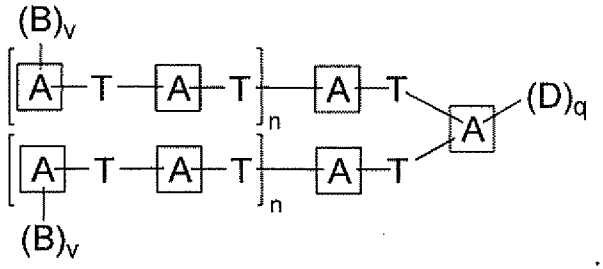
Vb

In certain embodiments, each occurrence of [A] in an m-bracketed moiety is substituted by a number of B moieties corresponding to the value of $v \geq 1$. For example, when k = 2, each occurrence of p = 1, and each occurrence of m = 2, the conjugate may be of the formula (Vc):



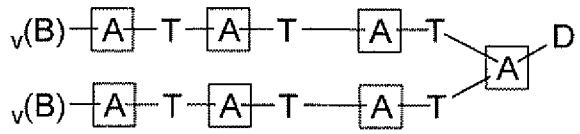
Vc

In other embodiments, only terminal occurrences of [A] in an m-bracketed moiety are substituted by a number of B moieties corresponding to a value of $v \geq 1$. For example, when k = 2, each occurrence of p = 1, and each occurrence of m = 2 (and v = 0 for the first m-bracketed moiety in each n-branch), the conjugate may be of the formula (Vd):



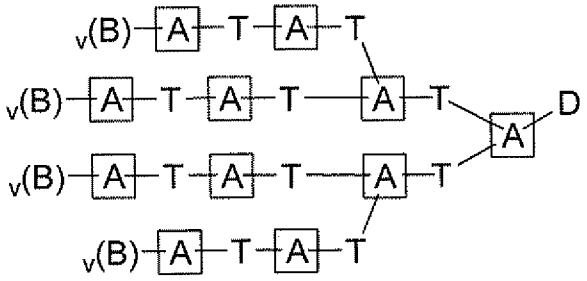
Vd

By way of further example, when q =1 and n = 1 in both k-branches of the previous formula, the conjugate may be of the formula (Ve):



Ve

Alternatively, when q =1 and n = 2 in both k-branches of the previous formula, the conjugate may be of the formula (Vf):



Vf

In various embodiments, the present disclosure also provides conjugates which include affinity ligands and/or a drug or detectable label which are non-covalently bound the conjugate framework.

For example, in some embodiments, the present disclosure provides conjugates of any of the foregoing formulas, wherein:

each of [A], T, D, k, q, k + q, p, n, m and v is defined as described above and herein;

–B is –T–LRP^B–X;

each occurrence of X is independently an affinity ligand; and

each occurrence of LRP^B is independently a ligand-receptor pair which forms a non-covalent bond between T and X with a dissociation constant in human serum of less than 1 pmol/L.

In yet other embodiments, the present disclosure provides conjugates of any of the foregoing formulas, wherein:

each of [A], T, B, k, q, k + q, p, n, m and v is defined as described above and herein;

–D is –T–LRP^D–W;

each occurrence of W is independently a drug or a detectable label; and

each occurrence of LRP^D is independently a ligand-receptor pair which forms a non-covalent bond between T and W with a dissociation constant in human serum of less than 1 pmol/L.

In other embodiments, the present disclosure provides conjugates of any of the foregoing formulas wherein:

each of [A], T, k, q, k + q, p, n, m and v is defined as described above and herein;

–B is –T–LRP^B–X;

each occurrence of X is independently an affinity ligand;

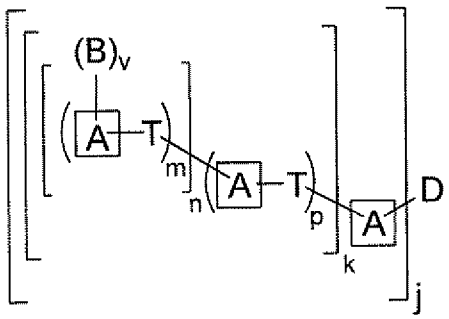
each occurrence of LRP^B is independently a ligand-receptor pair which forms a non-covalent bond between T and X with a dissociation constant in human serum of less than 1 pmol/L.

–D is –T–LRP^D–W;

each occurrence of W is independently a drug or a detectable label; and

each occurrence of LRP^D is independently a ligand-receptor pair which forms a non-covalent bond between T and W with a dissociation constant in human serum of less than 1 pmol/L.

In various embodiments, a conjugate of the present disclosure may have the general formula (VI):



VI

wherein [A], B, T, D, v, m, n, and p are as defined and described herein, k is an integer from 1 to 11, inclusive, and j is 1-4. Conjugates of formula (VI) may have multiple sites of conjugation of ligand to drug. It will be appreciated that, when q is 1, the subgenera described above (formulae Va-Vf) apply to conjugates of formula (VI) when j is 1. Likewise, similar subgenera can be contemplated by one skilled in the art for conjugates wherein j is 2, 3, or 4.

For purposes of exemplification and for the avoidance of confusion it is to be understood that an occurrence of: –[A]–D–[A]– in a conjugate of formula (VI) (i.e., when j is 2) could be represented as: –[A]–T–L^D–W–L^D–T–[A]– (when the drug is covalently bound to the conjugate

framework) or –[A]–T–LRP^D–W–LRP^D–T–[A]– (when the drug is non-covalently bound to the conjugate framework).

Description of Exemplary Groups

[A] *(node)*

In certain embodiments, each occurrence of [A] is independently an optionally substituted group selected from the group consisting of acyl, aliphatic, heteroaliphatic, aryl, heteroaryl, and heterocyclic. In some embodiments, each occurrence of [A] is the same. In some embodiments, the central [A] is different from all other occurrences of [A]. In certain embodiments, all occurrences of [A] are the same except for the central [A].

In some embodiments, [A] is an optionally substituted aryl or heteroaryl group. In some embodiments, [A] is 6-membered aryl. In certain embodiments, [A] is phenyl.

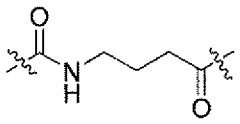
In certain embodiments, [A] is a heteroatom selected from N, O, or S. In some embodiments, [A] is nitrogen atom. In some embodiments, [A] is an oxygen atom. In some embodiments, [A] is sulfur atom. In some embodiments, [A] is a carbon atom.

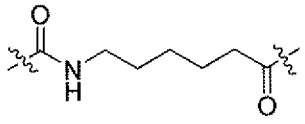
T (spacer)

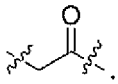
In certain embodiments, each occurrence of T is independently a bivalent, straight or branched, saturated or unsaturated, optionally substituted C_{1-20} hydrocarbon chain wherein one or more methylene units of T are optionally and independently replaced by -O-, -S-, -N(R)-, -C(O)-, -C(O)O-, -OC(O)-, -N(R)C(O)-, -C(O)N(R)-, -S(O)-, -$S(O)_2$-, -N(R)SO_2-, -SO_2N(R)-, a heterocyclic group, an aryl group, or a heteroaryl group. In certain embodiments, one, two, three, four, or five methylene units of T are optionally and independently replaced. In certain embodiments, T is constructed from a C_{1-10}, C_{1-8}, C_{1-6}, C_{1-4}, C_{2-12}, C_{4-12}, C_{6-12}, C_{8-12}, or C_{10-12} hydrocarbon chain wherein one or more methylene units of T are optionally and independently replaced by -O-, -S-, -N(R)-, -C(O)-, -C(O)O-, -OC(O)-, -N(R)C(O)-, -C(O)N(R)-, -S(O)-, -$S(O)_2$-, -N(R)SO_2-, -SO_2N(R)-, a heterocyclic group, an aryl group, or a heteroaryl group. In some embodiments, one or more methylene units of T is replaced by a heterocyclic group. In some embodiments, one or more methylene units of T is replaced by a triazole moiety. In certain

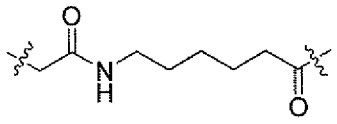
embodiments, one or more methylene units of T is replaced by -C(O)-. In certain embodiments, one or more methylene units of T is replaced by -C(O)N(R)-. In certain embodiments, one or more methylene units of T is replaced by -O-.

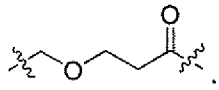
In some embodiments, T is .

In some embodiments, T is .

In some embodiments, T is .

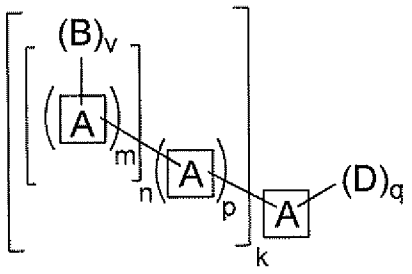
In some embodiments, T is .

In some embodiments, T is .

In some embodiments, T is .

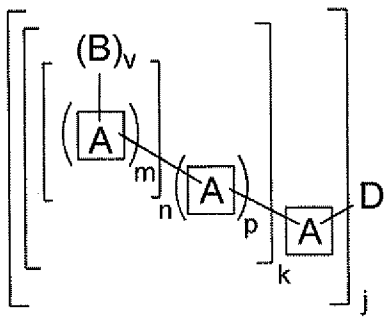
In certain embodiments, each occurrence of T is the same.

In certain embodiments, each occurrence of T (outside groups B and D) is a covalent bond and the conjugate is of the general formula (VII) or (VIII):



VII

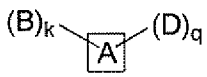
or



VIII

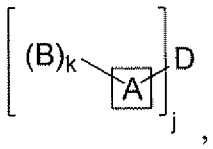
wherein A, B, D, v, m, n, p, k, and j are as defined and described for formula (V) or (VI), respectively.

In certain embodiments of general formulae (VII) and (VIII), each occurrence of [A] except for the central [A] is a covalent bond, each occurrence of v = 1, and the conjugate is of the formula (IX) or (X):



IX

or



X

wherein [A], B, D, q, k, and j are as defined and described for formula (V) or (VI), respectively.

In certain such embodiments for formula (IX), k = 2 and q = 1.

In other embodiments, k = 3 and q = 1.

In other embodiments, k = 2 and q = 2.

In certain such embodiments for formula (X), k = 1 and j = 2.

In other embodiments, k = 2 and j = 2.

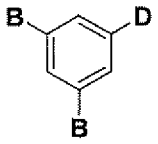
In other embodiments, k = 3 and j = 2.

In other embodiments, k = 1 and j = 3.

In other embodiments, k = 2 and j = 3.

In other embodiments, k = 3 and j = 3.

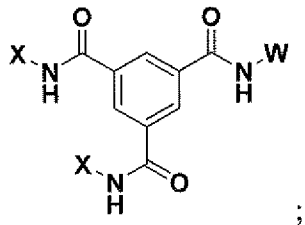
In some embodiments, the present disclosure provides conjugates of general formula (IXa):



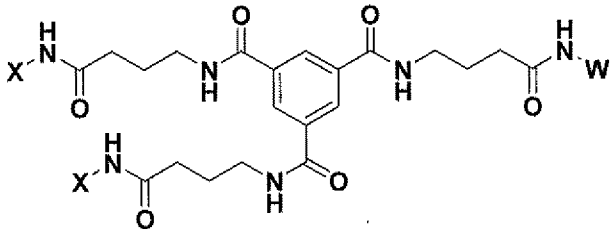
IXa

wherein B and D are as defined and described herein.

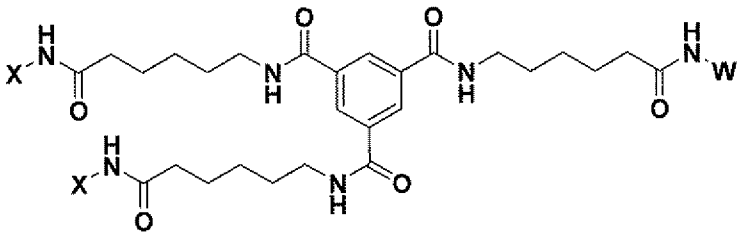
For example, in some embodiments, the present disclosure provides conjugates of formula:



;

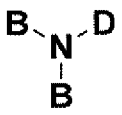


; or



wherein W and X is as defined and described herein.

In some embodiments, the present disclosure provides conjugates of general formula (IXb):

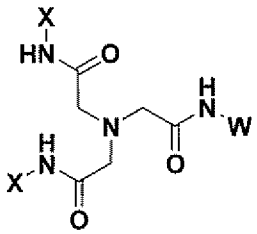


,

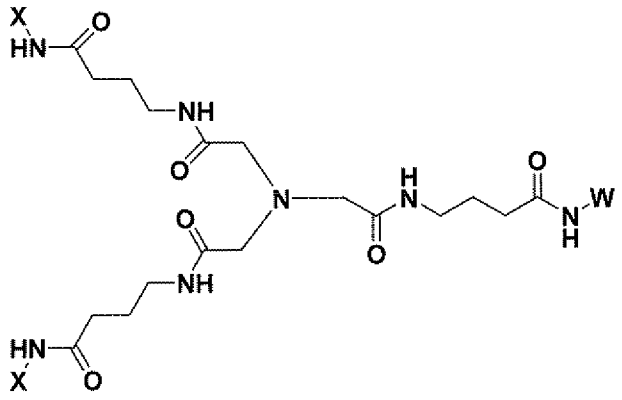
IXb

wherein B and D are as defined and described herein.

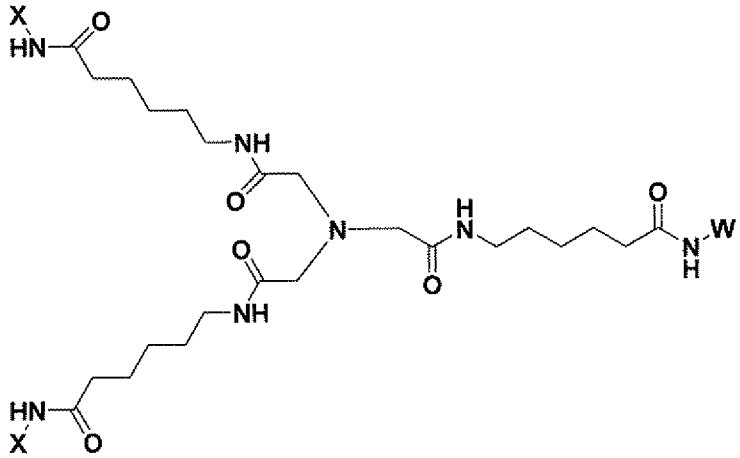
For example, in some embodiments, the present disclosure provides conjugates of formula:



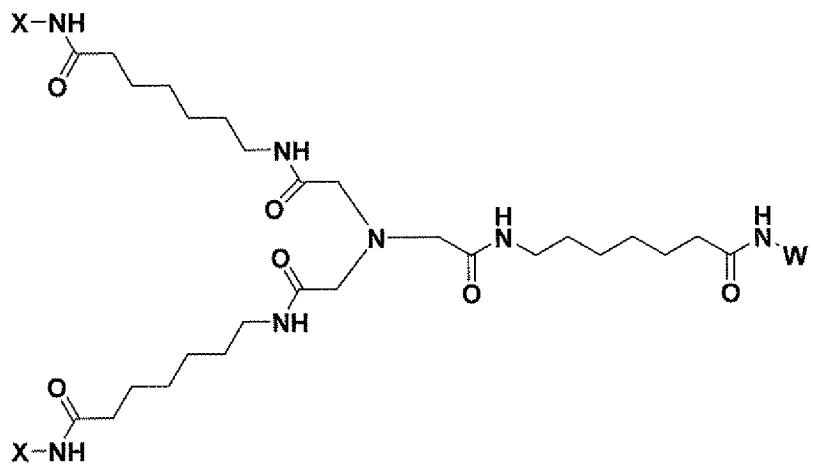
;



;

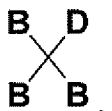


; or



wherein W and X are as defined and described herein.

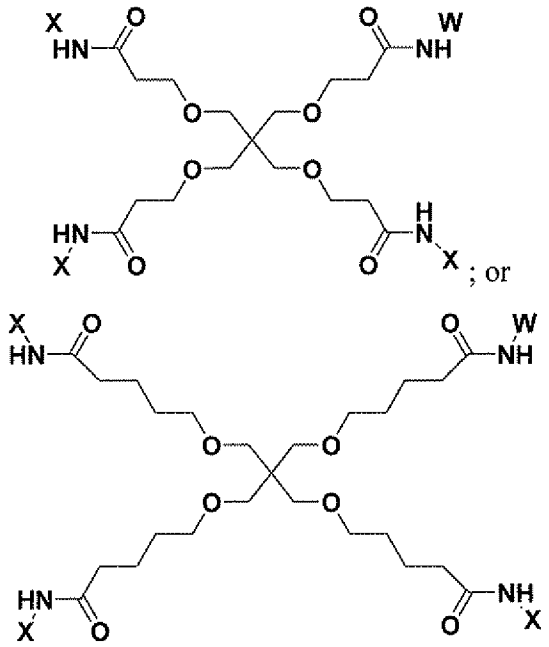
In some embodiments, the present disclosure provides conjugates of general formula (IXc):



IXc

wherein B and D are as defined and described herein.

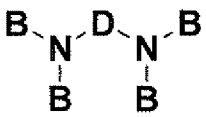
For example, in some embodiments, the present disclosure provides conjugates of formula:



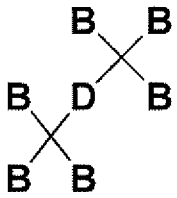
; or

wherein W and X are as defined and described herein.

It will be appreciated that similar subgenera to those of formulae (VIIa), (VIIb), and (VIIc), and species thereof, can be contemplated by one skilled in the art for conjugates of formula (VIII) wherein j is 2, 3, or 4. For example, when j is 2, in certain embodiments, the present disclosure provides conjugates of formula:



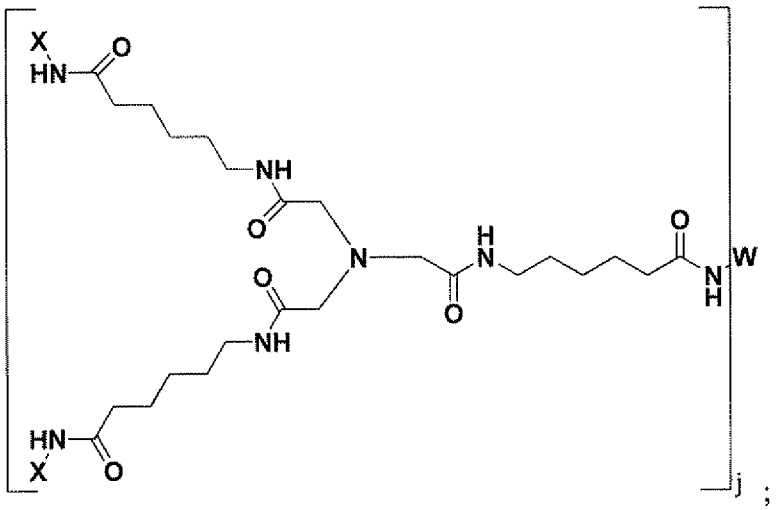
Xb-*i*

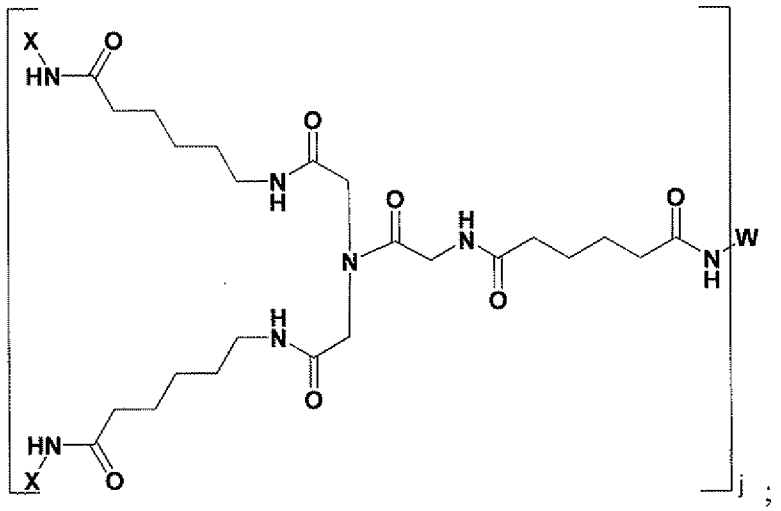


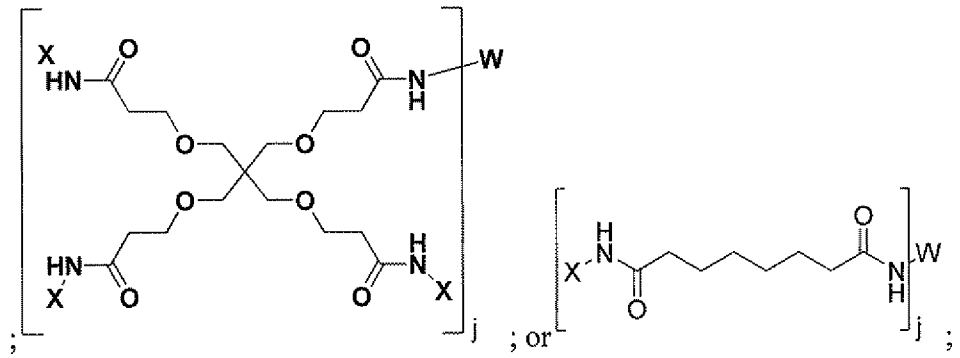
Xc-*i*

wherein B and D are as defined and described herein.

In certain embodiments, the present disclosure provides conjugates of formula:







wherein W, X, and j are as defined and described herein.

B (ligand)

In various embodiments, –B is –T–L^B–X where X is a ligand; and L^B is a covalent bond or a group derived from the covalent conjugation of an X with a T. Exemplary ligands were described above.

D (drug)

In various embodiments, –D is –T–L^D–W where W is a drug and L^D is a covalent bond or a group derived from the covalent conjugation of a W with a T. Exemplary drugs were described above.

D (detectable label)

As noted above, in various embodiments, the W in D is a detectable label. For example, a detectable label may be included in order to detect the location of conjugates within an organism, tissue or cell; when the conjugates are used in a sensor; etc. It is to be understood that a conjugate can comprise any detectable label known in the art. A conjugate can comprise more than one copy of the same label and/or can comprise more than one type of label. In general, the label(s) used will depend on the end application and the method used for detection.
The detectable label may be directly detectable or indirectly detectable, e.g., through combined action with one or more additional members of a signal producing system. Examples of directly detectable labels include radioactive, paramagnetic, fluorescent, light scattering, absorptive and colorimetric labels. Fluorescein isothiocyanate, rhodamine, phycoerythrin phycocyanin, allophycocyanin, γ-phthalaldehyde, fluorescamine, etc. are all exemplary fluorescent labels. Chemiluminescent labels, i.e., labels that are capable of converting a secondary substrate to a chromogenic product are examples of indirectly detectable labels. For example, horseradish peroxidase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenate, α-glycerophosphate dehydrogenase, triose phosphate isomerase, asparaginase, glucose oxidase, β-galactosidase, ribonuclease, urease, catalase, glucoamylase, acetylcholinesterase, luciferin, luciferase, aequorin and the like are all exemplary protein based chemiluminescent labels. Luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt, oxalate ester, etc. are exemplary non-protein based chemiluminescent labels. Another non-limiting and commonly used example of an indirectly detectable label is an affinity ligand, i.e., a label with strong

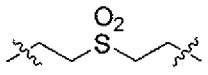
affinity for a secondary binding partner (e.g., an antibody or aptamer) which may itself be directly or indirectly detectable.

In general, a detectable label may be visualized or detected in a variety of ways, with the particular manner of detection being chosen based on the particular detectable label, where representative detection means include, e.g., scintillation counting, autoradiography, measurement of paramagnetism, fluorescence measurement, light absorption measurement, measurement of light scattering and the like.

In various embodiments, a pre-conjugated label may contain one or more reactive moieties (e.g., carboxyl or reactive ester, amine, hydroxyl, aldehyde, sulfhydryl, maleimidyl, alkynyl, azido, etc. moieties). As discussed below, these reactive moieties may, in certain embodiments, facilitate the conjugation process. Specific examples include peptidic labels bearing alpha-terminal amine and/or epsilon-amine lysine groups. It will be appreciated that any of these reactive moieties may be artificially added to a known label if not already present. For example, in the case of peptidic labels a suitable amino acid (e.g., a lysine) may be added or substituted into the amino acid sequence. In addition, as discussed in more detail below, it will be appreciated that the conjugation process may be controlled by selectively blocking certain reactive moieties prior to conjugation.

L^B and L^D (covalent conjugation)

One of ordinary skill will appreciate that a variety of conjugation chemistries may be used to covalently conjugate an X with a T and/or a W with a T (generally "components"). Such techniques are widely known in the art, and exemplary techniques are discussed below. Components can be directly bonded (i.e., with no intervening chemical groups) or indirectly bonded through a spacer (e.g., a coupling agent or covalent chain that provides some physical separation between the conjugated element and the remainder of the conjugate framework). It is to be understood that components may be covalently bound to a conjugate framework through any number of chemical bonds, including but not limited to amide, amine, ester, ether, thioether, isourea, imine, etc. bonds. In certain embodiments, L^B and/or L^D (generally "L" for the purposes of this section) is a covalent bond. In some embodiments, L is an optionally substituted moiety derived from conjugating an optionally substituted carbonyl-reactive, thiol-reactive, amine-reactive, or hydroxyl-reactive moiety of T with a carboxyl, thiol, amine, or hydroxyl group of X or W. In some embodiments, L is an optionally substituted moiety derived from conjugating an

optionally substituted carboxyl-reactive, thiol-reactive, amine-reactive, or hydroxyl-reactive moiety of X or W with a carboxyl, thiol, amine, or hydroxyl group of T. In some embodiments, L is . In some embodiments, L is a succinimide moiety.

In various embodiments, components may be covalently bound to a conjugate framework using "click chemistry" reactions as is known in the art. These include, for example, cycloaddition reactions, nucleophilic ring-opening reactions, and additions to carbon-carbon multiple bonds (e.g., see Kolb and Sharpless, *Drug Discovery Today* 8:1128-1137, 2003 and references cited therein as well as Dondoni, *Chem. Asian J.* 2:700-708, 2007 and references cited therein). As discussed above, in various embodiments, the components may be bound to a conjugate framework via natural or chemically added pendant groups. In general, it will be appreciated that the first and second members of a pair of reactive groups (e.g., a carboxyl group and an amine group which react to produce an amide bond) can be present on either one of the component and framework (i.e., the relative location of the two members is irrelevant as long as they react to produce a conjugate). Exemplary linkages are discussed in more detail below.

In various embodiments, carboxyl (or reactive ester) bearing components can be conjugated to -OH bearing frameworks (OBFs) using the procedure outlined by Kim et al., *Biomaterials* 24:4843-4851 (2003). Briefly, the OBF is dissolved in DMSO along with the carboxyl bearing component and reacted by means of N',N'-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) as catalysts under a dry atmosphere. Carboxyl bearing components can be conjugated to $-NH_2$ bearing frameworks (NBFs) using a carbodiimide (EDAC) coupling procedure. Using this procedure, the carboxyl bearing component is functionalized by reaction with EDAC in a pH 5 buffer followed by the addition of the NBF. In either of these cases (and in any of the following cases), the resulting products may be purified by any number of means available to those skilled in the art including, but not limited to, size exclusion chromatography, reversed phase chromatography, silica gel chromatography, ion exchange chromatography, ultrafiltration, and selective precipitation.

In various embodiments, amine bearing components can be coupled to -COOH bearing frameworks (CBFs). CBFs using activated ester moieties (e.g., see Hermanson in *Bioconjugate Techniques, 2nd edition*, Academic Press, 2008 and references cited therein). Briefly, a CBF with terminal activated carboxylic acid esters such as –NHS, –SSC, –NPC, etc. is dissolved in an anhydrous organic solvent such as DMSO or DMF. The desired number of equivalents of amine bearing component are then added and mixed for several hours at room temperature. Amine

bearing components can also be conjugated to CBFs to produce a stable amide bond as described by Baudys et al., *Bioconj. Chem.* 9:176-183, 1998. This reaction can be achieved by adding tributylamine (TBA) and isobutylchloroformate to a solution of the CBF and an amine bearing component in dimethylsulfoxide (DMSO) under anhydrous conditions. Amine bearing components can alternatively be coupled to OBFs through cyanalation using reagents including, but not limited to, cyanogen bromide (CNBr), N-cyanotriethylammonium tetrafluoroborate (CTEA), 1-Cyano-4-(Dimethylamino)-pyridinium tetrafluorborate (CDAP), and p-nitrophenylcyanate (pNPC). CNBr reactions can be carried out at mildly basic pH in aqueous solution. CDAP reactions are carried out in a mixture of DMSO and water at mildly basic pH using triethylamine (TEA) as a catalyst. In certain embodiments, amine bearing components can be conjugated to NBFs, e.g., through glutaraldehyde coupling in aqueous buffered solutions containing pyridine followed by quenching with glycine. In certain embodiments, amine bearing components can be conjugated to aldehyde bearing frameworks using a Schiff Base coupling procedure followed by reduction (e.g., see see Hermanson in *Bioconjugate Techniques, 2nd edition*, Academic Press, 2008 and references cited therein as well as Mei et al. in *Pharm. Res.* 16: 1680-1686, 1999 and references cited therein). Briefly, a framework with terminal activated aldehydes (e.g., acetaldehyde, propionaldehyde, butyraldehyde, etc.) is dissolved in an aqueous buffer with the pH at or below neutral to prevent unwanted aldehyde hydrolysis. The desired number of equivalents of an amine bearing component are then added and mixed at room temperature followed by addition of an excess of suitable reducing agent (e.g., sodium borohydride, sodium cyanobrohydride, sodium triacetoxyborohydride pyridine borane, triethylamine borane, etc.).

In various embodiments, hydroxyl bearing components can be conjugated to OBFs according to the divinylsulfone (DVS) procedure. Using this procedure, the OBF is added to a pH 11.4 bicarbonate buffer and activated with DVS followed by addition of a hydroxyl bearing component after which glycine is added to neutralize and quench the reaction. Hydroxyl bearing components may also be coupled to OBFs using activated ester moieties as described above to produce ester bonds.

In various embodiments, sulfhydryl bearing components can be coupled to maleimide bearing frameworks (MBFs) using a relatively mild procedure to produce thioether bonds (e.g., see Hermanson in *Bioconjugate Techniques, 2nd edition*, Academic Press, 2008 and references cited therein). Because the maleimide group is much less susceptible to hydrolysis than

activated esters, the reaction can be carried out under aqueous conditions. Briefly, an MBF is dissolved in a buffered aqueous solution at pH 6.5-7.5 followed by the desired number of equivalents of sulfhydryl bearing component. After mixing at room temperature for several hours, the thioether coupled conjugate may be purified. Sulfhydryl bearing components can also be conjugated to NBFs according to a method described by Thoma et al., *J. Am. Chem. Soc.* 121:5919-5929, 1999. This reaction involves suspending the NBF in anhydrous dimethylformamide (DMF) followed by the addition of 2,6-lutidine and acid anhydride and subsequent purification of the reactive intermediate. A sulfhydryl bearing component is then added to a solution of the intermediate in DMF with triethylamine.

In various embodiments, azide bearing components can be coupled to an alkyne bearing framework (ABF) using the copper(I)-catalyzed modern version of the Huisgen-type azide–alkyne cycloaddition to give a 1,4-di-substituted 1,2,3-triazole (e.g., see Dondoni, *Chem. Asian J.* 2:700 – 708, 2007 and references cited therein as well as Dedola et al., *Org. Biomol. Chem.* 5: 1006–1017, 2007). This reaction, commonly referred to as a "click" reaction, may be carried out for example in neat THF using N,N-diisopropylethylamine and $Cu(PPh_3)_3Br$ as the catalyst system (e.g., see Wu et al., *Chem. Commun.* 5775-5777, 2005). The reaction may also be carried out in a 3:1 (THF:water) mixture using sodium ascorbate and $CuSO_4 \cdot 5H_2O$ as the catalyst system (e.g., see Wu et al., *supra*). In either case, the azide bearing component is added to the ABF at the desired number of equivalents followed by mixing for 12-48 hours at room temperature. Alternatively, alkyne bearing components may be conjugated to an azide bearing framework using exactly the same conditions described above.

Certain components may naturally possess more than one of the same chemically reactive moiety. In some examples, it is possible to choose the chemical reaction type and conditions to selectively react the component at only one of those sites. For example, in the case where insulin is conjugated through reactive amines, in certain embodiments, the N-terminal α-Phe-B1 is a preferred site of attachment over the N-terminal α-Gly-A1 and ε-Lys-B29 to preserve insulin bioactivity (e.g., see Mei et al., *Pharm. Res.* 16: 1680-1686, 1999 and references cited therein as well as Tsai et al., *J. Pharm. Sci.* 86: 1264-1268, 1997). In an exemplary reaction between insulin with hexadecenal (an aldehyde-terminated molecule), researchers found that mixing the two components overnight in a 1.5M pH 6.8 sodium salicylate aqueous solution containing 54% isopropanol at a ratio of 1:6 (insulin:aldehyde mol/mol) in the presence of sodium cyanoborohydride resulted in over 80% conversion to the single-substituted Phe-B1 secondary

amine-conjugated product (Mei et al., *Pharm. Res.* 16:1680-1686, 1999). Their studies showed that the choice of solvent, pH, and insulin:aldehyde ratio all affected the selectivity and yield of the reaction. In most cases, however, achieving selectivity through choice of chemical reaction conditions is difficult. Therefore, in certain embodiments it may be advantageous to selectively protect the component (e.g., insulin) at all sites other than the one desired for reaction followed by a deprotection step after the material has been reacted and purified. For example, there are numerous examples of selective protection of insulin amine groups available in the literature including those that may be deprotected under acidic (BOC), slightly acidic (citraconic anhydride), and basic (MSC) conditions (e.g., see Tsai et al., *J. Pharm. Sci.* 86: 1264-1268, 1997; Dixon et al., *Biochem. J.* 109: 312-314, 1968; and Schuettler et al., *D. Brandenburg Hoppe Seyler's Z. Physiol. Chem.* 360: 1721, 1979). In one example, the Gly-A1 and Lys-B29 amines may be selectively protected with tert-butoxycarbonyl (BOC) groups which are then removed after conjugation by incubation for one hour at 4 C in a 90% trifluoroacetic acid (TFA)/10% anisole solution. In one embodiment, a dry powder of insulin is dissolved in anhydrous DMSO followed by an excess of triethylamine. To this solution, approximately two equivalents of di-tert-butyl dicarbonate solution in THF is added slowly and the solution allowed to mix for 30-60 minutes. After reaction, the crude solution is poured in an excess of acetone followed by dropwise addition of dilute HCl to precipitate the reacted insulin. The precipitated material is centrifuged, washed with acetone and dried completely under vacuum. The desired di-BOC protected product may be separated from unreacted insulin, undesired di-BOC isomers, and mono-BOC and tri-BOC byproducts using preparative reverse phase HPLC or ion exchange chromatography (e.g., see Tsai et al., *J. Pharm. Sci.* 86: 1264-1268, 1997). In the case of reverse phase HPLC, a solution of the crude product in 70% water/30% acetonitrile containing 0.1% TFA is loaded onto a C8 column and eluted with an increasing acetonitrile gradient. The desired di-BOC peak is collected, rotovapped to remove acetonitrile, and lyophilized to obtain the pure product.

LRP^B and LRP^D (non-covalent conjugation)

One of ordinary skill will appreciate that a variety of conjugation chemistries may be used to non-covalently conjugate an X with a T and/or a W with a T (generally "components"). Such techniques are widely known in the art, and exemplary techniques are discussed below. In certain embodiments, the dissociation constant (K_d) of the non-covalent linkage in human serum

is less than 1 pmol/L. For example, a component may be non-covalently bound to a conjugate framework via a non-covalent ligand-receptor pair as is well known in the art (e.g., without limitation a biotin-avidin based pair). In such an embodiment, one member of the ligand receptor-pair is covalently bound to the component while the other member of the pair is covalently bound to the conjugate framework. When the component and conjugate framework are combined, the strong non-covalent interaction between the ligand and its receptor causes the component to become non-covalently bound to the conjugate framework. Typical ligand/receptor pairs include protein/co-factor and enzyme/substrate pairs. Besides the commonly used biotin/avidin pair, these include without limitation, biotin/streptavidin, digoxigenin/anti-digoxigenin, FK506/FK506-binding protein (FKBP), rapamycin/FKBP, cyclophilin/cyclosporin and glutathione/glutathione transferase pairs. Other suitable ligand/receptor pairs would be recognized by those skilled in the art, e.g., monoclonal antibodies paired with a epitope tag such as, without limitation, glutathione-S-transferase (GST), c-myc, FLAG® and further those described in Kessler pp. 105-152 of *Advances in Mutagenesis"* Ed. by Kessler, Springer-Verlag, 1990; "*Affinity Chromatography: Methods and Protocols (Methods in Molecular Biology)*" Ed. by Pascal Baillon, Humana Press, 2000; and "*Immobilized Affinity Ligand Techniques*" by Hermanson et al., Academic Press, 1992.

k and q

For conjugates of general formula (V), k is an integer from 2 to 11, inclusive, defining at least two k-branches within the conjugate. In certain embodiments, k = 2 or 3. q is an integer from 1 to 4, inclusive, and defines the number of D groups which are bound to the central A group. In certain embodiments, q = 1. In some embodiments, q = 2. k + q is an integer from 3 to 6, inclusive. In certain embodiments, k + q = 3 or 4.

For conjugates of general formula (VI), when j is 2, 3, or 4, k is an integer from 1 to 11, inclusive. In certain embodiments, k is 1, 2, or 3. q is an integer from 1 to 4, inclusive, and defines the number of D groups which are bound to the central A group. In certain embodiments, q = 1. In some embodiments, q = 2. k + q is an integer from 3 to 6, inclusive. In certain embodiments, k + q = 3 or 4.

p and m

Each occurrence of p is independently an integer from 1 to 5, inclusive. In certain embodiments, each occurrence of p is the same. In certain embodiments, p = 1, 2 or 3. In certain embodiments, p = 1.

Each occurrence of m is independently an integer from 1 to 5, inclusive. In certain embodiments, each occurrence of m is the same. In certain embodiments, m = 1, 2 or 3. In certain embodiments, m = 1.

n and v

Each occurrence of n is independently an integer from 0 to 5, inclusive, with the proviso that within each k-branch at least one occurrence of n is ≥ 1. Branches within a given k-branch are referred to herein as n-branches.

In certain embodiments, each occurrence of [A] in a p-bracketed moiety is substituted by a number of n-bracketed moieties corresponding to a value of n ≥ 1, e.g., see formula (Va) above. In some such embodiments, each occurrence of n in the conjugate is the same. In some of these embodiments, n = 1 or 2.

In other embodiments, only terminal occurrences of [A] in a p-bracketed moiety are substituted by a number of n-bracketed moieties corresponding to a value of n ≥ 1, e.g., see formula (Vb) above. In certain embodiments, each k-branch includes just one occurrence of n ≥ 1 (i.e., all other occurrences of n = 0). In some such embodiments, each occurrence of n in the conjugate is the same. In some of these embodiments, n = 1 or 2.

Each occurrence of v is independently an integer from 0 to 5, inclusive, with the proviso that within each k-branch at least one occurrence of v is ≥ 1.

In certain embodiments, each occurrence of [A] in an m-bracketed moiety is substituted by a number of B moieties corresponding to the value of v ≥ 1, e.g., see formula (Vc) above. In some such embodiments, each occurrence of v in the conjugate is the same. In some of these embodiments, v = 1 or 2.

In other embodiments, only terminal occurrences of [A] in an m-bracketed moiety are substituted by a number of B moieties corresponding to a value of v ≥ 1, e.g., see formula (Vd) above. In certain embodiments, each k-branch includes just one occurrence of v ≥ 1 (i.e., all other occurrences of v = 0). In some such embodiments, each occurrence of v in the conjugate is the same. In some of these embodiments, v = 1 or 2. In certain embodiments, each n-branch includes at least one occurrence of v ≥ 1. In certain embodiment, each n-branch includes just one occurrence of v ≥ 1 (i.e., all other occurrences of v = 0). In some such embodiments, each occurrence of v in the conjugate is the same. In some of these embodiments, v = 1 or 2.

j

j of formula (VI) is an integer from 1 to 4, inclusive, and defines the number of conjugations to the D group. In certain embodiments, j = 1. In certain embodiments, j = 2. In some embodiments, j = 3. In other embodiments, j = 4.

Loading levels

In general, the amount of drug (or detectable label) that is loaded onto a conjugate will depend on the molecular weight of the drug and can be controlled by adjusting the molecular weight of the conjugate framework and/or the level of chemical activation (i.e., when pendant groups are added to the framework). In various embodiments, the drug and/or detectable label loading level may be in the range of 5 to 99% w/w of drug and/or detectable label to conjugate (e.g., including drug). In various embodiments, loading levels within the narrower range of 50 to 99% may be used, e.g., in the range of 80 to 99%.

Other

In various embodiments, a biodegradable framework may be used. In various embodiments, a non-biodegradable framework may be used, e.g., when biodegradability is not relevant to the application and/or when the resulting framework or conjugate is sufficiently well excreted that biodegradability is not necessary. In various embodiments, the conjugate framework (or spacer when present, e.g., between a drug and framework) is susceptible to digestion by an enzyme. In various embodiments, the enzyme is present at the site of administration. One skilled in the art will recognize that a number of enzymes are present in patients that could cleave a conjugate framework. Without limitation, these include saccharidases, peptidases, and nucleases. Exemplary saccharidases include, but are not limited to, maltase, sucrase, amylase, glucosidase, glucoamylase, and dextranase. Exemplary peptidases include, but are not limited to, dipeptidyl peptidase-IV, prolyl endopeptidase, prolidase, leucine aminopeptidase, and glicyl glycine dipeptidase. Exemplary nucleases include, but are not limited to, deoxyribonuclease I, ribonuclease A, ribonucelase T1, and nuclease S1.

One skilled in the art will also recognize that, depending on the choice of enzyme, there are a number of conjugate frameworks that are susceptible to enzymatic cleavage. For example, in cases where saccharidase degradation is desired, frameworks which include polysaccharides can be used (e.g., without limitation, a conjugate that includes a polysaccharide comprising repeating chains of 1,4-linked alpha-D-glucose residues will be degraded by alpha-amylases).

Without limitation, suitable polysaccharides include glycogen and partially digested glycogen derived from any number of sources, including but not limited to, sweet corn, oyster, liver (human, bovine, rabbit, rat, horse), muscle (rabbit leg, rabbit abdominal, fish, rat), rabbit hair, slipper limpet, baker's yeast, and fungus. Other polysaccharide polymers and spacers that one could use include carboxylated polysaccharides, -NH_2 pendant polysaccharides, hydroxylated polysaccharides, alginate, collagen-glycosaminoglycan, collagen, mannan, amylose, amylopectin, cellulose, hyaluronate, chondroitin, dextrin, chitosan, etc. In cases where peptidase cleavage is desired, polypeptides that contain amino acid sequences recognized by the cleaving enzyme can be used (e.g., without limitation, a conjugate that includes a [-Glycine-Proline-] sequence will be degraded by prolidase). In certain embodiments one could use co-polymers of aminated and non-aminated amino acids, co-polymers of hydroxylated and non-hydroxylated amino acids, co-polymers of carboxylated and non-carboxylated amino acids, co-polymers of the above or adducts of the above. In cases where nuclease degradation is desired, polynucleotides can be used (e.g., without limitation, a conjugate that includes a polynucleotide containing an oligomer of sequential adenosine residues will be degraded by ribonuclease A).

In various embodiments, the pharmacokinetic and/or pharmacodynamic behavior of a conjugate (i.e., conjugated drug and/or drug which has been released from a conjugate by chemical or enzymatic degradation) may be substantially the same as the corresponding unconjugated drug (e.g., when both are administered subcutaneously). For example, from a pharmacokinetic (PK) perspective, the serum concentration curve may be substantially the same as when an equivalent amount of unconjugated drug is administered. Additionally or alternatively, the serum T_{max}, serum C_{max}, mean serum residence time (MRT), mean serum absorption time (MAT) and/or serum half-life may be substantially the same as when the unconjugated drug is administered. From a pharmacodynamic (PD) perspective, the conjugate may act on substances within the body in substantially the same way as the unconjugated drug. For example, in the case of an insulin conjugate, the conjugate may affect blood glucose levels in substantially the same way as unconjugated insulin. In this case, substantially similar pharmacodynamic behavior can be observed by comparing the time to reach minimum blood glucose concentration (T_{nadir}), the duration over which the blood glucose level remains below a certain percentage of the initial value (e.g., 70% of initial value or $T_{70\% BGL}$), etc. It will be appreciated that these PK and PD characteristics can be determined according to any of a variety

of published pharmacokinetic and pharmacodynamic methods (e.g., see Baudys et al., *Bioconjugate Chem.* 9:176-183, 1998 for methods suitable for subcutaneous delivery).

In one embodiment, a conjugate (i.e., in isolated form without modified lectin) produces pharmacokinetic (PK) parameters such as time to reach maximum serum drug concentration (T_{max}), mean drug residence time (MRT), serum half-life, and mean drug absorption time (MAT) that are within 40% of those values determined for the unconjugated drug. In various embodiments, a conjugate produces PK parameters that are within 35%, 30%, 25%, 20%, 15% or even 10% of those produced by the unconjugated drug. In some embodiments, a conjugate produces PK parameters that are within 20% of those produce by the unconjugated drug. For example, in embodiments involving an insulin conjugate for subcutaneous delivery the conjugate may produce an insulin T_{max} between 15-30 minutes, a mean insulin residence time (MRT) of less than 50 minutes, or a mean insulin absorption time (MAT) of less than 40 minutes, all of which are within 20% of those values determined from the human recombinant insulin treatment group. In certain embodiments, the conjugate may produce an insulin T_{max} between 20-25 minutes, a mean insulin residence time (MRT) of less than 45 minutes, and a mean insulin absorption time (MAT) of less than 35 minutes. In certain embodiment, the conjugate may produce a serum half-life of less than 120 minutes, e.g., less than 100 minutes.

In one embodiment, an inventive conjugate produces pharmacodynamic (PD) parameters such as time to reach minimum/maximum blood concentration of a substance (T_{nadir}/T_{max}) or duration over which the blood level of the substance remains below/above 70%/130% of the initial value ($T_{70\%\,BL}/T_{130\%\,AL}$). For example, in embodiments involving an insulin conjugate for subcutaneous delivery the conjugate may produce a glucose T_{nadir} between 45-60 minutes and a glucose $T_{70\%BGL}$ of less than 180 minutes, both of which are within 20% of those determined from the human recombinant insulin treatment group. In certain embodiments the conjugate may produce a glucose T_{nadir} between 50-55 minutes and a glucose $T_{70\%BGL}$ of less than 160 minutes. In various embodiments, a conjugate produces PD parameters that are within 40%, 35%, 30%, 25%, 20%, 15% or even 10% of those produced by the unconjugated drug. In some embodiments, a conjugate produces PD parameters that are within 20% of those produce by the unconjugated drug.

Intermediates for preparing conjugates

In one aspect, the invention provides reagents for preparing conjugates of the present disclosure. Thus, in various embodiments, a compound of general formula (V) is provided wherein:

each of [A], T, D, k, q, k + q, p, n, m and v is defined as described above and herein;

B is –T–$L^{B'}$; and

each occurrence of $L^{B'}$ is independently hydrogen, an alkyne-containing moiety, an azide-containing moiety, or an optionally substituted carbonyl-reactive, thiol-reactive, amine-reactive, or hydroxyl-reactive moiety.

In other embodiments, a compound of general formula (V) is provided wherein:

each of [A], T, B, k, q, k + q, p, n, m and v is defined as described above and herein;

D is –T–$L^{D'}$; and

each occurrence of $L^{D'}$ is independently hydrogen, an alkyne-containing moiety, an azide-containing moiety, or an optionally substituted carbonyl-reactive, thiol-reactive, amine-reactive, or hydroxyl-reactive moiety.

Methods for preparing conjugates

We have exemplified methods for preparing the aforementioned conjugates using insulin as an exemplary drug and aminoethylglucose (AEG), aminoethylmannose (AEM), aminoethylbimannose (AEBM), and/or aminoethyltrimannose (AETM) as exemplary affinity ligands. Without limitation, conjugates with two affinity ligands and one drug molecule and with short distances between all framework components may be prepared using tris(hydroxymethyl) aminomethane (Tris), Tris-succinimidyl aminotriacetate (TSAT), *tris*-Succinimidyl-1,3,5-benzenetricarboxylate (TSB), and Benzene-1, 3, 5-tricarboxy-(N-4-butyric-NHS-ester)amide (TSB-C4) as conjugate frameworks. If more space between framework components is desired then Succinimidyl (6-aminocaproyl)aminotriacetate (TSAT-C6), Succinimidyl (6-amino(PEO-6))aminotriacetate (TSAT-PEO-6), Benzene-1, 3, 5-tricarboxy-(N-6-aminocaproic-NHS ester)amide (TSB-C6), and Benzene-1, 3, 5-tricarboxy-(N-10-aminodecanoic-NHS ester)amide (TSB-C10) may be used. The TSAT-C6 spacer arm chemistry imparts more hydrophobic character to the conjugate as compared to TSAT-PEO-6.

For example, for purposes of illustration, in one embodiment, both the affinity ligand (e.g., AEG, AEM, AEMB and AETM) and insulin may be reacted to a TSAT-C6 framework through the terminal activated esters to produce insulin-TSAT-C6-AEG-2, insulin-TSAT-C6-AEM-2, insulin-TSAT-C6-AEMB-2, and insulin-TSAT-C6-AETM-2 conjugates. The various affinity

ligands are synthesized ahead of time as discussed in the Examples. In addition, the A1 and B29 amino groups of insulin are BOC-protected as described in the Examples so that each insulin can only react at the Phe-B1 α-amino group. Approximately one equivalent of BOC-insulin as a 40-50 mg/ml solution in DMSO is added at room temperature to a 50 mg/ml solution of TSAT-C6 in DMSO containing excess triethylamine and allowed to react for approximately one hour. Next, an excess of AEG, AEM, AEBM, and/or AETM (2-10 equivalents) as a 100 mg/ml solution in DMSO is added and allowed to react for an additional 2 hours. After reaction, the DMSO solution is superdiluted by 10x into a pH 5 saline buffer after which the pH is adjusted to 8.0 and the solution passed through a Biogel P2 column to remove low molecular reactants and salts. The material eluting in the void fraction is concentrated using a 3K ultrafiltration apparatus after which it is injected on a prep scale reverse phase HPLC column (C8, acetonitrile/water mobile phase containing 0.1% TFA) to purify the desired product from unreacted BOC2-insulin. The desired elution peak is collected pooled and rotovapped to remove acetonitrile followed by lyophilization to obtain a dry powder. Finally, the BOC protecting groups are removed by dissolving the lyophilized powder in 90% TFA/10% anisole for one hour at 4 C followed by 10x superdilution in HEPES pH 8.2 buffer containing 0.150M NaCl. The pH is adjusted to between 7.0 and 8.0 using NaOH solution after which the material is passed through a Biogel P2 column to remove anisole, BOC, and any other contaminating salts. The deprotected, purified aqueous conjugate solution is then concentrated to the desired level and stored at 4 C until needed.

It will be appreciated that this exemplary procedure may be used to produce other conjugates with different affinity ligands and drugs, different conjugation chemistries, different separations between framework components, and/or different valencies by substituting the TSAT-C6 framework with a different framework as described below.

For example, if yet more distance is required between framework components and/or a preserved charge is required at the site of conjugation, then an appropriately-sized amine-bearing diethyl acetal (e.g., aminopropionaldehyde diethyl acetal (APDA) or aminobutyraldehyde diethyl acetal (ABDA)) may be conjugated to one of the reactive groups on the frameworks listed here followed by complete reaction of the remaining reactive groups with the affinity ligand of interest (e.g. AEM, AEBM, or AETM). A reactive aldehyde group can then be revealed from the diethyl acetal under acidic conditions followed by a reductive amination with insulin to complete the drug conjugation step then ABDA-TSAT, ABDA-LCTSAT, etc. may be employed.

In yet another example, tetrakis-(N-succinimidyl carboxypropyl)pentaerythritol (TSPE), may be used to attach three affinity ligands and one drug molecule for increased multivalency. It will also be appreciated by those skilled in the art that any of the above teachings may be used to produce hyperbranched (e.g., dendrimer-like) conjugates with even higher order valencies. For example, Röckendorf and Lindhorst provide a comprehensive review of current approaches for producing hyperbranched structures in *Topics in Current Chemistry*. 217: 202-238, 2001.

Furthermore, ligands already containing a predetermined degree of multivalency may again be reacted according to the procedures described above to produce even higher orders of ligand multiplicity. For example, a divalent AEM-2, AEBM-2, or AETM-2 molecule containing a terminal reactive amine may be prepared by conjugating two of each affinity ligand to a suitable framework to which a reactive amine is also conjugated. A trivalent AEM-3, AEBM-3, or AETM-3 molecule containing a terminal reactive amine may be prepared by conjugating three of each affinity ligand to a suitable framework to which a reactive amine is also conjugated. The NH_2-divalent sugars may be reacted with the same frameworks described above to produce drug conjugates with 4 and 6 ligands per drug molecule. The NH_2-trivalent sugars may be reacted with the same frameworks described above to produce drug conjugates with 6 and 9 ligands per drug molecule.

In all cases, it should be recognized that a mixture of different ligands may be conjugated to the same drug via a multivalent framework by adjusting the framework chemistry, valency, and the ligand:framework stoichiometry. For example, Insulin-AEM-1-AEBM-1, Insulin-AEBM-1-AETM-1, Insulin AEM-2-AETM-2, and Insulin AEM-1-AETM-2 may all be synthesized according to this mixed ligand method.

Finally, in some cases, it may be desireable to conjugate the affinity ligand to the framework through a different means than the drug. For example, a divalent maleimide/monovalent activate ester functionalized framework (e.g., succinimidyl-3,5-dimaleimidophenyl benzoate (SDMB)) may be used to conjugate two sulfhydryl functionalized affinity ligands and one amine-functionalized drug in separate steps. For example, insulin or another amine-containing drug may be conjugated to the activated ester portion of the framework using methods described herein. In a separate step, the aminoethylsugar (AEM, AEBM, AETM) may be converted to a terminal sulfhydryl-bearing ligand by reaction with 4-iminothiolane. Finally, the framework-di-maleimide-insulin conjugate may be mixed with an excess of sulfhydryl-functionalized sugar to produce the resulting divalent-sugar-insulin conjugate.

Cross-linked materials

When conjugates and cross-linking agents are combined in the absence of the target molecule, a non-covalently cross-linked material is formed. In various embodiments, the material may be prepared in aqueous solution through self-assembly by mixing solutions of the cross-linking agent and conjugate. In various embodiments, particles of the material may be prepared by reverse emulsion. As described in more detail in U.S. Patent Application Publication No. 2004-0202719, this can be achieved by adding the aforementioned aqueous solution to a mixture of a hydrophobic liquid and a surfactant and agitating the mixture.

Once formed, the cross-linked material can be used for a variety of applications. When the material is placed in the presence of free target molecules these compete for the interactions between the cross-linking agents and the conjugates. Above a certain concentration of free target molecule, the level of competition becomes such that the material begins to degrade by releasing conjugates from the surface. In various embodiments, the extent and/or rate of release increases as the concentration of target molecule increases. As a result, conjugates are released from the material in a manner which is directly tied to the local concentration of the target molecule.

In general, the release properties of the material will depend on the nature of the cross-linking agents, conjugates, target molecule and conditions (e.g., pH, temperature, etc.). If the affinity of the cross-linking agents for the conjugates is much greater than for the target molecule then the material will only release conjugates at high concentrations of target molecule. As the relative affinity of the cross-linking agents for the conjugates is decreased, release of conjugates from the material will occur at lower target molecule concentrations. The release properties of the material can also be adjusted by varying the relative amounts of cross-linking agent to conjugate. Higher ratios of cross-linking agent to conjugate will lead to materials that release conjugates at higher target molecule concentrations. Lower ratios of cross-linking agent to conjugate will lead to materials that release conjugates at lower target molecule concentrations. It will be appreciated that, depending on the application, these variables will enable one to produce materials which respond to a wide variety of target molecule concentrations.

In various embodiments, the cross-linked material is insoluble when placed in pH 7 HEPES buffered saline at 37 C (25 mM HEPES containing 150 mM NaCl). In various embodiments, the cross-linked material remains substantially insoluble when target molecule is added to the buffer up to a threshold concentration called the set point. Above the set point, the

cross-linked material exhibits an increase in the extent and rate of release of conjugates. It will be appreciated that this transition may occur sharply or may occur gradually over a range of concentrations around the set point. In general, the desired set point and transition will depend on the nature of the target molecule and the intended application for the material. In particular, when the material is designed to respond to an increase in the level of a particular target molecule, the desired set point may be determined based on the normal physiological range of concentrations of the target molecule. It is to be understood that the amount of target molecule present in a patient may fluctuate based on internal and/or external factors. For example, in certain embodiments, the amount of target molecule may fluctuate naturally over time, e.g., in response to changes in hormonal cycles or metabolic pathways (lactate increasing during an endurance event, etc.). In certain embodiments, the fluctuations may result from an external event, e.g., an increase in glucose following a meal. In various embodiments, external factors may be used to artificially trigger the release of conjugates from a material of the present disclosure. For example, if release of conjugate is sensitive to an increase in glucose one could artificially release conjugates for a short period of time by ingesting a high-glucose drink.

In various embodiments, the target molecule is glucose. The normal physiological range of glucose concentrations in humans is 60 to 200 mg/dL. Glucose concentrations below 60 mg/dL are considered hypoglycemic. Glucose concentrations above 200 mg/dL are considered hyperglycemic. In various embodiments, a material of the present disclosure may remain substantially insoluble when placed in pH 7 HEPES buffered saline containing 20, 30, 40, 50, 60, 70, 80, 90, or 100 mg/dL glucose at 37 C for six hours using USP dissolution test method II at 50 rpm. In various embodiments, less than 1, 2, 4, 6, 8, or 10% of the material dissolves when placed in pH 7 HEPES buffered saline with 20, 30, 40, 50, 60, 70, 80, 90, or 100 mg/dL glucose at 37 C for six hours using USP dissolution test method II at 50 rpm. In various embodiments, at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100% of a material of the present disclosure dissolves when it is placed in pH 7 HEPES buffered saline with 100, 150, 200, 250, 300, 350 or 400 mg/dL glucose at 37 C for six hours using USP dissolution test method II at 50 rpm.

The following tables provide normal physiological ranges for other exemplary target molecules:

Metabolites	**Low**	**High**	**Unit**
Urea	7	18	mg/dL
Creatinine - male	0.7	1.3	mg/dL

Creatinine - female	0.6	1.1	mg/dL

Hormones	**Low**	**High**	**Unit**
Thyroid stimulating hormone (TSH)	0.4	4.7	mIU/L
Free thyroxine (FT4)	9	24	pmol/L
Free triiodothyronine (FT3)	2.5	5.3	pmol/L
Adrenocorticotropic hormone (ACTH)	1.3	15	pmol/L
Cortisol (morning)	250	850	nmol/L
Cortisol (afternoon)	110	390	nmol/L
Prolactin (male)	n/a	450	mIU/L
Prolactin (female)	n/a	580	mIU/L
Testosterone (male post-puberty)	8	38	nmol/L
Testosterone (male pre-puberty)	0.1	0.5	nmol/L
Testosterone (female)	0.3	2.5	nmol/L

It will be appreciated that the desired set point for these and other target molecules can be readily determined for a variety of different applications. It will also be appreciated that the set point may need to be adjusted for certain patients (e.g., based on patient gender, patients with abnormally low or high levels of a target molecule, etc.) or applications (e.g., a drug delivery system designed to release on a more frequent basis may require a lower threshold concentration than a system designed to release less frequently).

It will be appreciated that a material having a desired set point may be generated via routine experimentation using the materials and methods described herein. For example, the same cross-linking agent and conjugate can be combined to produce a series of materials with a gradually increasing ratio of cross-linking agent to conjugate (w/w). These materials will cover a spectrum of set points. Once a lead material with a suitable set point has been identified the process can be repeated with a finer resolution to yield an optimized material. Alternatively (or additionally) the same conjugate can be combined with a plurality of different cross-linking agents that have gradually increasing affinities for the conjugate. This will yield a plurality of materials with a spectrum of set points that can be further refined (e.g., by varying the w/w ratio of cross-linking agent to conjugate). Alternatively one could initiate the process by combining the same cross-linking agent with a plurality of different conjugates. In various embodiments, the conjugates may have varying affinities for the cross-linking agent (e.g., as a result of including different affinity ligands). In various embodiments, the conjugates may include the

same affinity ligands but have different molecular weights (e.g., as a result of different conjugate frameworks).

Uses

In another aspect, the present disclosure provides methods of using the materials. In general, the materials can be used to controllably release conjugates in response to a target molecule. As discussed below, the material can be brought into contact with the target molecule *in vitro* or *in vivo*.

In various embodiments, a material may be used as a component of an *in vitro* or *in vivo* chemical sensor. This aspect is described below in the context of glucose sensors; however, it will be appreciated from the foregoing that other chemical sensors may be prepared by simply using a different target molecule.

For example, in various embodiments, a material of the present disclosure may be used in glucose sensors that are based on fluorescence resonance energy transfer (FRET). FRET is based on the fact that when two different fluorophores are brought closely together this allows for energy transfer between the two fluorophores, resulting in a decrease in the fluorescence of one or both of the fluorophores, which is called fluorescence quenching (Ballerstadt et al., *Anal. Chim. Acta* 345:203-212, 1997). For example, in certain embodiments, in the absence of glucose, a mixture of a fluorescently labeled cross-linking agent and a fluorescently labeled conjugate will form an insoluble cross-linked material and the neighboring fluorophores will undergo FRET. In the presence of glucose, the average distance between the fluorescently labeled cross-linking agent and the fluorescently labeled conjugate will increase causing the level of FRET to decrease and thereby leading to an increase in the individual fluorescence signals. The level of fluorescence can thereby be directly correlated with the level of glucose. It is to be understood that alternative pairs of labels that produce a measurable response when brought in close proximity may be used instead of a pair of fluorescent labels. Thus, in certain embodiments, the invention provides a method comprising steps of: (I) mixing: (a) multivalent lectins with at least two binding sites for glucose, wherein the lectins include at least one affinity ligand which is capable of competing with glucose for binding with at least one of said binding sites and is covalently linked to a cysteine residue of the lectins and wherein the lectins include a first label which generates a measurable response when in close proximity to a second label; (b) conjugates that comprise an affinity ligand and the second label; (II) exposing a sample to the

mixture of multivalent lectins and conjugates, wherein: (a) if glucose is absent from the sample, the conjugates form a cross-linked material with the lectins through affinity binding to the multivalent lectins to produce a measurable response; (b) if glucose is present in the sample, the response is reduced because formation of cross-linked material is inhibited as a result of glucose from the sample competing with the conjugates for the binding sites on the multivalent lectins; and (III) detecting and optionally measuring the response with a sensor to determine the presence and optionally the amount of glucose in the sample. In certain embodiments, the first and second labels are fluorescent labels and the response is a fluorescent signal.

In certain embodiments, the two labels (e.g., fluorescent labels) may be located on different molecules that are brought into proximity by binding to the same multivalent lectin. Thus, in certain embodiments, the invention provides a method comprising steps of: (I) mixing: (a) multivalent lectins with at least two binding sites for glucose, wherein the lectins include at least one affinity ligand which is capable of competing with glucose for binding with at least one of said binding sites and is covalently linked to a cysteine residue of the lectins; (b) a first group of molecules that comprise an affinity ligand and a first label which generates a measurable response when in close proximity to a second label; and (c) a second group of molecules that comprise an affinity ligand and the second label; (II) exposing a sample to the mixture of multivalent lectins, and the first and second groups of molecules, wherein: (a) if glucose is absent from the sample, members of the first and second group of molecules are brought in close proximity through affinity binding to the multivalent lectins to produce a binding complex and a measurable response; (b) if glucose is present in the sample, the response is reduced because fewer of said binding complexes form as a result of glucose from the sample competing with the first and second molecules for the binding sites on the multivalent lectins; and (III) detecting and optionally measuring the response with a sensor to determine the presence and optionally the amount of glucose in the sample. In certain embodiments, the first and second labels are fluorescent labels and the response is a fluorescent signal.

In other exemplary embodiments, materials of the present disclosure may be used in viscosity-based glucose sensors (e.g., see U.S. Patent Nos. 6,267,002; 6,477,891; and 6,938,463). Conjugates and cross-linking agents are again combined to form a cross-linked material. Addition of glucose to the material now causes a concentration dependent reduction in viscosity which can be measured (e.g., as a function of shear rate using a microviscometer set up in a cone-and-plate geometry). The viscosity of the sample can thereby be directly correlated with

the level of glucose. It will be appreciated that these two exemplary glucose sensors do not require any drug to be present within the conjugates. It will also be appreciated that a viscosity-based sensor does not require a detectable label to be present within the conjugates.

In certain embodiments, the invention provides a method comprising steps of: (I) providing: (a) conjugates that comprises a plurality of affinity ligands, (b) multivalent lectins with at least two binding sites for glucose, wherein the lectins include at least one affinity ligand which is capable of competing with glucose for binding with at least one of said binding sites and is covalently linked to a cysteine residue of the lectins; (II) mixing the conjugates and lectins, wherein the viscosity of the resulting mixture is due to the binding between the conjugates and lectins; (III) contacting the mixture with a sample containing glucose which displaces conjugates from the lectins and causes a concentration dependent reduction in viscosity; and (IV) detecting and optionally measuring the resulting change in viscosity to determine the presence and optionally the amount of glucose in the sample.

In various embodiments, a material may be used to controllably deliver a drug to a patient. The invention encompasses treating a disease or condition by administering a material of the present disclosure. Although the materials can be used to treat any patient (e.g., dogs, cats, cows, horses, sheep, pigs, mice, etc.), they are most preferably used in the treatment of humans. A material can be administered to a patient by any route. In general the most appropriate route of administration will depend upon a variety of factors including the nature of the disease or condition being treated, the nature of the drug, the nature of the target molecule, the condition of the patient, etc. In general, the present disclosure encompasses administration by oral, intravenous, intramuscular, intra-arterial, subcutaneous, intraventricular, transdermal, rectal, intravaginal, intraperitoneal, topical (as by powders, ointments, or drops), buccal, or as an oral or nasal spray or aerosol. General considerations in the formulation and manufacture of pharmaceutical compositions for these different routes may be found, for example, in *Remington's Pharmaceutical Sciences*, 19th ed., Mack Publishing Co., Easton, PA, 1995.

In various embodiments, the material may be administered subcutaneously, e.g., by injection. The material can be dissolved in a carrier for ease of delivery. For example, the carrier can be an aqueous solution including, but not limited to, sterile water, saline or buffered saline. In general, a therapeutically effective amount of a drug in the form of a conjugate will be administered. By a "therapeutically effective amount" of a drug is meant a sufficient amount of the drug to treat (e.g., to ameliorate the symptoms of, delay progression of, prevent recurrence

of, delay onset of, etc.) the disease or condition at a reasonable benefit/risk ratio, which involves a balancing of the efficacy and toxicity of the drug. In general, therapeutic efficacy and toxicity may be determined by standard pharmacological procedures in cell cultures or with experimental animals, e.g., by calculating the ED_{50} (the dose that is therapeutically effective in 50% of the treated subjects) and the LD_{50} (the dose that is lethal to 50% of treated subjects). The ED_{50}/LD_{50} represents the therapeutic index of the drug. Although in general drugs having a large therapeutic index are preferred, as is well known in the art, a smaller therapeutic index may be acceptable in the case of a serious disease or condition, particularly in the absence of alternative therapeutic options. Ultimate selection of an appropriate range of doses for administration to humans is determined in the course of clinical trials.

In various embodiments, the drug is insulin and the average daily dose of insulin is in the range of 10 to 200 U, e.g., 25 to 100 U (where 1 Unit of insulin is ~ 0.04 mg). In certain embodiments, an amount of material with these insulin doses is administered on a daily basis. In certain embodiments, an amount of material with 5 to 10 times these insulin doses is administered on a weekly basis. In certain embodiments, an amount of material with 10 to 20 times these insulin doses is administered on a bi-weekly basis. In certain embodiments, an amount of material with 20 to 40 times these insulin doses is administered on a monthly basis. Those skilled in the art will be recognize that this same approach may be extrapolated to other approved drugs with known dose ranges, e.g., any of the approved insulin sensitizers and insulin secretagogues described herein.

It will be understood that the total daily usage of a drug for any given patient will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective amount for any particular patient will depend upon a variety of factors including the disease or condition being treated; the activity of the specific drug employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration and rate of excretion of the specific drug employed; the duration of the treatment; drugs used in combination or coincidental with the specific drug employed; and like factors well known in the medical arts. In various embodiments, a material of the present disclosure may be administered on more than one occasion. For example, the present disclosure specifically encompasses methods in which a material is administered by subcutaneous injection to a patient on a continuous schedule (e.g., once a day, once every two days, once a week, once every two weeks, once a month, etc.).

In certain embodiments, a material of the present disclosure may be used to treat hyperglycemia in a patient (e.g., a mammalian patient). In certain embodiments, the patient is diabetic. However, the present methods are not limited to treating diabetic patients. For example, in certain embodiments, a material may be used to treat hyperglycemia in a patient with an infection associated with impaired glycemic control. In certain embodiments, a material may be used to treat diabetes.

In various embodiments, a material of the present disclosure may be administered to a patient who is receiving at least one additional therapy. In various embodiments, the at least one additional therapy is intended to treat the same disease or disorder as the administered material. In various embodiments, the at least one additional therapy is intended to treat a side-effect of the primary drug. The two or more therapies may be administered within the same, overlapping or non-overlapping timeframes as long as there is a period when the patient is receiving a benefit from both therapies. The two or more therapies may be administered on the same or different schedules as long as there is a period when the patient is receiving a benefit from both therapies. The two or more therapies may be administered within the same or different formulations as long as there is a period when the patient is receiving a benefit from both therapies. In certain embodiments, a single material of the present disclosure may include more than one drug for treating the same disease or disorder. In certain embodiments, two or more separate materials of the present disclosure may be administered (as a mixture or separately) that include different drugs for treating the same disease or disorder. In certain embodiments, an unconjugated secondary drug may be included in a material of the present disclosure (i.e., a drug which is simply mixed with the components of the material and not covalently bound to the cross-linked material). For example, in certain embodiments, any of these approaches may be used to administer more than one anti-diabetic drug to a subject. Certain exemplary embodiments of this approach are described in more detail below in the context of insulin-related therapies; however, it will be appreciated from the foregoing that other therapies will benefit from such combination approaches.

Insulin sensitizers (e.g., biguanides such as metformin, glitazones) act by increasing a patient's response to a given amount of insulin. A patient receiving an insulin sensitizer will therefore require a lower dose of an insulin-based material of the present disclosure than an otherwise identical patient would. Thus, in certain embodiments, a material comprising insulin conjugates may be administered to a patient who is also being treated with an insulin sensitizer.

In various embodiments, the material of the present disclosure may be administered at up to 75% of the normal dose required in the absence of the insulin sensitizer. In various embodiments, up to 50, 40, 30 or 20% of the normal dose may be administered.

Insulin resistance is a disorder in which normal amounts of insulin are inadequate to produce a normal insulin response. For example, insulin-resistant patients may require high doses of insulin in order to overcome their resistance and provide a sufficient glucose-lowering effect. In these cases, insulin doses that would normally induce hypoglycemia in less resistant patients fail to even exert a glucose-lowering effect in highly resistant patients. Similarly, the materials of the present disclosure are only effective for this subclass of patients when they release high levels of insulin-conjugates in a suitable timeframe. In certain embodiments, the treatment of this subclass of patients may be facilitated by combining the two approaches. Thus in certain embodiments, a traditional insulin-based therapy is used to provide a baseline level of insulin and a material of the present invention is administered to provide a controlled supplement of insulin when needed by the patient. Thus, in certain embodiments, a material comprising insulin conjugates may be administered to a patient who is also being treated with insulin. In various embodiments, the insulin may be administered at up to 75% of the normal dose required in the absence of the material of the present disclosure. In various embodiments, up to 50, 40, 30 or 20% of the normal dose may be administered. It will be appreciated that this combination approach may also be used with insulin resistant patients who are receiving an insulin secretagogue (e.g., a sulfonylurea, GLP-1, exendin-4, etc.) and/or an insulin sensitizer (e.g., a biguanide such as metformin, a glitazone).

Kits

In another aspect the present disclosure provides kits that include modified lectins and conjugates and other reagents for preparing a material. For example, a kit may include separate containers that include a plurality of conjugates and a plurality of modified lectins. When the conjugates and modified lectins of the kit are mixed a cross-linked material is formed. In various embodiments, the material is designed for subcutaneous delivery and the kit includes a syringe or pen. In various embodiments, a kit may include a syringe or pen which is pre-filled with a cross-linked material. The kit may also include instructions for mixing the conjugates and modified lectins to produce the cross-linked material.

In yet another aspect, the present disclosure provides libraries of conjugates and/or modified lectins. These libraries may be particularly useful for generating materials with a desired set point. In various embodiments, a library may include a plurality of modified lectins which produce different set points with the same conjugate. In various embodiments, a library may further include one or more conjugates which form cross-linked materials with modified lectins in the library. When the library includes more than one such conjugate, the different conjugates may have different molecular weights, a different number of affinity ligands per conjugate molecule and/or different affinity ligands. In various embodiments, a library may include one or more of the conjugates that include more than one type of affinity ligand. In various embodiments, a library may include a plurality of conjugates which produce different set points with the same modified lectin. In various embodiments, a library may further include one or more modified lectins which form cross-linked materials with conjugates in the library.

In yet another aspect, the present disclosure provides a kit that comprises: (a) a first container that includes modified lectins that include a first label which generates a measurable response when in close proximity to a second label; and (b) a second container that includes conjugates that comprise the second label.

In yet another aspect, the present disclosure provides a kit that comprises: (a) a first container that includes modified lectins; (b) a second container that includes a first group of molecules that comprise an affinity ligand and a first label which generates a measurable response when in close proximity to a second label; and (c) a third container that includes a second group of molecules that comprise an affinity ligand and the second label. In certain embodiments, the first and second molecules are in the same container.

EXAMPLES

I. Methods of making exemplary conjugates

This first set of examples describes various methods for making exemplary conjugates. The examples also include assays for purifying and assaying the starting ingredients and final products. It is to be understood that these methods can be modified to produce other conjugates that fall within the scope of the invention.

Example 1 – Synthesis of Azidoethylglucose (AzEG)

a. Synthesis of bromoethyleglucose

DOWEX 50Wx4 resin (Alfa Aesar, Ward Hill, MA) was washed with deionized water to remove color. A mixture of 225 gm D-glucose (1.25 mol; 1 equiv., Alfa Aesar) and 140 gm DOWEX 50Wx4 was treated with 2.2 L 2-bromoethanol (30.5 mol, 25 equiv.; 124.97 gm/mol; 1.762 gm/mL; BP = 150 C; Alfa Aesar) and the stirred mixture heated to 80 C for 4 hours. The reaction was monitored by TLC (20% methanol/dichloromethane (DCM)). Reaction was complete after about four hours, and it was allowed to cool to room temperature. The solution was filtered to remove the resin, and the resin washed with ethyl acetate and DCM. The resulting filtrate was stripped to an amber oil in a rotory evaporator. A total of 400 gm after stripping.

The amber oil was purified on silica gel (4 kg silica packed in DCM) in the following manner. The crude was dissolved in DCM and loaded onto the column, and then eluted with 2 x 4L 10% methanol/DCM; 2 x 4L 15% methanol/DCM; and 3 x 4L 20% methanol/DCM. Product containing fractions (on the basis of TLC) were pooled and stripped to dryness to afford 152 gm of 1-α-bromoethyl-glucose (42%).

b. Conversion of bromoethylglucose to azidoethylglucose (AzEM)

A 5L round bottom three-necked flask, equipped with a heating mantle, an overhead stirrer, and a thermometer, was charged with 150 gm bromoethylglucose (525 mmol). The oil was dissolved in 2 L water and treated with 68.3 gm sodium azide (1.05 mol, 2 equiv.; 65 gm/mol; Alfa-Aesar) followed by 7.9 gm sodium iodide (52.5 mmol, 0.08 equiv.; 149.89 gm/mol; Alfa-Aesar) and the solution warmed to 50 C and stirred overnight. The solution was cooled to room temperature and concentrated to dryness on the rotovap. The solid residue was digested with 3 x 500 mL of 5:1 vol. $CHCl_3$:MeOH at 40 C. The combined organic portions were filtered and evaporated to dryness to afford azidoethylglucose (86 gm) as an off-white solid. TLC (20% MeOH/DCM; char with H_2SO_4): single spot, indistinguishable from the starting material.

c. Repurification of azidoethylglucose

32 gm of azidoethylglucose was taken into 100 mL water. The turbid solution was filtered through a glass microfibre filter (Whatman GF/B). The golden filtrate was evaporated to a solid on a rotovapor. The solid was taken into methanol (100 mL) and the turbid solution was

again filtered through a glass microfibre filter. The resulting pale yellow filtrate was stripped to a solid under vacuum.

The solid was taken into a minimum of methanol (50 mL) and ethyl acetate (150 mL) was added slowly with stirring. The heavy slurry was cooled and filtered. The solid was air dried (hygroscopic) and put in a 60 C oven overnight. TLC has very little origin material. Yield 15.4 gm. The Mother Liquor was evaporated under vacuum to a yellow gum. No attempt was made to further purify this material at this time.

Example 2 – Synthesis of Azidoethylmannose (AzEM)

a. Synthesis of bromoethylmannose

DOWEX 50Wx4 resin (Alfa Aesar, Ward Hill, MA) is washed with deionized water to remove color. A mixture of 225 gm D-mannose (1.25 mol; 1 equiv., Alfa Aesar) and 140 gm DOWEX 50Wx4 is treated with 2.2 L 2-bromoethanol (30.5 mol, 25 equiv.; 124.97 gm/mol; 1.762 gm/mL; BP = 150 C; Alfa Aesar) and the stirred mixture heated to 80 C for 4 hours. The reaction is monitored by TLC (20% methanol/dichloromethane (DCM)). Reaction is complete after about four hours, and then allowed to cool to room temperature. The solution is filtered to remove the resin, and the resin washed with ethyl acetate and DCM. The resulting filtrate is stripped to an amber oil in a rotory evaporator.

The amber oil is purified on silica gel (4 kg silica packed in DCM) in the following manner. The crude is dissolved in DCM and loaded onto the column, and then eluted with 2 x 4L 10% methanol/DCM; 2 x 4L 15% methanol/DCM; and 3 x 4L 20% methanol/DCM. Product containing fractions (on the basis of TLC) are pooled and stripped to dryness to afford 152 gm of 1-α-bromoethyl-mannose (42%).

b. Conversion of bromoethylmannose to azidoethylmannose (AzEM)

A 5L round bottom three-necked flask, equipped with a heating mantle, an overhead stirrer, and a thermometer, is charged with 150 gm bromoethylmannose (525 mmol). The oil is dissolved in 2 L water and treated with 68.3 gm sodium azide (1.05 mol, 2 equiv.; 65 gm/mol; Alfa-Aesar) followed by 7.9 gm sodium iodide (52.5 mmol, 0.08 equiv.; 149.89 gm/mol; Alfa-Aesar) and the solution warmed to 50 C and stirred overnight. The solution is cooled to room temperature and concentrated to dryness on the rotovap. The solid residue is digested with 3 x

500 mL of 5:1 vol. $CHCl_3$:MeOH at 40 C. The combined organic portions are filtered and evaporated to dryness to afford azidoethylmannose as an off-white solid.

c. Repurification of azidoethylmannose

32 gm of azidoethylmannose is taken into 100 mL water. The turbid solution is filtered through a glass microfibre filter (Whatman GF/B). The filtrate is evaporated to a solid on a rotovapor. The solid is taken into Methanol (100 mL) and the turbid solution is again filtered through a glass microfibre filter. The resulting pale yellow filtrate is stripped to a solid under vacuum.

The solid is taken into a minimum of methanol (50 mL) and ethyl acetate (150 mL) is added slowly with stirring. The heavy slurry is cooled and filtered. The solid is air dried (hygroscopic) and put in a 60 C oven overnight. The Mother Liquor is evaporated under vacuum to a yellow gum.

Example 3 – Synthesis of Azidoethylmannobiose (AzEBM)

The AzEM compound from Example 2 is selectively protected using bezene dimethyl ether, purified by column chromatography and subsequently reacted with benzyl bromide to give 1-α-(2-azidoethyl)-4,6-benzaldehyde diacetal-3-benzyl-mannopyranoside. The product is subsequently glycosylated with 1-α-bromo-2,3,4,6-tetrabenzoylmannopyranoside using silver triflate chemistry under rigorously anhydrous conditions to give the protected-azidoethylmannobiose product. The intermediate product is then deprotected to remove the benzoyl groups to give AzEBM.

Example 4 – Synthesis of Azidoethylmannotriose (AzETM)

a. 1-α-bromo-2,3,4,6-tetrabenzoyl-mannose

To a 500 mL 3-neck flask containing a stir bar and nitrogen inlet was added 40 gm (60.9 mmole) of pentabenzoylmannose and 80 mL methylene chloride. The resulting solution was cooled in an ice bath to < 5 C, and 80 mL 33% HBr-acetic acid solution was added via an addition funnel at such a rate to maintain the reaction temperature < 10 C. Upon complete addition (~ 30 min.) the ice bath was removed and stirring was continued for 3 hours.

The reaction solution was diluted with an equal volume (160 mL) of DCM and extracted successively with water (2x 500 mL), saturated bicarbonate (2x 50 mL) and Brine (1x50 mL), dried over magnesium sulfate and the solvent evaporated to give 41 gm of solid foam. (Theoretical yield 40.1 gm) and was stored under N_2 in a freezer. This material was used without further purification. The reaction was monitored by TLC: silica gel (Hexane/Ethyl Acetate, 7/3) starting material R_f 0.65, product R_f 0.8 UV visualization. ^{1}H NMR ($CDCl_3$) δ 8.11(d, 2H),8.01(m, 4H), 7.84(d, 2H), 7.58(m, 4H), 7.41(m, 6H), 7.28(t, 2H), 6.58(s, 1H), 6.28(m, 2H), 5.8(m, 1H), 4.75(dd, 1H) 4.68 (dd, 1H) 4.5(dd, 1H).

b. 1-Azidoethyl-2,4-dibenzoylmannose

To a 1.0L, 3-neck flask containing a stir bar, nitrogen inlet and 300 mL of anhydrous acetonitrile was added 25 gm 1-azidoethylmannose (100.4 mmole), and 50 mL triethyl orthobenzoate (220 mmole, 2.2 equiv.). The resulting slurry was stirred at room temperature and 0.8mL (10 mmole) trifluoroacetic acid (TFA) was added neat. The solution cleared within 10 minutes and stirring was continued for an additional two hours , then 25 mL of 10% aqueous TFA was added and stirring was continued for an additional 2 hours to hydrolyze the intermediate to the ester isomers. The solvent was evaporated under vacuum to a viscous oil, which was triturated with 50 mL DCM and again evaporated to a viscous oil.

Toluene (70 mL) was added to the residue and the viscous solution was seeded with 2,4-dibenzoylazidoethylmannose. A fine precipitate formed within 15 minutes and stirring was continued overnight at room temperature. The resulting heavy suspension was set in the freezer for 2-4 hours, then filtered and the solid washed with ice cold toluene (2x10 mL). The solid was air dried to a constant weight to give 21 gm (TY 22.85 gm @ 50% isomeric purity) of ~95% isomeric purity. The product was taken into 40 mL toluene, stirred for 1 hour and then set in the freezer for an additional 2 hours. The solid was filtered and washed (2x10 mL) with ice cold toluene and air dried to a constant weight to give 18.5 gm of the single isomer product 2,4-

dibenzoylazidoethylmannose in 83% yield. The mother liquors contained the undesired isomer and a small amount of the desired isomer. The reaction was monitored by TLC: SG (Hexane/Ethyl Acetate 7/3) Starting Material R_f 0.0, orthoester intermediate R_f 0.9. (Hexane/Ethyl Acetate: 8/2) SM R_f 0.8, desired isomer R_f 0.4, un-desired isomer R_f 0.2. ^1H NMR 300MHz ($CDCl_3$) δ 8.12(t, 4H), 7.66(t, 2H), 7.5(m, 4H), 5.56(t, 1H), 5.48(m, 1H), 5.14(m, 1H), 4.5(dd, 1H), 4.0(m, 2H), 3.8(m, 3H), 3.56(m, 1H), 3.44(m, 1H).

c. Perbenzoylated-man(α-1,3)-man(α-1.6)-α-1-azidoethylmannopyranoside

To a 1.0 L 3-neck flask with a stir bar, nitrogen inlet was added 41 gm crude 1-bromo-tetrabenzoymannose (60.9 mmole, ~2.5 equiv.) in 185 mL DCM. To this was added 11.2 gm 2,4-dibenzoylazidoethylmannose (24.5 mmole) followed by 11.2 gm 4A sieves. The slurry was stirred a room temperature for 10 minutes and cooled to –15°C in a methanol/ice bath.

In a separate dark vessel was added 190 mL toluene followed by 15.1 gm silver-triflluoromethanesulfonate (AgOTf) (58.8 mmole, 2.4 equiv.) and was stirred into solution in the dark. This solution was transferred to a large addition funnel, and added drop-wise to the stirring suspension while protecting the reaction from light. The reaction temperature was maintained < -10 C by adjusting the AgOTf addition rate. Upon complete addition (~30 minutes) the cold bath was removed and the reaction stirred for an additional 2 hours until a single product remained by TLC (SG, Hexane/Ethyl Acetate: 7/3, Bromo R_f 0.9, azido R_f 0.4, trios product R_f 0.5, uv visualization).

Triethylamine (7 mL, 5.0 equiv.) was added followed by 200 mL DCM. The resulting slurry was filtered through a pad of silica gel and celite and washed with 2x 75 mL DCM. The solvent was evaporated under vacuum and the residue taken into ethyl acetate and washed sequentially with water (2x100 mL), bicarb (2x50 mL), brine (1x75 mL) and dried over magnesium sulfate. The solvent was evaporated under vacuum to give 39 gm of solid foam (TY 39.5 gm). ^1H NMR 300MHz ($CDCl_3$) δ 8.3(d, 2H), 8.2(m, 8H), 7.85(d, 4H), 7.75(dd, 4H), 7.3-7.65(m, 30H), 7.2(t, 2H), 6.05(m, 4H), 5.9(t, 2H), 5.63(m, 2H), 5.38(s, 2H), 5.18(d, 1H), 4.65(m, 4H), 4.5(m, 2H), 4.35(m, 4H), 3,8(m, 2H), 3.54(m, 2H).

d. Man(α-1,3)-man(α-1.6)-α-1-azidoethylmannopyranoside

To a stirring suspension of 3.0 gm perbenzoylated-man (α-1,3)-man(α-1.6)-α-1-azidoethylmannopyranoside (1.86 mmole) in 40 mL methanol was added 0.2 mL 4.28M sodium methoxide in methanol. The resulting suspension was stirred 20 hours at room temperature

giving a clear solution. The completion of the reaction was monitored by TLC, (SG, hexane/ethyl acetate: 8/2 SM R_f 0.4, product R_f 0.0).

The methanol was evaporated under vacuum giving an oily semi-solid. The residue was taken into ethyl acetate (50 mL) and stirred for 3 hours. The solid was filtered, washed with fresh ethyl acetate (2x20 mL) and air dried to a constant weight to give 1.09 gm (TY 1.07 gm) of product. The mother liquors contained residual methyl benzoate, the de-protection by-product.

Example 5 – Synthesis of aminoethyl-sugars (AEG, AEM, AEBM, AETM) from azidoethyl-sugars (AzEG, AzEM, AzEBM, AzETM)

The azido-terminated compounds from Examples 1-4 are readily hydrogenated at room temperature by using palladium/carbon catalyst, a small amount of acetic acid, and ethanol as a solvent to give the corresponding amine-terminated compounds. Figure 10 shows the chemical structures of AEG, AEM, AEBM, AETM. The process is identical to the one described for AETM below, except that those skilled in the art will understand that the amounts of reagents, solvents, etc. should be scaled to the number of moles of sugar-ligand to be hydrogenated.

a. Man (α-1,3)-Man(α-1.6)-α-1-aminoethylmannopyranoside ("aminoethyltrimannose", AETM)

To a solution of 5.3 gm (9.25 mmole) man(α-1,3)-man(α-1.6)-α-1-azidoethylmannopyranoside in 100 mL water and 50 mL ethanol was added 0.8 gm 5% Pd/C. The vigorously stirring suspension was hydrogenated at 30-40 psi for 48 hours or until no starting material was apparent by TLC (SG, Methanol, SM R_f 0.75, Pdt R_f 0.0, PMA vis.). The suspension was filtered over celite, which was rinsed with ethanol (2x50 mL) and the filtrate concentrated under vacuum.

HPLC of this material (C18, 3% Acetonitrile/97% 0.1% H_3P0_4, 220 nm, 2 ml/min) gave uv adsorption of the injection column void material, Rt 2.5 minutes, indicative of benzoate ester.

The filtrate was diluted with 70 mL water and 12 mL of 1N NaOH and the solution stirred overnight at room temperature (HPLC: no uv material at column void Rt 2.5 min., uv material at Rt 10.5 minutes co-eluting with benzoic acid). 2 gm of decolorizing charcoal were added and the stirring suspension heated to 80 C, cooled to room temperature and filtered over celite. The filtrate pH was adjusted to 8.0 with 2N HCl and the colorless solution concentrated under vacuum to about 50% volume.

The solution was loaded onto a resin column (Dowex 50W, 50 gm) and washed with water until eluting fractions were neutral to pH (6x75 mL) removing any residual acid by-products. The amine product was washed off the column with 0.25N ammonium hydroxide (6x75 mL) and the fractions containing the amine product-ninhydrin detection were combined and concentrated to 25-30 mL under vacuum. This concentrated solution was added drop-wise to 300 mL stirring ethanol and stirring continued for an additional 2 hours. The product was filtered, washed with fresh ethanol (2x50 mL) and air dried to a constant weight. The resulting white amorphous solid was dried further in a vacuum oven at 80 C for 5 hours to give 4.1 gm of a white granular solid (TY 5.1 gm). The NMR was clean of any aromatic protons. ^{1}H NMR 300 MHz (D_2O) δ 5.08(s, 1H), 4.87(s, 1H), 4.81(s, 1H), 4.8-3.6(m, 18H), 2.9(m, 2H).

Example 6 – Dipropargyl sugar synthesis and production of AE-ligand

a. Synthesis of diethyl diproparglymalonate

Diethylmalonate (122.5g, 0.7648 mol) was added to absolute ethanol (800ml) containing sodium ethoxide (prepared from sodium metal, 38.5 g, 1.67mol). After 30 min, propargyl bromide (200g, 1.68 mol) was slowly added to the stirred suspension, keeping the temperature under 60 C. The mixture was refluxed overnight (15 hours). The precipitated salts were removed by filtration and washed with ethanol. Solvent was removed *in vacuo*, and the residue diluted with water and extracted with ethanol (2x 200ml). The combined extracts were dried over MgSO4, filtered, washed with Et2O and the solvent removed *in vacuo* to afford a golden colored oil. The oil was placed on high vacuum (40 C) for 3 hours and allowed to stand. Solids began to crystallize forming an oily solid. Let stand overnight (16 hours). Cyclohexane was charged to flask, solids broken-up, filtered, and washed with cyclohexane to afford white crystalline product (81 gm, 44.8% yield). Reaction was followed by GC.

b. Synthesis of dipropargylmalonic acid

Diethyl dipropargyl malonate (80 gm, 0.339 mol) was refluxed in 600 ml of 10% alcoholic potassium hydroxide overnight (15 hours). Solvent was removed *in vacuo* and the residue was acidified with 3N HCl. The residue was extracted with Et2O (2x 300ml). The combined extracts were dried over MgSO4, filtered, washed with Et2O and concentrated *in vacuo* to an oil. Placed on high vac (40 C) for 2 hours and let stand to afford dipropargylmalonic acid as an oil (46 gm, 75.4 % yield). Reaction was followed by GC.

c. Synthesis of dipropargylacetic acid

The dipropargylmalonic acid (26 gm, 0.443 mol) was heated neat at 135 C until CO_2 stopped evolving. It was then allowed to cool to an oil. The oil was distilled at 0.5 psi. The remaining oily residue in the distillation flask and solid were combined (15.7 gm, 79.9% yield) and was used as is in the next step.

d. Synthesis of [2-(3-prop-2-ynyl-hex-5-ynoylamino)-ethyl]-carbamic acid t-butyl ester

N-boc-ethylenediamine (18.3 gm, 0.1143 mol) in 50 ml of CH_3CN was added slowly via an addition funnel to a stirred solution containing dipropargylacetic acid (15.56 gm, 0.1143 mol), TBTU (36.74 gm, 0.114 mol) and DIPEA (29.6 gm, 0.229 mol) in 300ml of CH_3CN at 0 C. Precipitation occurred. The ice bath was removed and the product was stirred at ambient temperature overnight (16 hours). The reaction was now totally homogeneous. The solution was concentrated *in vacuo* and the residue was diluted with 800ml of water. The resulting solids were filtered, washed copiously with water, and vacuum dried to give 14.3 gm of crude product. Re-crystallization (2x) from DCM, filtration and washing with hexanes affords the product (9.85 gm, 31% yield, 98% purity by HPLC (214nm)).

e. Click reaction of azidosugar to [2-(3-prop-2-ynyl-hex-5-ynoylamino)-ethyl]-carbamic acid t-butyl ester

To 1,1 dipropargyl-acetyl-(-1N, 2N-BOC-1,2-diaminoethyl)amide (DP, 418 mg, 1.5 mmole) in DCM (20 mL) was added drop-wise TFA (4 mL) over 5 minutes at 0 C. The darkening solution was stirred at room temperature overnight. The volatiles were evaporated under reduced pressure. Toluene (20 mL) was added to the residue and stripped under reduced pressure two times. The resulting dark oil was used without further purification.

To this residue was added THF (20 mL) and water (20 mL) with stirring for 15 minutes. Copper Sulfate (225 mg, 0.9 mmole) was added followed by sodium ascorbate (180 mg, 0.9 mmole). The resulting mixture was heated to 55-60 C for 6 hours and then stirred at room temperature for 18 hours. The solution was evaporated under reduced pressure to approx. half volume and filtered through a microfibre glass filter. The resulting clear solution was placed on a resin column (Dowex 50X-2) which was washed with water (6x 75 mL) until neutral pH, and then washed with 10% NH_4OH (8 x 75 mL). The fractions staining positive with Ninhydrin were

combined and evaporated under reduced pressure to a glassy solid. The glass residue was taken into water (250 mL) and treated with 0.5 gm charcoal and heated to reflux. The cooled slurry was filtered over celite and a microfibre filter. The resulting pale yellow solution was evaporated to a glassy solid under reduced pressure and methanol was added and evaporated (2x) to give a off white foam (0.9 gm, TY 1.0 gm).

Example 7 – Tripropargyl sugar synthesis and production of AE-ligand

a. 2-(2-BOC-aminoethyl)thioacetamide-tris[(propargyloxy)methyl] aminomethane

To a solution of t-butyl N-(2-mercaptoethyl)carbamate (Frontrun Organix, Ipswich, MA; 177.26 mg, 1 mmole) in ethanol (5 mL) was added NaOH (1.1 mmole) with stirring at room temperature. To this solution was added 2-bromoacetamide-tris[(propargyloxy)methyl]aminomethane (356 mg, 1.0 mmole, see *J. Org. Chem.* 73, 5602, 2008) and stirring was continued for 20 hours (TLC SG 8/2 hexane/ethyl acetate, pdt R_f 0.4). The solvent was evaporated under vacuum and the residue was taken into ethyl acetate (40 mL) and washed successively with water (25 mL), 0.5 N NaOH (25 mL) and Brine (25 mL), dried over Na_2SO_4 filtered and concentrated to an oil (360 mg, TY 452.3 mg). NMR $CDCl_3$, (ppm): 7.05(s, 1H, N-H); 5.25 ((s, 1H,N-H); 4.85 (s,6H); 3.85(s, 6H); 3.3 (m,2H); 3.15 (s, 2H); 2.7 (m, 2H); 2.42 (s, 3H); 1.22 (s, 9H).

b. 2-(2-aminoethyl)thioacetamide-tris[(triazolo-1-(2-ethylmannose) 4-methoxy)methyl] aminomethane

To a stirring solution of 2-(2-BOC-aminoethyl)thioacetamide-tris[(propargyloxy)methyl] aminomethane (1 gm, 2.21 mmole) in DCM (40 mL) at room temperature was added TFA (4 mL) dropwise. The resulting solution was stirred overnight. The solvents were removed under vacuum and the residue taken into toluene (15 mL) and evaporated to dryness.

The residue was taken into THF (40 mL), water (40 mL) and stirred into solution. Azidoethylmannose (3.75 eq., 2.0 gm, 8.3 mmole) was added followed by copper sulfate (500 mg, 2.0 mmole) and sodium ascorbate (400 mg, 2.0 mmole) and the resultant mixture stirred at 55-60 C (oil bath) for 6 hours, cooled to room temperature and stirred overnight. The resulting mixture was concentrated under vacuum to one half volume and filtered thru a micro-glass filter. The filtrate was loaded on a resin column (Dowex 50w 50x4-100) and eluted with water (6x75 mL) until neutral. The column was then eluted with 15% Ammonium Hydroxide (10x75 mL)

and the fractions positive to ninhydrin were pooled and concentrated to a glassy foam (1.29 gm, TY (MW 1099 g/mol), 53% over two steps).

Example 8 – Synthesis of NH_2-B1-BOC2(A1,B29)-insulin

In a typical synthesis, 4 g of powdered insulin (Sigma Aldrich, St. Louis, MO) is dissolved in 100 ml of anhydrous DMSO at room temperature followed by the addition of 4 ml of triethylamine (TEA). The solution is stirred for 30 minutes at room temperature. Next, 1.79 ml (2.6 equivalents) of di-tert-butyl-dicarbonate/THF solution (Sigma Aldrich, St. Louis, MO) is slowly added to the insulin-TEA solution and mixed for approximately one hour. The reaction is quenched via the addition of 4 ml of a stock solution containing 250 ul of ethanolamine in 5 ml of DMSO followed by mixing for five minutes. After quenching, the entire solution is poured into 1600 ml of acetone and mixed briefly with a spatula. Next, 8 x 400 µl aliquots of a 18.9% HCl:water solution are added dropwise over the surface of the mixture to precipitate the reacted insulin. The precipitated material is then centrifuged and the supernatant decanted into a second beaker while the precipitate cake is set aside. To the supernatant solution, another 8 x 400 µl aliquots of a 18.9% HCl:water solution are added dropwise over the surface of the mixture to obtain a second precipitate of reacted insulin. This second precipitate is centrifuged and the supernatant is discarded. The combined centrifuge cakes from the two precipitation steps are washed once with acetone followed by drying under vacuum at room temperature to yield the crude powder which typically contains 60% of the desired BOC2 product and 40% of the BOC3 material.

A preparative reverse phase HPLC method is used to isolate the pure BOC2-insulin from the crude powder. Buffer A is deionized water containing 0.1% TFA and Buffer B is acetonitrile containing 0.1% TFA. The crude powder is dissolved at 25 mg/ml in a 70%A/30%B mixture and syringe filtered prior to injection on the column. Before purification, the column (Waters SymmetryPrep C18, 7 um, 19 x 150 mm) is equilibrated at 15 ml/minutes with a 70%A/30%B mobile phase using a Waters DeltraPrep 600 system. Approximately 5 ml of the crude powder solution is injected onto the column at a flow rate of 15 ml/minutes over the course of 5 minutes after which a linear gradient is employed from 70%A/30%B to 62%A/38%B over the course of the next 3.5 minutes and held there for an additional 2.5 minutes. Using this method, the desired BOC2 peak elutes at approximately 10.6 minutes followed closely by the BOC3 peak. Once collected, the solution is rotovapped to remove acetonitrile and lyophilized to obtain pure BOC2-insulin powder. Identity is verified by LC-MS (HT Laboratories, San Diego, CA) and site of conjugation determined by N-terminal sequencing (Western Analytical, St. Louis, MO).

Example 9 – Synthesis of benzene-1,3,5-tricarboxy-(N-ω-aminoacid-NHS ester) amide frameworks

A solution of 1,3,5-benzenetricarbonyl chloride (1 gm, 3.8 mmole) in dichloromethane (DCM) (5 mL) is added drop-wise to a vigorously stirring solution of an ω-aminoacid (3.1 equivalents) in 1N NaOH (25 mL) in an ice bath. The ice bath is removed and stirring is continued for 4 hours at room temperature. 2N HCl (~15 mL) is added dropwise to approximately pH 2 and the resulting slurry is stirred for an additional 2 hours. The precipitate is filtered, washed with cold water (2x20 mL) and dried in air under vacuum and then in a 60 C oven overnight. The resulting white solid is used without further purification. Yield for each ω-aminoacid (4-aminobutyric acid: yield 1.6 gm, 91%; 6-aminocaproic acid: yield 1.9 gm, 92%)

The above material is taken into DMSO (5 mL) containing N-hydroxysuccinimide (3.1 mmole, 3.1 equiv.) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDCI, 3.6 mmole, 3.6 equiv.) is added at room temperature. The resulting solution is stirred for 24 hours, diluted with water (125 mL) and extracted with ethyl acetate (3x50 mL). The combined organic phase is washed with water (2x50 mL), brine (1x50 mL) and dried over $MgSO_4$. The solvent is evaporated and the semi-solid residue triturated with acetonitrile (10 mL). The solid is filtered and washed with cold solvent, dried in air under vacuum and then in a 60 C oven overnight. The product is free of urea bi-product. Benzene-1,3,5-tricarboxy-(N-6-aminocaproic-NHS ester)amide (TSB-C6): 304 mg, 36%, mp 140-142 C. Benzene-1, 3, 5-tricarboxy-(N-4-butyric-NHS-ester)amide (TSB-C4): 245 mg, 45%, mp 182-184 C.

Example 10 – Dendritic framework synthesis

a. Hydrogenation of nitro-group containing, alkyne-terminally functionalized dendrons

Dendrons containing either n=2, 4, or 8 terminal alkynes and a nitropropionic acid core are obtained (e.g., from Polymer Factory, Sweden) and used without further purification. The dendron is dissolved in 100 mL a 50:50 vol. mixture of DCM and ethanol, and 0.8 gm of 5% Pd/C is added. The vigorously stirring suspension is hydrogenated at 30-40 psi for 48 hours or until no starting material is apparent by TLC. The suspension is filtered over celite, which is rinsed with ethanol (2x50 mL) and the filtrate concentrated under vacuum.

The filtrate is diluted with 70 mL water and 12 mL of 1N NaOH and the solution stirred overnight at room temperature. 2 gm of decolorizing charcoal are added and the stirring

suspension heated to 80 C, cooled to room temperature and filtered over celite. The filtrate pH is adjusted to 8.0 with 2N HCl and the colorless solution concentrated under vacuum to about 50% volume.

The solution is loaded onto a resin column (Dowex 50W, 50 gm) and washed with water until eluting fractions are neutral to pH (6x75 mL) removing any residual acid by-products. The amine product is washed off the column with 0.25N ammonium hydroxide (6x75 mL) and the fractions containing the amine product (ninhydrin detection) are combined and evaporated to vacuum using a rotary evaporator.

b. Reaction of dendron (amine, alkyne-4) with azidoethyl mannose

The dendron product containing the amino core and four terminal alkyne groups obtained after hydrogenation (8.3 mmol) is taken into THF (40 mL), water (40 mL) and stirred into solution. Azidoethylmannose (4.75 eq., 2.53 gm, 10.51 mmole) is added followed by copper sulfate (500 mg, 2.0 mmole) and sodium ascorbate (400 mg, 2.0 mmole) and the resultant mixture stirred at 55-60 C (oil bath) for 6 hours, cooled to room temperature and stirred overnight. The resulting mixture is concentrated under vacuum to one half volume and filtered thru a micro-glass filter. The filtrate is loaded on a resin column (Dowex 50w 50x4-100) and eluted with water (6x 75 mL) until neutral. The column is then eluted with 15% ammonium hydroxide (10x75 mL) and the fractions positive to ninhydrin are pooled and concentrated to a glassy foam.

Example 11 – Amine-functionalized drug conjugation with multivalent activated esters in organic solvent (drug added first)

A framework containing N-terminal activated esters is dissolved at 60 mM in 1.0 ml of anhydrous DMSO followed by the addition of 400 ul (excess) of triethylamine (TEA). The solution is stirred rapidly for 10 minutes at room temperature. The amine-bearing drug is then dissolved separately in 7.9 ml of DMSO at a concentration of 7.4 mM. Once dissolved, the entire drug solution is added dropwise over the course of 10 minutes to the framework/DMSO/TEA solution followed by room temperature mixing for two hours. The remaining activated esters are then reacted with amine-functionalized affinity ligands in the following manner. A 370 mM solution of affinity ligand is prepared in an appropriate volume of dry DMSO. Once dissolved, enough solution is added to provide a number of reactive

equivalents equal to three times the number of initial activated ester groups, N, minus one. For example, if there are N=3 initial activated ester groups per framework, then (3x(3-1)x60mM/370 mM)=0.973 ml of affinity ligand solution are added. If there are N=4 initial activated ester groups per framework, then (3x(4-1)x60mM/370 mM)=1.46 ml of affinity ligand solution are added, and so on. After the affinity ligand solution is added, the solution is stirred for one more hour at room temperature to ensure complete reaction.

The resulting solution is then superdiluted by 10x into a 20 mM pH 5.0 HEPES buffered saline solution containing 0.150 M NaCl followed by pH adjustment with dilute HCl to a final pH of 8.0. The aqueous solution is first purified by size exclusion using an appropriate solid phase for the desired separation of conjugated and unconjugated materials. The solution passing through the column void volume is then concentrated using an appropriately sized ultrafiltration membrane to approximately 10 ml. This solution is further purified to obtain the desired product using preparative reverse phase HPLC on a Waters C8, 7 um, 19 x 150 mm column. Buffer A is deionized water containing 0.1% TFA and Buffer B is acetonitrile containing 0.1% TFA. Before purification, the column is equilibrated at 15 ml/minutes with a 80%A/20%B mobile phase using a Waters DeltraPrep 600 sytem. Approximately 5 ml of the crude solution is injected onto the column over the course of 2 minutes at a flow rate of 15 ml/minutes after which a linear gradient is employed from 80%A/20%B to 75%A/25%B over the next 5 minutes followed by a slower linear gradient from 75%A/25%B to 62%A/38%B over the next 22 minutes. The retention time of the desired peak will vary depending on the drug, framework, and affinity ligand used. Once collected, the solution is rotovapped to remove acetonitrile and lyophilized to obtain pure conjugate whose identity may be verified by LC-MS (HT Laboratories, San Diego, CA).

Example 12 – B1-insulin conjugates with multivalent sugars - homogeneous ligand

Using the method described in Example 11 and the amine-bearing drug, NH_2-B1-BOC2(A1,B29)-insulin (MW=6,008 g/mol) of Example 8, drug conjugates were prepared with the following frameworks and affinity ligands. Tris-Succinimidyl-1,3,5-benzenetricarboxylate (TSB), tris-Succinimidyl aminotriacetate (TSAT), tris-Succinimidyl (6-aminocaproyl)aminotriacetate (TSAT-C6), and tetrakis-(N-succinimidyl carboxypropyl)pentaerythritol TSPE activated ester frameworks were purchased from Molecular Biosciences (Boulder, CO) and used without further purification. The TSB-C4 and TSB-C6 frameworks were synthesized according to Example 9. The AEM, AEBM, and AETM affinity

ligands were synthesized according to Examples 1-4. The appropriately sized size exclusion medium is Biogel P2 (Bio-Rad Laboratories, Hercules, CA), and the appropriately sized ultrafiltration membrane molecular weight cutoff is 3 kD.

In all cases, the BOC protecting groups were removed by dissolving the lyophilized powder obtained according to Example 11 in 90% TFA/10% anisole for one hour at 4 C followed by 10x superdilution in 25 mM HEPES pH 8.2 buffer containing 0.150M NaCl. The pH was adjusted to between 7.0 and 8.0 using NaOH solution after which the material is passed through a Biogel P2 column to remove anisole, BOC and other low MW byproducts of deprotection, as well as any other contaminating salts. The deprotected, purified aqueous conjugate solution was then concentrated using Amicon 3K membranes (Millipore, Billerica, MA) to approximately 58 U of insulin/ml (based on A280 measurements) and stored at 4 C until needed. Because the starting NH_2-B1-BOC2(A1,B29)-insulin material only possesses one free amine group at the Phe-B1 terminus, the Phe-B1 is the only site of insulin conjugation to the framework as verified in each deprotected final product by N-terminal sequencing.

Synthesis Conditions				Product Characterization		
Framework	**Framework MW**	**Affinity ligand**	**AE-sugar MW**	**Purity (HPLC)**	**MW (LC-MS)**	**Sugar/Insulin**
TSB	501	AEM	223	97%	6410	2.0
TSB	501	AEBM	385	94%	6734	2.0
TSB	501	AETM	547	96%	7057	2.0
TSB-C4	755	AEM	223	95%	6665	2.0
TSB-C4	755	AEBM	385	97%	6989	2.0
TSB-C4	755	AETM	547	95%	7313	2.0
TSB-C6	882	AEM	223	99%	6791	2.0
TSB-C6	882	AEBM	385	99%	7114	2.0
TSB-C6	882	AETM	547	95%	7438	2.0
TSAT	482	AEM	223	98%	6390	2.0
TSAT	482	AEBM	385	95%	6714	2.0

Synthesis Conditions				Product Characterization		
Framework	Framework MW	Affinity ligand	AE-sugar MW	Purity (HPLC)	MW (LC-MS)	Sugar/Insulin
TSAT	482	AETM	547	94%	7038	2.0
TSAT-C6	822	AEM	223	97%	6730	2.0
TSAT-C6	822	AEBM	385	99%	7054	2.0
TSAT-C6	822	AETM	547	97%	7378	2.0
TSPE	813	AEM	223	98%	6829	3.0
TSPE	813	AEBM	385	97%	7314	3.0
TSPE	813	AETM	547	94%	7802	3.0

Example 13 – B1-insulin conjugates with multivalent sugars – mixed ligands

Using the method described in Example 11 and the amine-bearing drug, NH_2-B1-BOC2(A1,B29)-Insulin (MW=6,008 g/mol) of Example 8, insulin conjugates were prepared which possessed a mixture of sugar affinity ligands connected to the framework. The TSAT-C6 and TSPE activated ester frameworks were purchased from Molecular Biosciences (Boulder, CO) and used without further purification. The AEM, AEBM, and AETM were synthesized according to Examples 1-4. The appropriately sized size exclusion medium is Biogel P2 (Bio-Rad Laboratories, Hercules, CA), and the appropriately sized ultrafiltration membrane molecular weight cutoff is 3 kD.

In all cases, the BOC protecting groups were removed by dissolving the lyophilized powder obtained according to Example 11 in 90% TFA/10% anisole for one hour at 4 C followed by 10x superdilution in 25 mM HEPES pH 8.2 buffer containing 0.150M NaCl. The pH was adjusted to between 7.0 and 8.0 using NaOH solution after which the material was passed through a Biogel P2 column to remove anisole, BOC and other low MW byproducts of deprotection, as well as any other contaminating salts. The deprotected, purified aqueous conjugate solution was then concentrated using Amicon 3K membranes (Millipore, Billerica, MA) to the desired level and stored at 4 C until needed. Because the starting NH_2-B1-BOC2(A1,B29)-insulin material only possesses one free amine group at the Phe-B1 terminus,

the Phe-B1 is the only site of insulin conjugation to the framework as verified in each deprotected final product by N-terminal sequencing.

Framework	Framework MW	Mixed Affinity ligand	AE-sugar MW	Purity (HPLC)	MW (LC-MS)	Sugar / Insulin
TSPE	813	AEM/AETM (33/67 mol/mol)	223/547	94%	7478	1.0 AEM, 2.0 AETM
TSPE	813	AEM/AETM (67/33 mol/mol)	223/547	94%	7152	2.0 AEM, 1.0 AETM
TSAT-C6	822	AEM/AEBM (50/50 mol/mol)	223/385	96%	6892	1.0 AEM, 1.0 AEBM
TSAT-C6	822	AEBM/AETM (50/50 mol/mol)	385/547	95%	7216	1.0 AEBM, 1.0 AETM

Example 14 – B1-insulin conjugates with multivalent sugars using premade multivalent sugars

Using the method described in Example 11 and the amine-bearing drug, NH_2-B1-BOC2(A1,B29)-insulin (MW=6,008 g/mol) of Example 8, the following insulin conjugates are prepared from pre-synthesized multivalent amine-containing affinity ligands. The disuccinimidyl suberate (DSS) and TSAT-C6 activated ester frameworks are purchased from Molecular Biosciences (Boulder, CO) and used without further purification. Divalent AEM-2, AEBM-2, and AETM-2 molecules containing a terminal reactive amine are prepared by conjugating two of each affinity ligand to a suitable framework to which a reactive amine is also conjugated. Trivalent AEM-3, AEBM-3, and AETM-3 molecules containing a terminal reactive amine are prepared by conjugating three of each affinity ligand to a suitable framework to which a reactive amine is also conjugated. The appropriately sized size exclusion medium is Biogel P2 (Bio-Rad Laboratories, Hercules, CA), and the appropriately sized ultrafiltration membrane molecular weight cutoff is 3 kD.

In all cases, the BOC protecting groups are removed by dissolving the lyophilized powder obtained according to Example 11 in 90% TFA/10% anisole for one hour at 4 C followed by 10x superdilution in 25 mM HEPES pH 8.2 buffer containing 0.150M NaCl. The pH is adjusted to between 7.0 and 8.0 using NaOH solution after which the material is passed through a Biogel P2 column to remove anisole, BOC and other low MW byproducts of deprotection, as well as any other contaminating salts. The deprotected, purified aqueous conjugate solution is then

concentrated using Amicon 3K membranes (Millipore, Billerica, MA) to the desired level and stored at 4 C until needed. Because the starting NH_2-B1-BOC2(A1,B29)-Insulin material only possesses one free amine group at the Phe-B1 terminus, the Phe-B1 is the only site of insulin conjugation to the framework as verified in each deprotected final product by N-terminal sequencing.

Synthesis Conditions				Expected Product Characterization	
Framework	**Framework MW**	**Affinity Ligand**	**AE-sugar MW**	**MW (LC-MS)**	**Sugar/ Insulin**
DSS	368	AEM-2	676	6621	2.0 AEM
DSS	368	AEBM-2	1000	6945	2.0 AEBM
DSS	368	AETM-2	1324	7269	2.0 AETM
DSS	368	AEM-3	1085	7031	3.0 AEM
DSS	368	AEBM-3	1571	7517	3.0 AEBM
DSS	368	AETM-3	2057	8003	3.0 AETM
TSAT-C6	822	AEM-2	676	7637	4.0 AEM
TSAT-C6	822	AEBM-2	1000	8285	4.0 AEBM
TSAT-C6	822	AETM-2	1324	8933	4.0 AETM
TSAT-C6	822	AEM-3	1085	8046	6.0 AEM
TSAT-C6	822	AEBM-3	1571	9018	6.0 AEBM
TSAT-C6	822	AETM-3	2057	9990	6.0 AETM

Example 15 – B1-insulin conjugates with multivalent sugars using dendritic framework – homogeneous ligand

0.1 gm (0.098 mmol) dendron containing an amino core and four terminal alkyne groups prepared in Example 10b is dissolved at 100 mg/ml in anhydrous DMSO. The solution is added dropwise to a solution containing disuccinimidyl suberate (DSS, Molecular Biosciences, 0.098 mmol) and triethylamine (400 uL) and allowed to react for 1 hour at room temperature. This mixture is then added dropwise to a 50 mg/ml solution containing the NH_2-B1-BOC2(A1,B29)-insulin (MW=6,008 g/mol) of Example 8 (0.588g, 0.098 mmol) and allowed to react for 2 hours.

The resulting conjugate is superdiluted in water, and the pH adjusted to 8.0. The solution is desalted using BioGel P2, followed by concentration using Amicon 3k ultrafiltration devices. The resulting solution is purified by reverse phase chromatography, rotovapped to remove acetonitrile, and lyophilized. The BOC protecting groups are removed by dissolving the lyophilized powder in 90% TFA/10% anisole for one hour at 4 C followed by 10x superdilution in 25 mM HEPES pH 8.2 buffer containing 0.150M NaCl. The pH is adjusted to between 7.0 and 8.0 using NaOH solution after which the material is passed through a Biogel P2 column to remove anisole, BOC and other low MW byproducts of deprotection, as well as any other contaminating salts. The deprotected, purified aqueous conjugate solution is then concentrated using Amicon 3K membranes (Millipore, Billerica, MA) to the desired level and stored at 4 C until needed. Because the starting NH_2-B1-BOC2(A1,B29)-insulin material only possesses one free amine group at the Phe-B1 terminus, the Phe-B1 is the only site of insulin conjugation to the framework and is verified in each deprotected final product by N-terminal sequencing.

Example 16 – Synthesis of NH_2-B29-BOC2(A1,B1)-insulin

a. Fmoc-1-(B29)-insulin

In a typical synthesis, 4 gm of powdered insulin (Sigma Aldrich, St. Louis, MO) is dissolved in 100 ml of anhydrous DMSO at room temperature followed by the addition of 4 ml of triethylamine (TEA). The solution is stirred for 30 minutes at room temperature. Next, 1.2 equivalents of 9-fluorenylmethyl *N*-succinimidyl carbonate (Fmoc-NHS) (Sigma Aldrich, St. Louis, MO) is slowly added to the insulin-TEA solution as a 1.0 M solution of the Fmoc-NHS in THF. The reaction is mixed for approximately one hour. The reaction is quenched via the addition of 4 ml of a stock solution containing 250 ul of ethanolamine in 5 ml of DMSO followed by mixing for five minutes. After quenching, the entire solution is poured into 1600 ml of acetone and mixed briefly with a spatula. Next, 8 x 400 µl aliquots of a 18.9% HCl:water solution are added dropwise over the surface of the mixture to precipitate the reacted insulin. The precipitated material is then centrifuged and the supernatant decanted into a second beaker while the precipitate cake is set aside. To the supernatant solution, another 8 x 400 µl aliquots of a 18.9% HCl:water solution are added dropwise over the surface of the mixture to obtain a second precipitate of reacted insulin. This second precipitate is centrifuged and the supernatant is discarded. The combined centrifuge cakes from the two precipitation steps are washed once with acetone followed by drying under vacuum at room temperature to yield the crude powder

which typically contains 20% of the Fmoc1 product, 65% of the Fmoc2 product, and 15% of unreacted insulin.

A preparative reverse phase HPLC method is used to isolate the pure desired Fmoc1-insulin from the crude powder. Buffer A is deionized water containing 0.1% TFA and Buffer B is acetonitrile containing 0.1% TFA. The crude powder is dissolved at 25 mg/ml in a 70%A/30%B mixture and syringe filtered prior to injection on the column. Before purification, the column (Waters SymmetryPrep C18, 7 um, 19 x 150 mm) is equilibrated at 15 ml/minutes with a 70%A/30%B mobile phase using a Waters DeltraPrep 600 system. Approximately 5 ml of the crude powder solution is injected onto the column at a flow rate of 15 ml/minutes over the course of 5 minutes after which a linear gradient is employed from 70%A/30%B to 62%A/38%B over the course of the next 3.5 minutes and held there for an additional 2.5 minutes. Using this method, the desired Fmoc1 peak elutes at approximately 3 minutes after the unreacted RHI peak, followed closely by the Fmoc2-insulin peak. Once collected, the solution is rotovapped to remove acetonitrile and lyophilized to obtain pure Fmoc1-insulin powder. Identity is verified by LC-MS (HT Laboratories, San Diego, CA) and site of conjugation determined by N-terminal sequencing (Western Analytical, St. Louis, MO).

b. BOC2(A1,B1)-Fmoc-(B29)-insulin

In a typical synthesis, 1 g of Fmoc1-(B29)-insulin is dissolved in 25 ml of anhydrous DMSO at room temperature followed by the addition of 1 ml of triethylamine (TEA). The solution is stirred for 30 minutes at room temperature. Next, 0.379 ml (2.2 equivalents) of di-tert-butyl-dicarbonate/THF solution (Sigma Aldrich, St. Louis, MO) is slowly added to the insulin-TEA solution and mixed for approximately one hour. The reaction is quenched via the addition of 1 ml of a stock solution containing 250 ul of ethanolamine in 5 ml of DMSO followed by mixing for five minutes. After quenching, the entire solution is poured into 400 ml of acetone and mixed briefly with a spatula. Next, 8 x 100 µl aliquots of a 18.9% HCl:water solution are added dropwise over the surface of the mixture to precipitate the reacted insulin. The precipitated material is then centrifuged and the supernatant decanted into a second beaker while the precipitate cake is set aside. To the supernatant solution, another 8 x 100 µl aliquots of a 18.9% HCl:water solution are added dropwise over the surface of the mixture to obtain a second precipitate of reacted insulin. This second precipitate is centrifuged and the supernatant is discarded. The combined centrifuge cakes from the two precipitation steps are washed once

with acetone followed by drying under vacuum at room temperature to yield the crude powder which typically contains greater than 90% of the desired BOC2-Fmoc-1 product.

A preparative reverse phase HPLC method is used to isolate the pure BOC2-Fmoc-1-insulin from the crude powder. Buffer A is deionized water containing 0.1% TFA and Buffer B is acetonitrile containing 0.1% TFA. The crude powder is dissolved at 25 mg/ml in a 70%A/30%B mixture and syringe filtered prior to injection on the column. Before purification, the column (Waters SymmetryPrep C18, 7 um, 19 x 150 mm) is equilibrated at 15 ml/minutes with a 70%A/30%B mobile phase using a Waters DeltraPrep 600 system. Approximately 5 ml of the crude powder solution is injected onto the column at a flow rate of 15 ml/minutes over the course of 5 minutes after which a linear gradient is employed from 70%A/30%B to 62%A/38%B over the course of the next 3.5 minutes and held there for an additional 2.5 minutes. Using this method, the desired BOC2-Fmoc-1 peak elutes at approximately 5 minutes after the Fmoc1-insulin starting material. Once collected, the solution is rotovapped to remove acetonitrile and lyophilized to obtain pure BOC2(A1,B1)-Fmoc(B29)-insulin powder. Identity is verified by LC-MS (HT Laboratories, San Diego, CA) and site of conjugation determined by N-terminal sequencing (Western Analytical, St. Louis, MO).

c. NH_2-(B29)-BOC2(A1,B1)-insulin

The Fmoc protecting group of the BOC2(A1,B1)-Fmoc(B29) is removed by dissolving the lyophilized powder obtained according to the previous step in 20% piperidine in dimethylformamide (DMF) for 30 minutes at 4 C followed by 10x superdilution in 25 mM HEPES pH 8.2 buffer containing 0.150M NaCl. The pH is adjusted to between 7.0 and 8.0 using NaOH solution after which the material is passed through a Biogel P2 column to remove Fmoc, DMF, and any other contaminating salts. The NH_2-(B29)-BOC2(A1,B1)-insulin is lyophilized into a powder if needed or used directly in aqueous solution if desired.

Example 17 – Synthesis of NH_2-B29-BOC2(A1,B1)-insulin conjugates

All of the multivalent-affinity ligand-drug conjugates described in previous examples using the NH_2-B1-BOC2(A1,B29)-insulin of Example 8 may be prepared instead using the NH_2-B29-BOC2(A1,B1)-insulin of Example 16. All of the resulting conjugates will possess the same MW and degree of substitution characteristics, but the site of conjugation to the insulin molecule

will be at the epsilon B29 amino group and not the N-terminal Phe-B1. This can be confirmed by N-terminal sequencing.

Example 18 – Amine-functionalized drug conjugation with multivalent activated esters in organic solvent (drug added last)

This example describes an alternative to the method described in Example 11 in which the drug is added to the framework before the affinity ligand(s). In this example the affinity ligand(s) are added to the framework before the drug.

A framework containing N terminal activated esters is dissolved at 60 mM in 1 ml of anhydrous DMSO followed by the addition of 400 ul (excess) of triethylamine (TEA). The solution is stirred rapidly for 10 minutes at room temperature. In parallel, a 122 mM solution of affinity ligand is prepared in an appropriate volume of anhydrous DMSO. Once dissolved, enough affinity ligand solution is added dropwise over the course of ten minutes to provide a number of reactive equivalents equal to exactly the number of activated ester groups on the framework, N, minus one. For example, if there are N=3 activated ester groups on the framework, then (1x(3-1)x60mM/122 mM)=0.98 ml of affinity ligand solution are added. If there are N=4 activated ester groups on the framework, then (1x(4-1)x60mM/122 mM)=1.5 ml of affinity ligand solution are added, and so on. After the affinity ligand solution is added, the solution is stirred for two hours at room temperature.

The amine-bearing drug is then dissolved separately in 7.5 ml of anhydrous DMSO at a concentration of 8.1 mM. Once dissolved, the entire drug solution is added over the course of one minute to the framework/DMSO/affinity ligand/TEA solution followed by room temperature mixing for an additional two hours to ensure complete reaction.

The resulting solution is then superdiluted by 10x into a 20 mM pH 5.0 HEPES buffered saline solution containing 0.150 M NaCl followed by pH adjustment with dilute HCl to a final pH of 8.0. The aqueous solution is first purified by size exclusion using an appropriate solid phase for the desired separation of conjugated and unconjugated materials. The solution passing through the column void volume is then concentrated using an appropriately sized ultrafiltration membrane to approximately 10 ml. This solution is further purified to obtain the desired product using preparative reverse phase HPLC on a Waters SymmetryPrep C18, 7 um column, 19 x 150 mm. Buffer A is deionized water containing 0.1% TFA and Buffer B is acetonitrile containing 0.1% TFA. Before purification, the column is equilibrated at 15 ml/minutes with a 80%A/20%B mobile phase using a Waters DeltraPrep 600 sytem. Approximately 5 ml of the crude solution is

injected onto the column over the course of 2 minutes at a flow rate of 15 ml/minutes after which a linear gradient is employed from 80%A/20%B to 75%A/25%B over the next 5 minutes followed by a slower linear gradient from 75%A/25%B to 62%A/38%B over the next 22 minutes. The retention time of the desired peak will vary depending on the drug, framework, and affinity ligand used. Once collected, the solution is rotovapped to remove acetonitrile and lyophilized to obtain pure conjugate whose identity may be verified by LC-MS (HT Laboratories, San Diego, CA).

Example 19 – B29-insulin conjugates with multivalent sugars produced in organic solvent from unprotected insulin

This example makes use of the fact that in the unprotected insulin case, the Lys-B29 epsilon-amino moiety is the most reactive amine, followed by the A1 and then the B1. Therefore, when unprotected insulin is used as the amine-containing drug the resulting conjugate should be predominantly substituted at the Lys-B29 position. Using the method described in Example 18 and recombinant human insulin (MW=5808 Da, Sigma Aldrich, St. Louis, MO) as the amine-containing drug, the following insulin conjugates were prepared using the TSAT-C6 activated ester framework purchased from Molecular Biosciences (Boulder, CO). The AEM and AETM were synthesized as described previously. The appropriately sized size exclusion medium was Biogel P2 (Bio-Rad Laboratories, Hercules, CA), and the appropriately sized ultrafiltration membrane molecular weight cutoff was 3kDa.

Synthesis Conditions				**Product Characterization**		
Framework	**Framework MW**	**Affinity ligand**	**AE-sugar MW**	**Purity (HPLC)**	**MW (LC-MS)**	**Sugar/ Insulin**
TSAT-C6	822	AEM	223	85%	6729	2.0
TSAT-C6	822	AETM	547	85%	7378	2.0

According to N-terminal sequencing, approximately 85 % of the AEM-containing framework was conjugated to insulin via the Lys-B29 and approximately 87 % of the AETM-containing framework was conjugated to insulin via the Lys-B29.

Example 20 – Amine-functionalized drug conjugation with multivalent activated esters in aqueous solvent (drug added last)

This example describes an alternative to the method described in Example 18 in which the reaction is performed in aqueous solvent instead of organic solvent.

The framework containing N terminal activated esters is dissolved at 60 mM in 6.25 ml of anhydrous DMSO followed by the addition of 2 ml (excess) of triethylamine (TEA). The solution is stirred rapidly for 10 minutes at room temperature. In parallel, a 448 mM solution of affinity ligand is prepared in an appropriate volume of anhydrous DMSO. Once dissolved, enough affinity ligand solution is added dropwise over the course of ten minutes to provide a number of reactive equivalents equal to 1.5 times the number of activated ester groups on the framework, N, minus one. For example, if there are N=3 activated ester groups on the framework, then (1.5x(3-1)x60mM/448 mM)x6.25ml = 2.5 ml of affinity ligand solution are added. If there are N=4 activated ester groups on the framework, then (1.5x(4-1)x60mM/448 mM)x6.25ml = 3.8 ml of affinity ligand solution are added, and so on. After the affinity ligand solution is added, the solution is stirred for one hour at room temperature.

The amine-bearing drug is then dissolved separately at 17.2 mM in 2.67 ml of a 0.1M, pH 11 sodium carbonate buffer and the pH subsequently adjusted to 10.8 with 1.0N sodium hydroxide. Once dissolved, the entire framework/DMSO/affinity ligand/TEA solution is added dropwise over the course of 75 minutes to the drug/carbonate buffer solution. During the addition, the pH of the resulting mixture is adjusted every 5 minutes to 10.8 if necessary using dilute HCl or NaOH. The solution is allowed to stir for an additional 15 minutes after the dropwise addition to ensure complete reaction.

The resulting solution is then superdiluted by 10x into a 20 mM pH 5.0 HEPES buffered saline solution containing 0.150 M NaCl followed by pH adjustment with dilute HCl to a final pH of 8.0. The aqueous solution is first purified by size exclusion using an appropriate solid phase for the desired separation of conjugated and unconjugated materials. The solution passing through the column void volume is then concentrated using an appropriately sized ultrafiltration membrane to approximately 40 ml. This solution is further purified to obtain the desired product using preparative reverse phase HPLC on a Waters SymmetryPrep C18, 7 um, 19 x 150 mm column. Buffer A is deionized water containing 0.1% TFA and Buffer B is acetonitrile containing 0.1% TFA. Before purification, the column is equilibrated at 15 ml/minutes with a 80%A/20%B mobile phase using a Waters DeltraPrep 600 sytem. Approximately 5 ml of the

crude solution is injected onto the column over the course of 2 minutes at a flow rate of 15 ml/minutes after which a linear gradient is employed from 80%A/20%B to 75%A/25%B over the next 5 minutes followed by a slower linear gradient from 75%A/25%B to 62%A/38%B over the next 22 minutes. The retention time of the desired peak will vary depending on the drug, framework, and affinity ligand used. Once collected, the solution is rotovapped to remove acetonitrile and lyophilized to obtain pure conjugate whose identity may be verified by LC-MS (HT Laboratories, San Diego, CA).

Example 21 – B29-AEM-2-insulin conjugate synthesized in aqueous solvent from unprotected insulin

This example makes use of the fact that in the unprotected insulin case, the Lys-B29 epsilon-amino moiety is the most reactive amine, followed by the A1 and then the B1. Therefore, when unprotected insulin is used as the amine-containing drug the resulting conjugate should be predominantly substituted at the Lys-B29 position. Using the method described in Example 20 and recombinant human insulin (MW = 5808, Sigma Aldrich, St. Louis, MO) as the amine-containing drug, an AEM-2 insulin conjugate was prepared using the TSAT-C6 activated ester framework purchased from Molecular Biosciences (Boulder, CO). The AEM used as the insulin analog was synthesized as described previously. The appropriately sized size exclusion medium was Biogel P2 (Bio-Rad Laboratories, Hercules, CA), and the appropriately sized ultrafiltration membrane molecular weight cutoff was 3 kD. The final product (95% pure by HPLC) was found to have the desired MW of 6729 g/mol (LC-MS), representing a total of 2.0 AEM molecules conjugated per insulin, with greater than 85% of the conjugate molecules conjugated at the Lys-B29 site (N-terminal sequencing).

Example 22 – Generalized amine-functionalized drug conjugation with aldehyde-containing framework

a. Framework functionalized with more than one affinity ligand and one terminal aldehyde

First, a framework containing N terminal activated esters is dissolved at 60 mM in 27.0 ml of anhydrous DMSO followed by the addition of 800 ul (excess) of triethylamine (TEA). The solution is stirred rapidly for 10 minutes at room temperature. A stock solution of amine-bearing diethyl acetal is prepared at 580 mM in 5 ml of anhydrous DMSO. Once dissolved, 2.9 ml of the diethyl acetal solution are added dropwise over the course of 5 minutes to the framework/DMSO/TEA solution followed by room temperature mixing for an additional 15 minutes. The remaining activated esters are then reacted with amine-functionalized affinity ligands in the following manner. A 370 mM solution of affinity ligand is prepared in an appropriate volume of dry DMSO. Once dissolved, enough solution is added to provide a number of reactive equivalents equal to 1.5 times the number of initial activated ester groups, N, minus one. For example, if there are N=3 initial activated ester groups per framework, then (1.5x(3-1)x60mMx27/370 mM)= 13 ml of affinity ligand solution are added. If there are N=4

initial activated ester groups per framework, then (1.5x(4-1)x60mMx27/370 mM)= 20 ml of affinity ligand solution are added, and so on. After the affinity ligand solution is added, the solution is stirred for an additional hour and 45 minutes at room temperature to ensure complete reaction. After reaction, the entire solution is diluted by a factor of ten with diethyl ether, mixed vigorously, and centrifuged to separate the dense bottom phase containing the desired material from the supernatant. After discarding the supernatant, the same volume of ethanol is added to generate a solid precipitated mass. After centrifuging and discarding the supernatant, the material is washed extensively with ethanol and ether and then dried under vacuum to yield the crude framework containing multiple affinity ligands and a diethyl acetal group.

b. Conjugation of amine-functionalized drug with terminal aldehyde

Once dried, the aldehyde group is generated from the diethyl acetal by dissolving the collected material in 60 ml of DI water with the solution pH adjusted to 1.0. The solution is mixed for 30 minutes after which 6 ml of a 200 mM HEPES pH 8.2 buffer containing 1.5 M NaCl is added and the solution pH adjusted to 6.5 using dilute NaOH solution. 48 mmol of the amine containing drug are added to the solution and the pH readjusted to 6.5 if necessary. Separately, a stock solution of reducing agent is prepared by dissolving 1.5 g of sodium cyanoborohydride (Sigma Aldrich, St. Louis, MO) in 15 ml of a 20 mM HEPES pH 7.0 buffer containing 0.150 M NaCl and the pH carefully adjusted to 6.5 with dilute HCl solution. 13 ml of the cyanoborohydride stock solution are added to the drug/framework/aldehyde solution and allowed to react overnight at room temperature.

The resulting aqueous solution is first purified by size exclusion using an appropriate solid phase for the desired separation of conjugated and unconjugated materials. The solution passing through the column void volume is then concentrated using an appropriately sized ultrafiltration membrane to approximately 10 ml. This solution is further purified to obtain the desired product using preparative reverse phase HPLC on a Waters SymmetryPrep C18, 7 um column, 19 x 150 mm. Buffer A is deionized water containing 0.1% TFA and Buffer B is acetonitrile containing 0.l% TFA. Before purification, the column is equilibrated at 15 ml/minutes with a 80%A/20%B mobile phase using a Waters DeltraPrep 600 sytem. Approximately 5 ml of the crude solution is injected onto the column over the course of 2 minutes at a flow rate of 15 ml/minutes after which a linear gradient is employed from 80%A/20%B to 75%A/25%B over the next 5 minutes followed by a slower linear gradient from

75%A/25%B to 62%A/38%B over the next 22 minutes. The retention time of the desired peak will vary depending on the drug, framework, and affinity ligand used. Once collected, the solution is rotovapped to remove acetonitrile and lyophilized to obtain pure conjugate whose identity may be verified by LC-MS (HT Laboratories, San Diego, CA).

Example 23 – AEM-2-framework containing a terminal reactive aldehyde group and subsequent insulin conjugation at B1

a. TSAT functionalized with 2 AEM and 1 aminobutyraldehyde diethyl acetal (ABDA)

This material is synthesized according to the method described in Example 22a using TSAT (Molecular Biosciences, Boulder, CO) as the multivalent activated ester framework and 4-aminobutyraldehyde diethyl acetal (Sigma Aldrich, St. Louis, MO) as the amine-bearing diethyl acetal. AEM (MW = 223 g/mol), synthesized as described previously was used as the affinity ligand.

b. Conjugation of TSAT-AEM-2-ABDA with NH_2-B1-BOC2(A1,B29)-insulin

This material was synthesized using the method described in Example 22b and the TSAT-AEM-2-ABDA produced in (a) above along with the amine-bearing drug, NH_2-B1-BOC2(A1,B29)-insulin (MW = 6,008 g/mol), synthesized according to Example 8. The appropriately sized size exclusion medium is Biogel P2 (Bio-Rad Laboratories, Hercules, CA), and the appropriately sized ultrafiltration membrane molecular weight cutoff is 3 kD. Because the starting NH_2-B1-BOC2(A1,B29)-insulin material only possesses one free amine group at the Phe-B1 terminus, the Phe-B1 is the only site of insulin conjugation to the framework. The BOC protecting groups are removed by dissolving the lyophilized powder in 90% TFA/10% anisole for one hour at 4 C followed by 10x superdilution in 25 mM HEPES pH 8.2 buffer containing 0.150M NaCl. The pH is adjusted to between 7.0 and 8.0 using NaOH solution after which the material is passed through a Biogel P2 column to remove anisole, BOC and other low MW byproducts of deprotection, as well as any other contaminating salts. The deprotected, purified aqueous conjugate solution is then concentrated using Amicon 3K membranes (Millipore, Billerica, MA) to the desired level and stored at 4 C until needed.

The final product (95% pure by HPLC) was found to have the desired MW of 6462 g/mol (LC-MS), representing a total of 2.0 AEM molecules conjugated per insulin, 99% of which were conjugated at the Phe-B1 site (N-terminal sequencing).

Example 24 – AEM-3-framework containing a terminal reactive aldehyde group and subsequent insulin conjugation at B1

a. TSPE functionalized with 3 AEM and 1 aminobutyraldehyde diethyl acetal (ABDA)

This material is synthesized according to the method described in Example 22a using TSPE (Molecular Biosciences, Boulder, CO) as the multivalent activated ester framework and 4-aminobutyraldehyde diethyl acetal (Sigma Aldrich, St. Louis, MO) as the amine-bearing diethyl acetal. AEM (MW = 223 g/mol), synthesized as described previously, was used as the affinity ligand.

b. Conjugation of TSPE-AEM-3-ABDA with NH_2-B1-BOC2(A1,B29)-Insulin

This material was synthesized using the method described in Example 22b and the TSPE-AEM-3-ABDA produced in (a) above along with the amine-bearing drug, NH_2-B1-BOC2(A1,B29)-insulin (MW = 6,008 g/mol), synthesized according to Example 8. The appropriately sized size exclusion medium is Biogel P2 (Bio-Rad Laboratories, Hercules, CA), and the appropriately sized ultrafiltration membrane molecular weight cutoff is 3 kD. Because the starting NH_2-B1-BOC2(A1,B29)-insulin material only possesses one free amine group at the Phe-B1 terminus, the Phe-B1 is the only site of insulin conjugation to the framework. The BOC protecting groups are removed by dissolving the lyophilized powder in 90% TFA/10% anisole for one hour at 4 C followed by 10x superdilution in 25 mM HEPES pH 8.2 buffer containing 0.150M NaCl. The pH is adjusted to between 7.0 and 8.0 using NaOH solution after which the material is passed through a Biogel P2 column to remove anisole, BOC and other low MW byproducts of deprotection, as well as any other contaminating salts. The deprotected, purified aqueous conjugate solution is then concentrated using Amicon 3K membranes (Millipore, Billerica, MA) to the desired level and stored at 4 C until needed.

The final product (95% pure by HPLC) was found to have the desired MW of 6897 g/mol (LC-MS), representing a total of 3.0 AEM molecules conjugated per insulin, 99% of which were conjugated at the Phe-B1 site (N-terminal sequencing).

Example 25 – AEM-3-scaffold containing a terminal reactive aldehyde group and subsequent insulin conjugation at B1 using unprotected insulin

a. TSPE functionalized with 3 AEM and 1 aminobutyraldehyde diethyl acetal (ABDA)

This material is synthesized according to the method described in Example 22a using TSPE (Molecular Biosciences, Boulder, CO) as the multivalent activated ester scaffold and 4-aminobutyraldehyde diethyl acetal (Sigma Aldrich, St. Louis, MO) as the amine-bearing diethyl acetal. AEM (MW = 223 g/mol), synthesized as described previously, was used as the indicator analog.

b. Conjugation of TSPE-AEM-3-ABDA with NH_2-B1-BOC2(A1,B29)-Insulin

This material was synthesized using the method described in Example 22b and the TSPE-AEM-3-ABDA produced in (a) above along with the amine-bearing drug, unmodified insulin (MW = 5,808 g/mol, Sigma-Aldrich, St. Louis, MO). The appropriately sized size exclusion medium is Biogel P2 (Bio-Rad Laboratories, Hercules, CA), and the appropriately sized ultrafiltration membrane molecular weight cutoff is 3 kD. Although the starting unprotected insulin material possesses three free amine groups, the Phe-B1 is the predominant site of insulin conjugation to the scaffold due to the fact that the Phe-B1 (pKa ~ 6.8) is the most reactive amine at pH 6.5. The lyophilized powder is dissolved in 25 mM HEPES pH 8.2 buffer containing 0.150M NaCl. The pH is adjusted to between 7.0 and 8.0 using NaOH solution after which the material is then concentrated using Amicon 3K membranes (Millipore, Billerica, MA) to the desired level and stored at 4 C until needed.

The final product (95% pure by HPLC) was found to have the desired MW of 6897 g/mol (LC-MS), representing a total of 3.0 AEM molecules conjugated per insulin, >85% of which were conjugated at the Phe-B1 site (N-terminal sequencing).

Example 26 – Mixed framework chemistry and corresponding separate conjugation of drug and affinity ligands

Succinimidyl-3,5-dimaleimidophenyl benzoate (SDMB) can be purchased from Molecular Biosciences (Boulder, CO) and used in the following example without further purification. SDMB is dissolved at 60 mM in 1.0 ml of anhydrous DMSO followed by the addition of 400 ul (excess) of triethylamine (TEA). The solution is stirred rapidly for 10 minutes at room temperature. The amine-bearing drug is then dissolved separately in 7.5 ml of anhydrous DMSO at a concentration of 8.1 mM. Once dissolved, the entire SDMB solution is added dropwise over the course of ten minutes to the DMSO-drug solution followed by room temperature mixing for an additional two hours to ensure complete reaction.

The resulting solution is then superdiluted by 10x into a 20 mM pH 5.0 HEPES buffered saline solution containing 0.150 M NaCl followed by pH adjustment with dilute HCl to a final pH of 8.0. The aqueous solution is first purified by size exclusion using an appropriate solid phase for the desired separation of conjugated and unconjugated materials. The solution passing through the column void volume is then concentrated using an appropriately sized ultrafiltration membrane to approximately 10 ml.

Separately, 6.0 mmol of an amine-containing affinity ligand is dissolved in a 20 mM pH 8.2 HEPES buffered saline solution containing 0.150 M NaCl at a concentration of 450 mM. To this solution, 6.6 mmol of iminothiolane (Sigma-Aldrich, St. Louis, MO) is added and allowed to react at pH 8.2 for 30 minutes at room temperature to convert the amine-terminal groups to terminal sulfhydryl groups. The resulting material is mixed with the 10 ml solution of drug-framework-di-maleimide conjugate produced in the previous step. The maleimide groups are allowed to react with the indicator-anolog sulfydryl groups at pH 8.2 for 2 hours to ensure complete reaction. The resulting solution is then purified by size exclusion using an appropriate solid phase for the desired separation of conjugated and unconjugated materials. The solution passing through the column void volume is then concentrated using an appropriately sized ultrafiltration membrane to approximately 10 ml.

Finally, this solution is further purified to obtain the desired product using preparative reverse phase HPLC on a Waters SymmetryPrep C18, 7 um column, 19 x 150 mm. Buffer A is deionized water containing 0.1% TFA and Buffer B is acetonitrile containing 0.1% TFA. Before purification, the column is equilibrated at 15 ml/minutes with a 80%A/20%B mobile phase using a Waters DeltraPrep 600 sytem. Approximately 5 ml of the crude solution is injected onto the column over the course of 2 minutes at a flow rate of 15 ml/minutes after which a linear gradient is employed from 80%A/20%B to 75%A/25%B over the next 5 minutes followed by a slower linear gradient from 75%A/25%B to 62%A/38%B over the next 22 minutes. The retention time of the desired peak will vary depending on the drug, framework, and affinity ligand used. Once collected, the solution is rotovapped to remove acetonitrile and lyophilized to obtain pure conjugate whose identity may be verified by LC-MS (HT Laboratories, San Diego, CA).

Example 27 – Insulin-conjugated to aminoethylsugars using mixed framework chemistry

Using the method described in Example 26 and the amine-bearing drug, NH_2-B1-BOC2(A1,B29)-insulin (MW = 6,008 g/mol), synthesized according to Example 8, the

following specific drug conjugates are obtained. AEM (MW = 223 g/mol), AEBM (MW = 385 g/mol), and AETM (MW = 547 g/mol) were synthesized as previously described and used as the affinity ligands in the synthesis. The appropriately sized size exclusion medium is Biogel P2 (Bio-Rad Laboratories, Hercules, CA), and the appropriately sized ultrafiltration membrane molecular weight cutoff is 3 kD.

In all cases, the BOC protecting groups are removed by dissolving the lyophilized powder obtained according to Example 26 in 90% TFA/10% anisole for one hour at 4 C followed by 10x superdilution in 25 mM HEPES pH 8.2 buffer containing 0.150M NaCl. The pH is adjusted to between 7.0 and 8.0 using NaOH solution after which the material is passed through a Biogel P2 column to remove anisole, BOC and other low MW byproducts of deprotection, as well as any other contaminating salts. The deprotected, purified aqueous conjugate solution is then concentrated using Amicon 3K membranes (Millipore, Billerica, MA) to approximately 58 U of insulin/ml (based on A280 measurements) and stored at 4 C until needed. Because the starting NH_2-B1-BOC2(A1,B29)-insulin material only possesses one free amine group at the Phe-B1 terminus, the Phe-B1 will be the only site of insulin conjugation to the framework. This can be verified in each deprotected final product by N-terminal sequencing.

Synthesis Conditions			**Expected Product Characterization**	
Affinity Ligand	**AE-sugar MW**	**AE-iminothiolane intermediate MW**	**MW (LC-MS)**	**Sugar/ Insulin**
AEM	223	360	6822	2.0 AEM
AEBM	385	522	7146	2.0 AEBM
AETM	547	684	7470	2.0 AETM

Example 28 – Generalized click chemistry for drug conjugation with complementary frameworks

A framework (8.3 mmol) containing at least one amino functionality and one or more terminal alkyne groups is taken into THF (40 mL), water (40 mL) and stirred into solution. An azidoethyl group-bearing drug (10.51 mmole) is added followed by copper sulfate (500 mg, 2.0

mmole) and sodium ascorbate (400 mg, 2.0 mmole). The resulting mixture is stirred at 55-60 C (oil bath) for 6 hours, cooled to room temperature, stirred overnight and concentrated under vacuum to one half volume and filtered thru a micro-glass filter. The filtrate is loaded on a resin column (Dowex 50w 50x4-100) and eluted with water (6x 75 mL) until neutral. The column is then eluted with 15% ammonium hydroxide (10x75 mL) and the fractions positive to ninhydrin are pooled and concentrated to a glassy foam.

Example 29 – Conjugates prepared using natural insulins from other species such as bovine and porcine

Insulins from other species which contain at least one reactive amine functionality (e.g., bovine and porcine insulin) may be coupled using any of the methods used to conjugate recombinant human insulin. Those skilled in the art will appreciate that the molecular weights of the resulting conjugates made from bovine or porcine insulins will differ from those made from recombinant human insulin by the amounts listed in the following table.

Type of Insulin	Molecular Weight (g/mol)	Difference in MW human insulin (g/mol)
Human insulin	5808	-
Porcine insulin	5778	-30
Bovine insulin	5733	-75

Those skilled in the art will also appreciate that the resulting conjugates made from bovine or porcine insulin may have chromatographic peak retention times that differ slightly from those conjugates made from human insulin, due to the small differences in structures between the insulins.

Example 30 – Conjugates prepared with insulin analogs such as Lispro, Aspart, Glulysine, Glargine, and Detemir

All known insulin analogs which contain at least one reactive amine functionality (e.g., Lispro, Aspart, Glulisine, Glargine, and Detemir) may be coupled using any of the methods used to conjugate recombinant human insulin. Those skilled in the art will appreciate that the

molecular weights of the resulting conjugates made from insulin analogs will differ from those made from recombinant human insulin by the amounts listed in the following table.

Type of Insulin	Molecular Weight (g/mol)	Difference in MW human insulin (g/mol)
Human insulin	5808	-
Insulin lispro	5808	-
Insulin aspart	5832	+24
Insulin glulisine	5823	+15
Insulin glargine	6063	+255
Insulin detemir	5913	+105

Those skilled in the art will also appreciate that the resulting conjugates made from insulin analogs may have chromatographic peak retention times that differ slightly from those conjugates made from human insulin, due to the small differences in structures between the insulins.

The use of insulin glulisine (which does not contain a B29 lysine, but rather a B3 lysine) will give predominantly B3 conjugates when using unprotected insulin glulisine. However, if B1-insulin glulisine conjugates are desired, then BOC-(A1,B3)-insulin glulisine is first synthesized using the same protocol as BOC-(A1,B29)-human insulin as described in Example 8.

Example 31 – Conjugates prepared with peptidic insulin secretagogue conjugates

Peptidic insulin secretagogues (e.g., without limitation GLP-1 or the GLP-1 analog exanitide) which contain an N-terminal amine functionality may be coupled using any of the methods used to conjugate insulin.

II. In vitro assays of exemplary conjugates

This second set of examples describes various experiments investigating the *in vitro* properties of some exemplary conjugates.

Example 32 – Synthesis of insulin-glycogen conjugates

This comparative example describes the synthesis of an insulin-glycogen conjugate according to U.S. Patent Application Publication No. 20070099820. Briefly, 1 gm of commercially available, unpurified oyster glycogen (Type II, Sigma-Aldrich, St. Louis, MO) is dissolved in deionized water at a concentration of 10 mg/ml. Solid CNBr is added to the resulting solution at a CNBr to glycogen mass ratio of 0.68 and the pH maintained constant at 10.7 +/- 0.2 using 3N sodium hydroxide (NaOH) solution. After stirring for 15 minutes, another equal mass of solid CNBr equal is added and the pH maintained constant at 10.7 +/- 0.2 while stirring for 45 minutes. Insulin is then added to the solution at an insulin to glycogen mass ratio of 0.60 and the pH adjusted to 9.15 using solid sodium bicarbonate. The solution is stirred overnight, ultrafiltered exhaustively against deionized water using a 50 kDa MWCO polyethersulfone disc membrane filter (Millipore, Bedford, MA), and lyophilized. The resulting powder is then purified from unconjugated insulin by gel filtration HPLC (Waters, Milford, MA) using a 1 M acetic acid mobile phase over a Superdex^{TM} 30 HiLoad 16/60 (Amersham Biosciences, Piscataway, NJ) packed column. The insulin glycogen fraction is then lyophilized to obtain the conjugate as a pure white powder. The resulting purified material contained 1.0 wt% of insulin per insulin-glycogen conjugate as measured using amino acid analysis (UCLA Biopolymers Laboratory, Los Angeles, CA).

Example 33 – Liquid chromatography analysis

This example describes the differences between the RP-HPLC profiles of insulin-glycogen synthesized according to Example 32 and an exemplary conjugate synthesized according to the present invention. 100 ul of a 5 mg/ml solution of insulin-glycogen synthesized according to Example 32 and 100 ul of a 1 mg/ml solution of exemplary conjugate were injected separately onto a Waters Symmetry C8 5um column (4.6 mm x 250 mm), equilibrated with a 80% Water/20% Acetonitrile (CH3CN) mobile phase (each containing 0.1% TFA). The exemplary conjugate used in this study was synthesized using TSAT-C6 as the framework, AEM as the affinity ligand, and NH_2-B1-BOC2(A1,B29)-insulin as the drug.

The samples were eluted at 1.0 ml/minutes using the following gradient method: 0-5 minutes – constant 80% Water/20% CH3CN, 5-35 minutes – linear gradient to 50% Water/50% CH3CN. The elution profiles in Figure 1 show a single spike for the exemplary conjugate indicating a single chemically distinct species as compared to a broad and heterogenous elution

profile for the insulin-glycogen conjugate, indicating a broad distribution of different chemical and/or molecular weight entitites.

Example 34 – Molecular weight distribution analysis

This example describes the difference in MW and MW distribution between the insulin-glycogen synthesized according to Example 32 and the same exemplary conjugate. The MW and MW distribution of the insulin-glycogen conjugate was determined by injecting 1 ml of a 25 mg/ml solution in pH 7 HEPES buffered saline onto an Ultrahydrogel Size Exclusion Column (Waters Corporation, Millford, MA) equilibrated with HEPES buffered saline. The column was eluted over the course of 30 minutes at 0.5 ml per min, and the elution profile was measured as an absorbance at 280 nm. In separate experiments using the same protocol, dextran MW standards of 1000, 5000, 12000, 25000, 50000, 80000, 150000, 270000, and 410000 g/mol (Sigma-Aldrich, St. Louis, MO) were injected to establish a calibration curve of MW versus retention time. Based on the calibration curve and the elution profile of the insulin-glycogen conjugate, the average MW was determined to be 500,000 g/mol with 67% of the distribution eluting over the broad range of 250,000 to 1,000,000 g/mol (data not shown). In contrast, the exemplary conjugate was determined to have just a single MW of exactly 6,730 g/mol as determined by LC/MS (HT Laboratories, San Diego, CA) (data not shown).

Example 35 – Chemical and physical stability of conjugates

This example compares the stability of an exemplary conjugate with that of unconjugated insulin under accelerated conditions according to the method described in Hinds et al. (*Bioconj. Chem.* 11:195-201, 2000) at 37 C and a mechanical agitation rate of 150 strokes/min. Pharmaceutical grade recombinant human insulin (RHI) was selected as the control for the accelerated stability study. Holcombe et al. (*Diabetes Care* 27:1241-1242, 2004) describes that under non-accelerated conditions RHI stability is maintained for at least 30 days at room temperature (RT) and considerably longer when refrigerated. Figure 2 shows the results from the aggregation stability assay for RHI and two exemplary conjugates in pH 7.4 phosphate buffered saline (PBS) at 50 U/ml. In all cases, the % remaining in solution was determined by centrifuging (4500xg, 5 min) the solution at a given time point, measuring the A280 of the supernatant, and dividing the supernatant A280 by that of the original starting solution. Conjugate 1 was synthesized using TSAT-C6 as the framework, AEM as the affinity ligand, and

NH_2-B1-BOC2(A1,B29)-insulin as the drug. Conjugate 2 was synthesized using TSPE as the framework, AEM as the affinity ligand, and NH_2-B1-BOC2(A1,B29)-insulin as the drug.

After 48 hours of continuous agitation at 37 C, less than 6% of the RHI remained stable in solution, while the majority of the RHI precipitated out as insoluble aggregates. After the same amount of time, the both conjugates remained substantially more stable, as 96%- 99% of the IPC's remained intact and soluble in the PBS solution. The data conclusively show that the conjugates are significantly more stable than RHI under these conditions.

RP-HPLC was used to assess the chemical stability of the conjugates (see Figure 3a). After 48 hours of accelerated stability the conjugate solutions were analyzed using a C8-reverse phase column using a water-acetonitrile elution gradient. The retention times of the pre- and post-stability conjugate samples are shown along with the percentage of unconjugated (free) insulin and desamido insulin found in the resulting LC traces. No detectable amounts of free insulin or desamido were observed, indicating that (i) the covalent linkage between the sugars and the insulin molecule is stable, and (ii) no significant chemical degradation of the conjugate occurs during the accelerated stability test (AST). Prior to and in parallel with the AST, the conjugate was also subjected to a 90-day non-accelerated stability test that included daily thermal cycling between 4°C and RT. At the conclusion of the parallel study, RP-HPLC demonstrated that the conjugate was still chemically and physically stable (data not shown).

Further confirmation of the conjugate chemical stability in HEPES buffer is provided from the LC-MS data obtained before and after subjecting the conjugate to the AST. Interestingly, the 48 hour AST conjugate samples in PBS showed that substantial degradation had occurred, while the 48 hour AST conjugate samples in HEPES buffer were completely intact and stable (see Figure 3b). Conjugate HS-1-60-1 stored in HEPES has a MW of 6730 Da before and after the AST, demonstrating that both mannose residues, the multimeric scaffold, and insulin are all chemically unchanged and quite stable. To ensure conjugate stability, all buffers used for storage, *in vitro* testing, and *in vivo* testing contain HEPES as the buffering agent.

The LC-MS data greatly enhances FDA manufacturing regulatory compliance, as the LC-MS test can readily act as the chemical identity assay of the conjugate. Since the drug (e.g., insulin), multimeric scaffold, and conjugate all have discrete molecular weights, the resulting affinity ligand ratio can be readily calculated by subtracting the scaffold MW from the conjugate MW to give the remaining mass due to the sugar groups. In the case of conjugate HS-1-60-1, the mannose:insulin molar ratio is calculated as exactly 2.0.

Example 36 – Functional stability of conjugates

After demonstrating that the conjugate was chemically and physically stable, a 72 hour AST conjugate was assessed for its subcutaneous bioactivity *in vivo* vs. fresh conjugate using Sprague-Dawley rats at 5 U/kg (see Figure 4).

Analysis of the 72 hour HEPES AST conjugate data showed that the time to reach the glucose nadir (T_{nadir}) was 60 minutes, and the time to return to 70% of the fasting blood glucose values ($T_{70\%BG}$) was less than 128 ± 15 min. A comparison of fresh conjugate vs. 72 hour AST conjugate bioactivity curves at each timepoint using the student t-test (n=4 for each group) showed no significant differences (all p-values > 0.21). These results were within specified targets for the formulation, indicating that preserved conjugate chemical stability translates into preserved *in vivo* functional performance.

III. In vivo assays of exemplary conjugates

This third set of examples describes various experiments investigating the *in vivo* properties of some exemplary conjugates.

Example 37 – Conjugate bioactivity versus RHI and dextran or glycogen conjugates

a. Insulin-dextran bioactivity

This comparative example evaluates the *in vivo* pharmacodynamic profile of subcutaneously administered insulin-dextran (Sigma-Aldrich, MW ~ 70K). As shown below, the insulin-dextran conjugates synthesized according to U.S. Patent Publication No. 20040202719 act relatively slowly after subcutaneous injection, because the high MW of the conjugate polymer significantly hinders the absorption rate into systemic circulation. Insulin-dextran was synthesized using a modified cyanogen bromide (CNBr) coupling reaction. Briefly, 500 mg of dextran (MW = 70K, Sigma-Aldrich) was dissolved in 50 ml of deionized water. 56 mg of solid CNBr was added to the resulting solution and the pH was maintained at 10.7 ± 0.2 using 5 N NaOH solution. After stirring for 15 min, another 56 mg of solid CNBr was added and the pH was maintained at 10.7 ± 0.2 while stirring for 45 minutes. 300 mg of recombinant human insulin (RHI) was then added to the solution, and the pH was adjusted to 9.15 using solid sodium bicarbonate. The solution was stirred overnight, ultrafiltered exhaustively against DI water using a 10K MWCO polyethersulfone disc membrane filter (Millipore, Bedford, MA), and lyophilized.

The resulting powder was then purified from unconjugated insulin by high performance liquid chromatography (Waters, Milford, MA) using a 1 M acetic acid mobile phase over a Superdex™ 75 packed column (Amersham Biosciences, Piscataway, NJ). The insulin-dextran fraction was then lyophilized to obtain the conjugate as a pure powder. The degree of insulin conjugation was 10 % (w/w) as determined by amino acid analysis (UCLA Biopolymers Laboratory, Los Angeles, CA).

Subcutaneous injections of the insulin-dextran were administered using 0.25 ml of a sterilized 1x PBS solution (20 U of equivalent insulin/ml) behind the neck of fasted normal non-diabetic rats (Male Sprague-Dawley, 200-250 g, n = 4). Blood samples were collected via tail vein bleeding at -15 and 0 minutes, and at 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 minutes after injection. Blood glucose values were measured using commercially available test strips (Precision Xtra, Abbott Laboratories, Abbott Park, IL). As shown in Figure 5, the times to reach the glucose nadir (T_{nadir}) concentration was found to be about 3 hours after injection, and the serum glucose levels remain depressed for at least five hours post injection.

b. Insulin-glycogen bioactivity

This example evaluates the *in vivo* pharmacodynamic profile of subcutaneously administered insulin-glycogen. The insulin-glycogen conjugate was synthesized according to Example 32. The bioactivity of the insulin-glycogen conjugate was evaluated by injecting a 2.5 equivalent U of insulin/kg dose behind the neck of fasted normal non-diabetic rats (Male Sprague-Dawley, 200-250 g, n = 4). Blood samples were collected via tail vein bleeding at -15 and 0 minutes, and at 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 minutes after injection. Blood glucose values were measured using commercially available test strips (Precision Xtra, Abbott Laboratories, Abbott Park, IL). As compared to the insulin-dextran conjugates above, the high MW insulin-glycogen conjugates lower glucose levels much more rapidly and to a greater extent (see Figure 6). This rapid action and elimination profile is due to the rapid enzymatic digestion of the high MW glycogen polymer chain following subcutaneous injection.

c. A comparison of conjugate and RHI bioactivity

This example evaluates and compares the *in vivo* pharmacodynamic profile of a subcutaneously administered exemplary conjugate and recombinant human insulin (RHI). The

exemplary conjugate was synthesized using TSAT-C6 as the scaffold, AEM as the indicator analog, and NH_2-B1-BOC2(A1,B29)-insulin as the drug. In each case, the conjugate or RHI was injected at 3.5 U/kg behind the neck of fasted normal non-diabetic rats (Male Sprague-Dawley, 400-500 g, n = 6). Blood samples were collected via tail vein bleeding at 0 minutes and at 30, 60, 90, 120, 150, 180, 210, 240, and 300 minutes after injection. Blood glucose values were measured using commercially available test strips (Precision Xtra, Abbott Laboratories, Abbott Park, IL). As shown in Figure 7, the glucose depression profiles for RHI and the exemplary conjugate are nearly identical despite the inability for the exemplary conjugate to be enzymatically digested *in vivo*. The rapid action and elimination profiles of the conjugate are most likely due to the fact that the conjugate is only 14% larger than RHI making any effect of increased MW almost negligible in terms of pharmacodynamic properties.

Example 38 – PK comparison with RHI

This example describes and compares the serum insulin profiles obtained for a subcutaneously administered exemplary conjugate and recombinant human insulin (RHI). The exemplary conjugate was synthesized using TSAT-C6 as the framework, AEM as the affinity ligand, and NH_2-B1-BOC2(A1,B29)-insulin as the drug. In each case, the conjugate or RHI was injected at 3.5 U/kg behind the neck of fasted normal non-diabetic rats (Male Sprague-Dawley, 400-500 g, n = 6). Blood samples were collected via tail vein bleeding at 0 minutes and at 30, 60, 90, 120, 150, 180, 210, 240, and 300 minutes after injection. Blood from each timepoint was centrifuged at 4 C to collect the serum. Serum insulin concentrations were subsequently measured with a commercially available ELISA kit (Human Insulin ELISA, Mercodia, Uppsala, Sweden). As can be seen in Figure 8, the pharmacokinetic profile for the conjugate is statistically indistinguishable from that of RHI, demonstrating that this conjugate is rapidly absorbed into and eliminated from serum following a subcutaneous injection.

Example 39 – PK and bioactivity of a B29-substituted version of the AEM-2-TSAT-C6-insulin conjugate

This example describes the serum insulin and blood glucose depression profiles obtained for a subcutaneously administered exemplary conjugate. The exemplary conjugate was synthesized using TSAT-C6 as the framework, AEM as the affinity ligand, and recombinant human insulin as the drug (to produce a B29-substituted conjugate instead of a B1-substituted

conjugate as in Examples 37 and 38). In this case, the conjugate was injected at 5 U/kg behind the neck of fasted normal non-diabetic rats (Male Sprague-Dawley, 400-500 g, n = 3). Blood samples were collected via tail vein bleeding at 0 minutes and at 30, 60, 90, 120, 150, 180, 210, 240, and 300 minutes after injection. Blood glucose values were measured using commercially available test strips (Precision Xtra, Abbott Laboratories, Abbott Park, IL). In addition, blood from each timepoint was centrifuged at 4 C to collect the serum. Serum insulin concentrations were subsequently measured with a commercially available ELISA kit (Human Insulin ELISA, Mercodia, Uppsala, Sweden). As can be seen in Figure 9, the pharmacokinetic profile for the B29-substituted conjugate is statistically indistinguishable from that of RHI as well as the B1-substituted conjugate from Example 38, demonstrating that this conjugate is also rapidly absorbed into and eliminated from serum following a subcutaneous injection.

Example 40 – PK and bioactivity comparison with Lispro

This example compares the serum insulin and blood glucose profiles obtained for a subcutaneously administered exemplary conjugate and insulin lispro. Insulin lispro (HUMALOG®) is a rapid acting insulin analog in which the penultimate lysine and proline residues on the C-terminal end of the B-peptide have been reversed. This modification blocks the formation of insulin multimers. Data from soluble recombinant human insulin (RHI) is also provided for comparison (see Example 38 and Figure 8).

The exemplary conjugate was synthesized using TSAT-C6 as the framework, AEM as the affinity ligand, and NH_2-B1-BOC2(A1,B29)-insulin as the drug. In each case, the conjugate or insulin lispro was injected at 3.5 U/kg behind the neck of fasted normal non-diabetic rats (Male Sprague-Dawley, 400-500 gm, n = 6). Blood samples were collected via tail vein bleeding at 0 minutes and at 30, 60, 90, 120, 150, 180, 210, 240, and 300 minutes after injection. Blood glucose values were measured using commercially available test strips (Precision Xtra, Abbott Laboratories, Abbott Park, IL). In addition, blood from each timepoint was centrifuged at 4 C to collect the serum. Serum insulin concentrations were subsequently measured with a commercially available ELISA kit (Human Insulin ELISA, Mercodia, Uppsala, Sweden). As can be seen in Figure 12, the pharmacokinetic profile for the conjugate is statistically indistinguishable from that of insulin lispro.

Example 41 – Effect of affinity ligand on bioactivity

This example compares the blood glucose profiles obtained for a series of subcutaneously administered exemplary conjugates. The exemplary conjugates were synthesized using TSAT-C6 as the framework, and NH_2-B1-BOC2(A1,B29)-insulin as the drug. The affinity ligand composition was varied across the conjugates to cover a range of affinities: AEM-2, AEBM-2, AETM-1 AEBM-1 and AETM-2 (from lowest to higest affinity). In each case, the conjugates were injected at 5 U/kg (3.5 U/kg for AEM-2) behind the neck of fasted normal non-diabetic rats (Male Sprague-Dawley, 400-500 gm, n = 6). Blood samples were collected via tail vein bleeding at 0 minutes and at 30, 60, 90, 120, 150, 180, 210, 240, and 300 minutes after injection. Blood glucose values were measured using commercially available test strips (Precision Xtra, Abbott Laboratories, Abbott Park, IL). In addition, blood from each timepoint was centrifuged at 4 C to collect the serum. Serum insulin concentrations were subsequently measured with a commercially available ELISA kit (Human Insulin ELISA, Mercodia, Uppsala, Sweden).

As can be seen in Figure 13, the glucose lowering response decreased as the affinity of the ligand increased. This data provided the first indication that the nature of the affinity ligand may affect the bioactivity of the conjugate. Figures 14-16, show the blood glucose levels alongside the serum insulin levels for each of the four conjugates tested. These results show quite clearly that the reduced glucose response for conjugates with higher affinity ligands result from the reduced PK profile of the conjugate (compare Figure 14 for AEM-2 with Figure 16 for AETM-2). We have demonstrated that this reduced PK profile (and associated bioactivity) can be reversed by an increase in the physiological glucose concentration (i.e., the level of conjugate in circulation rises with increasing glucose concentration). It will be appreciated that, in certain embodiments, this glucose dependence can be used to further tune the *in vivo* properties of a conjugate.

IV. Binding-site modified lectins

This fourth set of examples describes the preparation and testing of a variety of binding-site modified lectins.

Example 42 – Recombinant Con A (rCon A) production in *E. coli*

Preparation of rCon A-expressing gene constructs

Gene synthesis of rCon A constructs was performed at GeneArt (Regensburg, Germany) as follows. Genes of interest coding for the expression of rCon A products are listed in the table

below. The genes were synthesized at GeneArt, then cut with NcoI (5' site) and BamHI (3' site) enzymes and then inserted into the same sites in the pET24d vector (GeneArt, Regensburg, Germany). The resulting plasmid was then amplified in *E. coli* via culture flasks and then extracted, purified, giving a ~1 mg/mL solution of the plasmid DNA in TE buffer. Nucleic acid sequences of exemplary rCon A contructs are given in the table below.

Construct ID	DNA sequence
SCI-1	atggctaccgtagcgcaagctgctgataccattgtggcggtggaactgga tacctatccgaacaccgatattggcgatccgagctatccgcatattggca tcgatatcaaaagcgtgcgcagcaaaaaaaccgcgaaatggaacatgcag aacggtaaagttggcaccgcgcacatcatctataactctgttggtaagag actaagtgctgttgtttcttatcctaacgctgactctgccactgtctctt acgacgttgacctcgacaatgtccttcctgaatgggttagagttggcctt tctgcttcaaccggactttacaaagaaaccaataccattctctcatggtc ttttacttctaagttgaagagcaattcaacacatgagacaaatgcactcc atttcatgttcaaccaatttagcaaagatcagaaggatttgatccttcaa ggtgacgccacaacaggaacagatggtaacttggaactcacaagggtgtc aagtaatgggagtccacagggaagcagtgtgggccgggctttgttctatg ccccagtccacatttgggaaagttctgctgtggtggcaagctttgaagct acctttacatttctcataaaatcacccgactctcacccagctgatggaat tgccttcttcatttcaaatattgacagttccatccctagtggttccactg gaaggctccttggactcttccctgatgcaaattga (SEQ ID NO:26)
SCI-2	atggctaccgtagcgcaagctgctgataccattgtggcggtggaactgga tacctatccgaacaccgatattggcgatccgagctatccgcatattggca tcgatatcaaaagcgtgcgcagcaaaaaaaccgcgaaatggaacatgcag aacggtaaagttggcaccgcgcacatcatctataactctgttaataagag actaagtgctgttgtttcttatcctaacgctgactctgccactgtctctt acgacgttgacctcgacaatgtccttcctgaatgggttagagttggcctt tctgcttcaaccggactttacaaagaaaccaataccattctctcatggtc ttttacttctaagttgaagagctgttcaacatgtgagacaaatgcactcc atttcatgttcaaccaatttagcaaagatcagaaggatttgatccttcaa ggtgacgccacaacaggaacagatggtaacttggaactcacaagggtgtc aagtaatgggagtccacagggaagcagtgtgggccgggctttgttctatg ccccagtccacatttgggaaagttctgctgtggtggcaagctttcaagct acctttacatttctcataaaatcacccgactctcacccagctgatggaat tgccttcttcatttcaaatattgacagttccatccctagtggttccactg gaaggctccttggactcttccctgatgcaaattga (SEQ ID NO:27)

Construct ID	DNA sequence
SCI-3	atggctaccgtagcgcaagctgctgataccattgtggcggtggaactgga tacctatccgaacaccgatattggcgatccgagctatccgcatattggca tcgatatcaaaagcgtgcgcagcaaaaaaaccgcgaaatggaacatgcag aacggtaaagttggcaccgcgcacatcatctataactctgttggtaagag actaagtgctgttgtttcttatcctaacgctgactctgccactgtctctt acgacgttgacctcgacaatgtccttcctgaatgggttagagttggcctt tctgcttcaaccggactttgtaaagaaaccaataccattctctcatggtc ttttacttctaagttgaagagcaattcaacacatgagacaaatgcactcc atttcatgttcaaccaatttagcaaagatcagaaggatttgatccttcaa ggtgacgccacaacaggaacagatggtaacttggaactcacaagggtgtc aagtaatgggagtccacagggaagcagtgtgggccgggctttgttctatg ccccagtccacatttgggaaagttctgctgtggtggcaagctttgaagct acctttacatttctcataaaatcacccgactctcacccagctgatggaat tgccttcttcatttcaaatattgacagttccatccctagtggttccactg gaaggctccttggactcttccctgatgcaaattga (SEQ ID NO:28)
SCI-4	atggctaccgtagcgcaagctgctgataccattgtggcggtggaactgga tacctatccgaacaccgatattggcgatccgagctatccgcatattggca tcgatatcaaaagcgtgcgcagcaaaaaaaccgcgaaatggaacatgcag aacggtaaagttggcaccgcgcacatcatctataactctgttggtaagag actaagtgctgttgtttcttatcctaacgctgactctgccactgtctctt acgacgttgacctcgacaatgtccttcctgaatgggttagagttggcctt tctgcttcaaccggactttactgtgaaaccaataccattctctcatggtc ttttacttctaagttgaagagcaattcaacacatgagacaaatgcactcc atttcatgttcaaccaatttagcaaagatcagaaggatttgatccttcaa ggtgacgccacaacaggaacagatggtaacttggaactcacaagggtgtc aagtaatgggagtccacagggaagcagtgtgggccgggctttgttctatg ccccagtccacatttgggaaagttctgctgtggtggcaagctttgaagct acctttacatttctcataaaatcacccgactctcacccagctgatggaat tgccttcttcatttcaaatattgacagttccatccctagtggttccactg gaaggctccttggactcttccctgatgcaaattga (SEQ ID NO:29)

DNA preparation for E. coli transformation

Transformation was performed at GeneArt (Regensburg, Germany), and the resulting transformed BL21(DE3) cells were delivered as a stab culture. Once the DNA was transformed into the *E. coli* strains, the resulting gene constructs coded for the amino acid sequences shown in the table below.

Construct ID	Amino acid sequence
SCI-1	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTA KWNMQNGKVGTAHIIYNSVGKRLSAVVSYPNADSATVS YDVDLDNVLPEWVRVGLSASTGLYKETNTILSWSFTSK LKSNSTHETNALHFMFNQFSKDQKDLILQGDATTGTDG NLELTRVSSNGSPQGSSVGRALFYAPVHIWESSAVVAS FEATFTFLIKSPDSHPADGIAFFISNIDSSIPSGSTGR LLGLFPDAN (SEQ ID NO:1)
SCI-2	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTA KWNMQNGKVGTAHIIYNSVNKRLSAVVSYPNADSATVS YDVDLDNVLPEWVRVGLSASGLYKETNTILSWSFTSKL KSCSTCETNALHFMFNQFSKDQKDLILQGDATTGTDGN LELTRVSSNGSPQGSSVGRALFYAPVHIWESSAVVASF QATFTFLIKSPDSHPADGIAFFISNIDSSIPSGSTGRL LGLFPDAN (SEQ ID NO:19)
SCI-3	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTA KWNMQNGKVGTAHIIYNSVGKRLSAVVSYPNADSATVS YDVDLDNVLPEWVRVGLSASTGLCKETNTILSWSFTSK LKSNSTHETNALHFMFNQFSKDQKDLILQGDATTGTDG NLELTRVSSNGSPQGSSVGRALFYAPVHIWESSAVVAS FEATFTFLIKSPDSHPADGIAFFISNIDSSIPSGSTGR LLGLFPDAN (SEQ ID NO:20)
SCI-4	ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTA KWNMQNGKVGTAHIIYNSVGKRLSAVVSYPNADSATVS YDVDLDNVLPEWVRVGLSASTGLYCETNTILSWSFTSK LKSNSTHETNALHFMFNQFSKDQKDLILQGDATTGTDG NLELTRVSSNGSPQGSSVGRALFYAPVHIWESSAVVAS FEATFTFLIKSPDSHPADGIAFFISNIDSSIPSGSTGR LLGLFPDAN (SEQ ID NO:21)

Example 43 - Preparation and purification of soluble rConA

Stab cultures described in Example 42 were streaked onto 2 X 100mm plates (YT kanamycin plates; Fisher Scientific) and incubated upside down in a 37 C incubator overnight. Individual colonies were grown in 5 mL of LB media with 50 µg/mL kanamycin (Kanamycin Sulfate; Fisher #BP906-5) and incubated at 125 rpm at 37 C overnight. Negative controls were also included. Next, 5 µl of the 5mL stock was added to 50 mL LB media with 5 g/mL

kanamycin (Kanamycin Sulfate; Fisher #BP906-5) and incubated at 37 C overnight. Glycerol stocks of samples were made by adding 350µl of growth medium to 150 µL of 50% glycerol. Glycerol stock samples were flash frozen in dry ice/ethanol and stored in -80 C freezer. Further scale-up was performed by innoculating 6 mL of the 50 mL culture into 1L LB media with Kanamycin 50 µg/mL (Kanamycin Sulfate; Fisher #BP906-5) and grown to OD 600nm of 0.400 at 37 C or 30 C. Samples were checked frequently (every 20 minutes) once 0.200 OD was reached. Once OD 0.400 was reached, samples were cooled to 30 C and induced with 500 µg/L of 1M IPTG (FC IPTG 0.5mM; Fisher #PR-V3951) and incubated overnight at 100 rpm at 30 C.

Cultures were decanted into 250 mL centrifuge tubes and centrifuged (Sorval RC-5B) at 7000 rpm (5000 x g) for 20 minutes to pellet bacteria. One small aliquot of supernatant was saved for analysis by SDS-PAGE prior to decanting the remaining supernatant into bleach. The bacterial pellets were stored at -80 C for future use in isolation of inclusion bodies and lysis/washes to free soluble protein.

Pellets were taken from -80 C and thawed on ice. 10 mL 1X TBS with 200 µL of 10 mg/mL Lysozyme (Fisher #PI89833) and 10 µL of 1M $MgCl_2$ was added to each 250 mL pellet and pipeted (sonication was used if needed). Samples were pipeted gently and mixed at Room Temperature for 10 minutes on a nutating mixer. Samples were spun at 15,000 x g for 20 minutes (Sorval RC-5B) and supernatants were removed by decanting. Supernatants containing soluble protein were saved for SDS-PAGE and Sephadex G75 purification (Wash #1). 10 mL of 1X TBS with 200 µL of 10 mg/mL Lysozyme (Fisher #PI89833), 10 µL of 1M $MgCl_2$ and 5 µL/10mL of DNase I (Fisher #PI-89835) was added to each pellet and pipteted and sonicated (for approximately 30 seconds, two times) to get a uniform solution and to remove clumps. Samples were placed at room temperature for 10 minutes on nutating mixer, then spun at 15,000 x g for 20 minutes (Sorval RC-5B). Supernatants were removed by decanting and were saved for SDS-PAGE and Sephadex G75 purification (Wash #2). Pellets were washed with 0.5X TBS (15ml wash/pellet), sonicated (for approximately 30 seconds, two times). Total volume was increased to 30 mL with 0.5X TBS. Samples were spun at 15,000 x g for 20 minutes (Sorval RC-5B). Supernatatnts were removed by decanting and were saved for SDS-PAGE and Sephadex G75 purification (Wash #3).

Example 44 - Affinity column purification of rCon A samples

rCon A samples synthesized according to Examples 42 and 43 were purified via affinity column chromatography to separate fully reacted material from unreacted and/or partially reacted material. Sephadex G75 (12g) was prepared in binding buffer (BB; 100mM HEPES; 150mM NaCl; pH to 7.4; 1mM $CaCl_2$ and 1mM $MnCl_2$ in 2L) and loaded onto a BioRad Econo Column (cat# 737-5031). The sephadex G75 column was washed twice with 125 mL BB. Soluble protein washes 1 thru 3 were loaded onto sephadex G75 column slowly using peristaltic pump. The column twice with 100 mL BB using peristaltic pump. rConA was eluted with 35 mL 20mM alpha methyl mannose in BB six times. Typically, the 6 fractions were collected in different tubes and the rConA generally was eluted in fractions 3 thru 5. The absorbance at 280 nm (A280) of each fraction was determined. Each of the fractions with A280 over background (.044) were combined and concentrated using a Millipore Ultra15 10K (4500 x g x 8 minutes in Beckman tabletop centrifuge). Samples were resuspended by pipetting carefully, and more pooled fractions were added if necessary and centrifuged. Samples were concentrated to approximately 1 mg/mL (A280 = .3) and stored at 4 C until required for future studies.

Example 45 - Gel formation with rCon A samples

rCon A synthesised and purified according to Examples 42-44 were tested for their ability to form gels with glycogen. Alpha methyl mannose present in the samples was initially removed as follows. Samples were added to BioSpin 30 columns (Bio-Rad, Hercules, CA) that had been previously equilibrated with deionized water. The BioSpin columns were centrifuged for 4 minutes at 1000 x g, and the resulting eluent contained rCon A samples that had been substantially desalted and alpha methyl mannose removed. Samples were combined and A280 measurements were obtained. Typically, the A280 measurements correlated with the A280 measurements obtained before removal of alpha methyl mannose.

The following assay was then used to measure gel formation with glycogen. Unmodified Oyster glycogen Type II (Sigma Aldrich, St. Louis, MO) was dissolved at 10 mg/ml in pH 7, 200 mM BES buffer (Sigma Aldrich, St. Louis, MO). 50 µL of the glycogen solution was then pipetted into a well of a 96-well microtiter plate (VWR Scientific, Bridgeport, NJ). rCon A sample were dissolved from a lyophilized powder at 10 mg/ml in pH 7, 100 mM BES buffer containing 1 mM manganese chloride and 1 mM calcium chloride. 50 µL of the rCon A solution was then added to the 50 µL glycogen solution in the microtiter plate well, and the plate was gently vibrated to ensure adequate mixing of the two liquids. The plate was allowed to develop

at room temperature for 10 minutes, after which time the turbidity of each plate well was assayed using a commercially available microplate reader (SpectraMAX, Molecular Devices, Mountain View, CA) at a wavelength of 490 nm (OD490). The OD490 value was used to determine the amount of glycogen precipitation for a given rCon A. This assay was also repeated using an initial 1 mg/ml glycogen solution instead of the 10 mg/ml glycogen solution. SCI-1, SCI-3 and SCI-4 samples formed gels with glycogen that were comparable to gels formed by native (plant-derived) Con A controls.

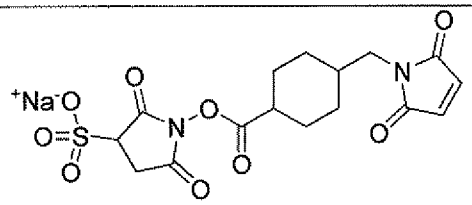
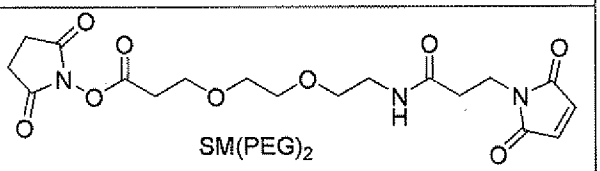
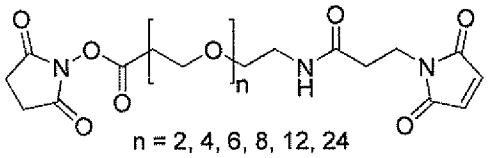
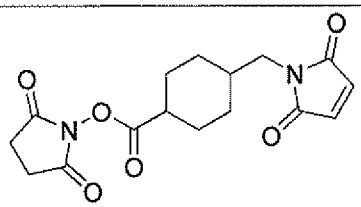
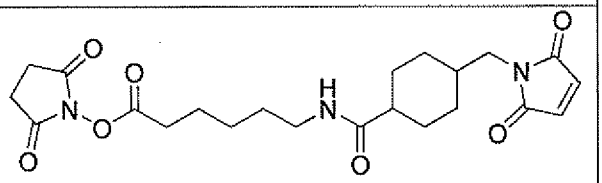
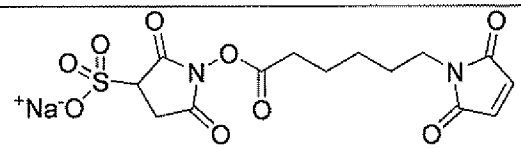
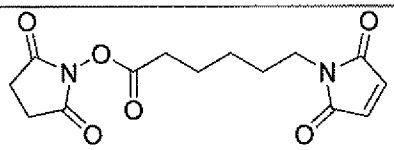
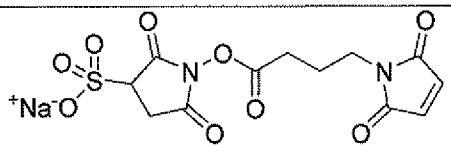
Example 46 – Chemical characterization of rCon A samples

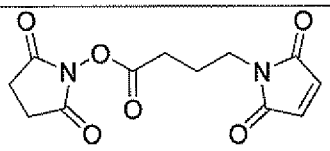
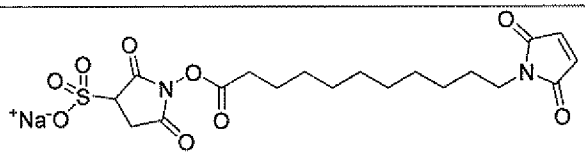
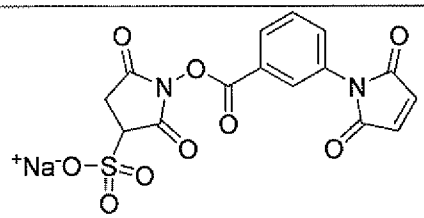
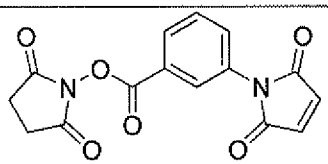
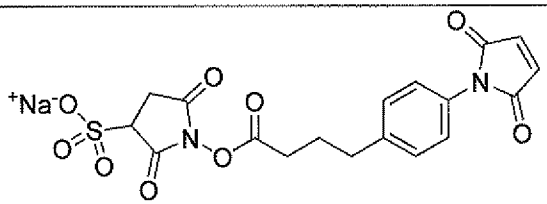
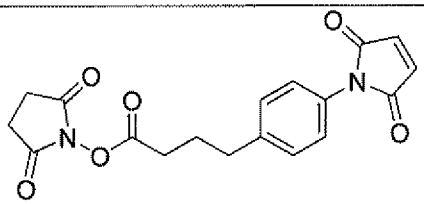
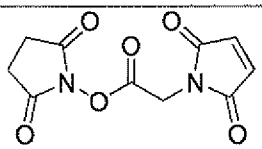
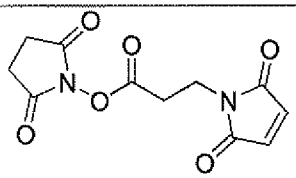
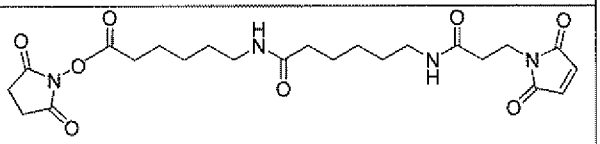
Denaturing polyacrylamide gel electrophoresis (PAGE) using sodium dodecyl sulfate (SDS) was performed on the rCon A samples to determine whether they contained soluble rCon A. Briefly, a 10-14% Tris-HCl pre-made gel (Criterion, Bio-Rad, Hercules, CA) and 1x Tris-glycine-SDS buffer (Bio-Rad, Hercules, CA) were used to perform the PAGE experiment. A broad-range molecular weight standard (Bio-Rad, Hercules, CA) and a 2 mg/mL sample of native concanavlin A lectin (Con A, Type VI, Sigma-Aldrich, St. Louis, MO) were also run as controls. 25 µL of each modified lectin or control sample was dissolved in 50 µL of 1x Laemmli Buffer (Bio-Rad, Hercules, CA) containing 5 µL of β-mercaptoethanol (Fisher Scientific), and the samples were heated in a boiling water bath for approximately 5 minutes. After the samples had cooled to room temperature, 10 µL of each sample was loaded into the wells of the pre-made PAGE gels. The samples were then run at 200 volts for 60 minutes. After the electrophoresis, the gels were fixed in a solution of deionized water:methanol:glacial acetic acid in a volume ratio of 60:30:10 for 30 minutes, follwed by two washes in deionized water. Finally, the gels were stained with 1x Bio-Safe Coomassie Blue stain (Bio-Rad, Hercules, CA) for 60 minutes. The final gels were imaged with a light table and digital camera to record the stained gel. The stained protein bands were assayed for their molecular weights by comparing against the molecular weight and native Con A control samples (see Figures 17 and 18).

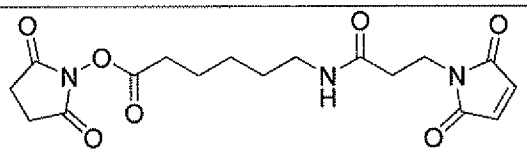
Example 47 - Synthesis of reactive linker coupled to affinity ligand and modified lectin

First, aminoethylmannose (AEM) was reacted with the precursors that are shown in the table below in order to produce a set of reactive linkers (the amino group of AEM reacts with the NHS group of each precursor to form a covalent bond). Each of these precursors is available

commercially (e.g., from Molecular Bioscience, Boulder, CO or Pierce/Thermo-Fisher Chemicals, Rockford, IL).

Precursor	Chemical Name	Chemical Structure
Sulfo-SMCC	Sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate	
SM(PEG)$_n$	Succinimidyl-[*N*-maleimidopropionamido]-#-ethyleneglycol) ester	 SM(PEG)$_2$  n = 2, 4, 6, 8, 12, 24
SMCC	Succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate	
LC-SMCC	Succinimidyl-4-[*N*-maleimidomethyl]cyclohexane-1-carboxy-[6-amidocaproate]	
Sulfo-EMCS	[*N*-ε-maleimidocaproyloxy] sulfosuccinimide ester	
EMCS	[*N*-ε-maleimidocaproyloxy] succinimide ester	
Sulfo-GMBS	*N*-[γ-maleimidobutyryloxy] sulfosuccinimide ester	

Precursor	Chemical Name	Chemical Structure
GMBS	*N*-[γ-maleimidobutyryloxy] succinimide ester	
Sulfo-KMUS	*N*-[κ-maleimidoundecanoyloxy] sulfosuccinimide ester	
Sulfo-MBS	*m*-maleimidobenzoyl-N-hydroxysulfosuccinimide ester	
MBS	*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester	
Sulfo-SMPB	Sulfosuccinimidyl 4-[*p*-maleimidophenyl]butyrate	
SMPB	Succinimidyl 4-[*p*-maleimidophenyl] butyrate	
AMAS	*N*-[α-maleimidoacetoxy] succinimide ester	
BMPS	*N*-[ß-maleimidopropyloxy] succinimide ester	
LC-SMPH	Succinimidyl-1-[β-maleimidopropionamido]-	

Precursor	Chemical Name	Chemical Structure
	hexanamido-6-hexanoate	
SMPH	Succinimidyl-6-[ß-maleimidopropionamido] hexanoate	

The process is described below using LC-SMPH as the precursor because this precursor was used in preparing the modified rCon A samples that are described in subsequent examples. However, it is to be understood that the same process was used with the other prescursors shown above. It will also be appreciated that any precursor containing a group such as an –NHS group that is capable of reacting with an amine-containing recognition element (e.g., an amino-terminal saccharide such as AEM) to form a reactive linker may be used in accordance with this process or a similar process.

As indicated above, aminoethylmannose (AEM) was initially reacted with the LC-SMPH precursor to form a sugar-maleimide molecule (the affinity ligand). AEM (45.28 mg) was added to a scintillation vial. Anhydrous DMSO (2 mL) and a small stir bar were added and stirred until all of the AEM was dissolved. TEA (0.8 mL) was added and stirred for one minute. LC-SMPH (100 mg) was dissolved in 2 mL of DMSO and slowly added to the reaction mixture and stirred for 1 hour at room temperature.

Thin Liquid Chromatography (TLC) was performed to measure the completeness of the reaction. Using a capillary tube, 5 drops of AEM solution, 5 drops of the reaction mixture, and 5 drops of the LC-SMPH were added on separate spots on the start line of the strip of TLC paper. Using a UV light source, it was noted which of the spots were UV active. The TLC strip was placed into a chamber containing 75/25 Ethanol / Ethyl Acetate and let run undisturbed for 10 minutes and then dried. The strip was checked for UV active spots using a UV light source and then dipped into H_2SO_4 for 2 seconds and dried until the sugar spots browned. Generally, if the reaction mixture spot is uv active the same way the LC-SMPH spot is, but brown like the AEM spot (with minimal streaking), then the reaction has gone to completion.

The reaction solution was then added drop wise to 40 mL of 1xS24 buffer (25 mM HEPES and 150 mM NaCl), keeping the pH between 6 and 9 using 1:10 HCl.

Next, the disulfide bonds of the rCon A synthesized and purified according to Examples 42-44 was reduced with DTT. Briefly, 200 mg of rCon A (40 mL at 5 mg/mL) containing alpha-methyl mannose was mixed with 11.87 mg of DTT. The mixture was incubated for 1 hour at room temperature and then DTT was removed using P6 Econo-Paks equilibrated with 1xS24.

Reduced rCon A was reacted with the sugar-maleimide molecule described above. In order to increase the likelihood that the conjugation reaction with rCon A went to completion, the sugar-maleimide solution was used at 20 times the molar concentration of the rCon A. Briefly, reduced rCon A was added drop-wise into the sugar-maleimide molecule solution while slowly stirring and incubated at room temperature for 2 hours. After the reaction, excess sugar-maleimide molecule was removed using P6 Econo-Paks equilibrated with 1xS24 buffer. Using Amicon Centrifuge tubes, the solution was concentrated down to 18 mg/mL for further testing.

Example 48 - Thiol Assay

A thiol assay was used to test the completeness of the reaction between the rCon A and the sugar maleimide molecule. Thiol determination was performed with Measure-iT™ thiol quantitation reagent (Invitrogen) according to the manufacturer's instructions. Briefly, stock and working solutions of the Measure-iT™ thiol quantitation standards (according to the table below) and quantitation reagent were prepared.

Concentration of Thiol Standard (uM)	Volume of Thiol Working Solution (uL)	Volume of Deionized H_2O (uL)
0	0	1000
2.75	25	975
5.5	50	950
11	100	900
22	200	800
33	300	700
44	400	600
55	500	500

Thiol quantitation reagent working solution (100 µL) was added to each well of a microwell plate. Thiol quantitation standards or unknown thiol samples were added to the wells, mixed and fluorescence was detected using a microplate reader. A standard curve was established in order to determine the unknown thiol concentration (Figure 19).

As shown in Figure 20, increasing the sugar maleimide to rCon A ratio was found to correlate with a decrease in unreacted disulfide bonds.

Example 49 - MAC Assay

Modified rCon A samples prepared in accordance with Example 47 were tested for their ability to agglutinate erythrocytes in the following MAC assay. The minimum agglutinating concentrations (MAC) of each modified rCon A was determined in V-well microtitre plates using a 2% v/v suspension of formaldehyde-stabilized rabbit erythrocytes according to the procedure of Crowley et al., *Methods. Enzymol.* 83:368-373, 1982. Formaldehyde-treated rabbit erythrocytes, prepared by published procedures (Nowak et al., *Biochim. Biophys. Acta* 393:115-123, 1975), from rabbit blood obtained from University of Michigan Unit for Laboratory Animal Medicine, were available from previous studies. The MAC was defined as the rCon A concentration in the highest dilution showing visible agglutination.

Briefly, 30 µL of an aqueous solution of each modified rCon A was added to the wells of a 96-well plate using dilutions so that the lectin concentration spanned from about 0.1 to 1000 ug/ml. A 30 µL aliquot of the formaldehyde-treated Rabbit erythrocytes was then pipetted into each well. At low lectin concentrations, there was insufficient lectin to form a network of crosslinked cells and the cells dropped to the bottom of the V-well forming what looks like a dark pin-point circle at the bottom of the plate when viewed from above. However, once the lectin concentration reached the minimum agglutination concentration (MAC), the lectin molecules began crosslinking the saccharide receptors on the erythrocyte surfaces, resulting in a network that cannot settle to the bottom of the V-well forming what looks like a large, opaque, diffuse circle when viewed from above. The lowest concentration that produces the large diffuse circle is the MAC value for a particular lectin.

The following table summarizes the MAC values for the different rCon A samples that were tested (see also Figures 21 and 22):

Con A	Recognition Element	Reactive Linker	MAC (ug/mL)
Native Con A	None	None	< 0.5
Acetylated native Con A	None	None	4.0
rCon A (SCI-3)	AEM	LC-SMPH	>1000
rCon A (SCI-4)	AEM	LC-SMPH	>1000

Example 50 – Mitogenicity assay

This example describes an assay that may be used to characterize and thereby compare the T-cell mitogenicity of different modified lectin compositions. Modifications and alternatives to this typical assay will be apparent to those skilled in the art. Peripheral blood mononuclear cells (PBMCs), rather than highly purified T-cells, are used for this assay since T-cell activation by lectins generally requires the presence of non-T-cell populations collectively termed accessory cells (e.g., monocytes, dendritic cells). In a typical assay, PBMCs are isolated from the whole blood of three healthy human donors and plated out separately at about 100,000 cells per well in a 96 well plate. Triplicate serial dilutions of different lectin compositions (e.g., native and treated) starting at 1000 (or 100) ug/ml concentration are then added to the wells. The plates are incubated for three days at 37 C, at which time 0.8 uCi of ^{3}H-thymidine is added to each well for an additional 18 hours. The degree of mitogenicity is then measured by ^{3}H-thymidine uptake by the proliferating PBMCs. In some cases, the mitogenicity of a novel lectin composition (e.g., a modified rCon A composition) is expressed as the % maximal native mitogenicity. The % maximal native mitogenicity is obtained by dividing the maximal CPM (counts per minute) value for the modified lectin composition over all measured concentrations by the maximal CPM value of the native lectin composition over all measured compositions.

In previous studies we have found a strong correlation between the MAC value and % Con A maximal mitogenicity, i.e., a significant increase in MAC value leads to a significant decrease in mitogenic effect. Therefore, MAC value is used in the present disclosure as a surrogate for determining potential reductions in mitogenicity for a given chemical modification.

V. Cross-linked materials for controllably releasing a conjugate

This fifth set of examples describes the preparation of exemplary cross-linked materials for controllable releasing conjugates. The examples also describe some of their *in vitro* and *in vivo* properties.

Example 51 – Cross-linked materials prepared from modified rCon A

0.50 ml of a 18 mg/ml modified rCon A solution in S14 buffer is added to a centrifuge tube and subsequently mixed with 0.111 ml of a 1.18 mg/ml zinc acetate dihydrate deionized water solution. 0.50 ml of a 2.3 mg/ml solution of C6-amine-AEBM-2-insulin in S14 buffer is

then added followed by rapid mixing to form a dispersion of insoluble particles. The dispersion is allowed to sit at room temperature for 20 min and then separated from the supernatant by centrifugation. The resulting cake is washed 2x with 1.0 ml of pH 7.4, 25 mM HEPES buffer containing 0.150 M sodium chloride (S24 buffer). During this process, the initial supernatant as well as the 2x wash solutions are collected in one large centrifuge tube. To the combined supernatant and wash solutions, 0.333 ml of a 1.18 mg/ml zinc acetate dihydrate deionized water solution is added. The solution is allowed to stand for 20 min after which any additional precipitated particles are isolated via centrifugation and combined with the particles remaining from the first two washing steps. This combined insoluble fraction is washed an additional 3x with 0.333 ml of S24 buffer. The remaining insoluble material is dispersed in 0.333 ml of S24 buffer and incubated overnight under mild agitation at 37 C. The next day, the remaining particles are again isolated by centrifugation and washed one additional time in 0.333 ml of S24. The resulting insoluble material is dispersed in a total volume of 0.30 ml using S24 and set aside for future studies. This process may be scaled up directly to produce any amount of desired product. C6-amine-AEBM-2-insulin may be substituted in the above synthesis with C6-amine-AETM-2-insulin (or any other conjugate) to produce a formulation with different stimuli-responsive performance characteristics.

Example 52 – IPGTT experiments in non-diabetic rats

0.300 ml of a given cross-linked material is injected subcutaneously into each of three normal male Sprague Dawley (SD) rats (Charles River Laboratories, location) weighing between 400 and 500 g. Prior to formulation injection, blood glucose values are measured via tail vein bleeding using a Precision Xtra glucometer (Abbott Laboratories, Alameda, CA) and approximately 100 ul of serum is obtained via tail vein bleeding to assay for background insulin levels. Food is removed from the rat cages during the duration of the study. Serum and blood glucose values are obtained at 30 min, 60 min, 90 min, and 120 min post-injection. At 120 min after the injection, an intraperitoneal injection of a 38% w/v glucose solution is injected to provide a 4 g/kg dose after which serum and blood glucose values are obtained at 135 min, 150 min, 180 min, 210 min, 240 min, and 300 min. Serum insulin concentrations are subsequently measured with a commercially available ELISA kit (Human Insulin ELISA, Mercodia, Uppsala, Sweden) using a standard curve generated from the pure insulin conjugate solution.

Example 53 – Effect of different animal sera on glucose-responsive dissolution of insulin-glycogen cross-linked materials and correlation to amylase activity

This example describes the *in vitro* dissolution in various animal sera as a function of glucose concentration for glucose-responsive formulations synthesized using an insulin-glycogen based conjugate. The insulin-glycogen conjugate is synthesized according to the following procedure. First, 62.5 ml of a 10 mg/ml recombinant human insulin solution (RHI) in pH 8.2, 25 mM HEPES buffer (Sigma-Aldrich, St. Louis, MA) is adjusted to pH 9.0 and cooled on ice to produce the RHI stock solution. Separately, 0.312 ml of triethylamine (TEA, Sigma-Aldrich, St. Louis, MA) is dissolved in 3 ml of DI water to produce the TEA stock solution. Separately, 0.300 g of cyanodimethylamino pyridinium tetrafluoroborate (CDAP, Sigma-Aldrich, St. Louis, MO) is dissolved in 1.2 ml of DMSO to produce the CDAP Stock solution. Separately, 100 mg of mannosamine-HCl (Sigma-Aldrich, St. Louis, MO) is dissolved in 1.5 ml of a 100 mM pH 9 HEPES saline buffered saline solution and pH adjusted to 9.0 to produce the mannosamine stock solution. Separately, 2.0 g of oyster Type IX glycogen (Sigma-Aldrich, St. Louis, MO) is dissolved in 40 ml of a 100 mM pH 9 HEPES saline buffered saline solution after which the solution is clarified by filtration and cooled on an ice bath. Next, 1 ml of the CDAP stock solution is added to the glycogen solution and mixed for one minute after which 1 ml of the TEA solution is added and the pH of the resulting solution adjusted to 9.0. After an additional 1 minute of stirring, 62 ml of the RHI solution are added and the resulting solution stirred for five minutes followed by addition of 1.065 ml of the mannosamine solution. The solution is stirred overnight at room temperature, ultrafiltered exhaustively against deionized water using a 50 kDa MWCO polyethersulfone disc membrane filter (Millipore, Bedford, MA), and lyophilized. The resulting powder is then purified 3x from unconjugated insulin by gel filtration HPLC (Waters, Milford, MA) using a 1 M acetic acid mobile phase over a Superdex^{TM} 30 HiLoad 16/60 (Amersham Biosciences, Piscataway, NJ) packed column. The insulin glycogen fraction is then lyophilized to obtain the conjugate as a pure white powder.

Glucose-responsive formulations are prepared using unmodified or modified rCon A as the multivalent crosslinking agent in the following manner. 200 ul of a 25 mg/ml insulin-glycogen conjugate solution in pH 7.0 HEPES buffered saline is mixed with 200 ul of a 25 mg/ml unmodified or modified rCon A solution in pH 7.0 HEPES buffered saline and allowed to stand for 20 minutes. Next, each formulation is centrifuged and washed 5x at room temperature

with 400 ul of pH 7.0 HEPES buffered saline. After the last wash and centrifugation, the supernatant is discarded and the remaining insoluble material dispersed in 50 ul of 1x PBS. The 24 x 50 ul dispersions are added to a 96-well plate along with 50 ul of serum from a particular animal species containing a specific amount of glucose according to the following format:

Insulin-glycogen / rCon A cross-linked material	**Species sera**			
Glucose Concentration (mg/dl)	pH 7, 1x PBS	Rat	Pig	Human
0	1	7	13	19
50	2	8	14	20
100	3	9	15	21
200	4	10	16	22
400	5	11	17	23
800	6	12	18	24

At the start of the experiment each well may appear white and opaque (as measured by a decrease in light transmission or increase in absorbance at 450 nm, A450). The 96-well plate is then incubated for 6 hours at 37 C after which the A450 value for each well is measured again. The % of the formulation remaining is calculated by dividing the A450 (final) by the A450 (initial) and multiplying by 100. If all the material has dissolved, the A450 value is close to zero indicating almost 0% remaining. Alternatively, if no material has dissolved, the A450 is close to the initial value indicating almost 100% remaining.

Example 54 – Glucose-responsive material using modified rCon A and an AEM-2 conjugate

This example describes the formation of glucose-responsive insoluble materials using a conjugate synthesized with TSAT-C6 as the framework, AEM as the affinity ligand, and NH_2-B1-BOC2(A1,B29)-insulin as the drug. 50 ul of a 2 mg/ml conjugate solution in pH 8.2 HEPES buffered saline is mixed with 50 ul of a 25 mg/ml modified rCon A solution in pH 7.0 HEPES buffered saline in each well of a 96-well microplate. Each well contains 5.5 ul of a concentrated

glucose solution of increasing concentrations to produce final concentrations equal to 0, 50, 100, 200, 400, 800, and 1600 mg/dl. The final well contains 5.5 ul of the highly potent alpha-methyl mannose sugar inhibitor such that the final concentration is 100 mM. The ability of the rCon A/conjugate mixture to precipitate in the presence of increasing glucose concentrations is then evaluated. When the combination forms an insoluble network, the well appears white and opaque (as measured by a decrease in light transmission or increase in absorbance at 450 nm, A450). When the glucose concentration is high enough, the contents of the entire well become soluble and clear (as measured by an increase in light transmission or decrease in absorbance at 450 nm, A450).

Example 55 – Testing performance across animal sera

This example describes the *in vitro* dissolution in various animal sera as a function of glucose concentration for the glucose-responsive formulation of Example 54. 24 x 50 ul dispersions are added to a 96-well plate along with 50 ul of serum from a particular animal species containing a specific amount of glucose according to the following format:

Insulin-glycogen / rCon A cross-linked material	**Species sera**			
Glucose Concentration (mg/dl)	pH 7, 1x PBS	Rat	Pig	Human
0	1	7	13	19
50	2	8	14	20
100	3	9	15	21
200	4	10	16	22
400	5	11	17	23
800	6	12	18	24

At the start of the experiment each well may appear white and opaque (as measured by a decrease in light transmission or increase in absorbance at 450 nm, A450). The 96-well plate is then incubated for 6 hours at 37 C after which the A450 value for each well is measured again. The % of the formulation remaining is calculated by dividing the A450 (final) by the A450

(initial) and multiplying by 100. If all the material has dissolved, the A450 value is close to zero indicating almost 0% remaining. Alternatively, if no material has dissolved, the A450 is close to the initial value indicating almost 100% remaining.

Example 56 – Glucose-responsive material using rCon A and an AEM-2 conjugate

This example describes an alternative method for forming glucose-responsive insoluble materials using a conjugate synthesized with TSAT-C6 as the framework, AEM as the affinity ligand, and NH_2-B1-BOC2(A1,B29)-insulin as the drug. 0.50 ml of a 2.3 mg/ml solution of conjugate in pH 8.2, 25 mM HEPES buffer containing 0.150 M sodium chloride (S14 buffer) is added to a centrifuge tube and subsequently mixed rapidly with 0.500 ml of a 25 mg/ml rCon A solution in pH 7.4, 25 mM HEPES buffer containing 0.150 M sodium chloride (S24 buffer) to form a dispersion of insoluble particles. The dispersion is allowed to sit at room temperature for 20 min and then separated from the supernatant by centrifugation. The resulting cake is washed 5x with 1.0 ml of pH 7.4, 25 mM HEPES buffer containing 0.150 M sodium chloride (S24 buffer). After the last wash, the remaining insoluble material is incubated overnight at 37 C. The next day, the remaining particles are again isolated by centrifugation and washed one additional time in 1.0 ml of S24. The resulting insoluble material is dispersed in a total volume of 0.30 ml using S24 and set aside for future studies. This process may be scaled up directly to produce any amount of desired product.

Example 57 – IPGTT experiments in non-diabetic rats

0.300 ml of the material prepared in Example 56 is injected subcutaneously into each of three normal male Sprague Dawley (SD) rats (Charles River Laboratories, Wilmington, MA) weighing between 400 and 500 g. Prior to formulation injection, blood glucose values are measured via tail vein bleeding using a Precision Xtra glucometer (Abbott Laboratories, Alameda, CA) and approximately 100 ul of serum is obtained via tail vein bleeding to assay for background insulin levels. Food is removed from the rat cages during the duration of the study. Serum and blood glucose values are obtained at 30 min, 60 min, 90 min, and 120 min post-injection. At 120 min after the injection, an intraperitoneal injection of a 38% w/v glucose solution is injected to provide a 4 g/kg dose after which serum and blood glucose values are obtained at 135 min, 150 min, 180 min, 210 min, 240 min, and 300 min. Serum insulin concentrations are subsequently measured with a commercially available ELISA kit (Human

Insulin ELISA, Mercodia, Uppsala, Sweden) using a standard curve generated from the pure insulin conjugate solution. Endogenous rat insulin does not cross-react on this assay; therefore, any results obtained will be due solely to the exogenously administered insulin conjugate and not from endogeneous insulin from the animal (See Human Insulin ELISA kit instructions, Mercodia, Uppsala, Sweden).

In a separate experiment, 0.300 ml of saline is injected subcutaneously into each of three normal male Sprague Dawley (SD) rats (Charles River Laboratories, Wilmington, MA) weighing between 400 and 500 g. Prior to saline injection, blood glucose values are measured via tail vein bleeding using a Precision Xtra glucometer (Abbott Laboratories, Alameda, CA) and approximately 100 ul of serum is obtained via tail vein bleeding to assay for background insulin levels. Food is removed from the rat cages during the duration of the study. Serum and blood glucose values are obtained at 30 min, 60 min, 90 min, and 120 min post-injection. At 120 min after the injection, an intraperitoneal injection of a 38% w/v glucose solution is injected to provide a 4 g/kg dose after which serum and blood glucose values are obtained at 135 min, 150 min, 180 min, 210 min, 240 min, and 300 min. Serum insulin concentrations are subsequently measured with a commercially available ELISA kit specific for Rat Insulin (Rat Insulin ELISA, Mercodia, Uppsala, Sweden). The results from this experiment will establish the glucose-responsive endogenous insulin secretion produced by the pancreas in a normal, non-diabetic rat.

In a separate experiment, 5 U/kg of recombinant human insulin (RHI, Sigma-Aldrich, St. Louis, MO) is injected subcutaneously into each of three normal male Sprague Dawley (SD) rats (Charles River Laboratories, Wilmington, MA) weighing between 400 and 500 g. Prior to the RHI injection, blood glucose values are measured via tail vein bleeding using a Precision Xtra glucometer (Abbott Laboratories, Alameda, CA) and approximately 100 ul of serum is obtained via tail vein bleeding to assay for background insulin levels. Food is removed from the rat cages during the duration of the study. Serum and blood glucose values are obtained at 30 min, 60 min, 90 min, and 120 min post-injection. At 120 min after the injection, an intraperitoneal injection of a 38% w/v glucose solution is injected to provide a 4 g/kg dose after which serum and blood glucose values are obtained at 135 min, 150 min, 180 min, 210 min, 240 min, and 300 min. Serum insulin concentrations are subsequently measured with a commercially available ELISA kit (Human Insulin ELISA, Mercodia, Uppsala, Sweden) using a standard curve generated from the pure insulin conjugate solution. Endogenous rat insulin does not cross-react on this assay; therefore, any results obtained will be due solely to the exogenously administered

insulin conjugate and not from endogeneous insulin from the animal (See Human Insulin ELISA kit instructions, Mercodia, Uppsala, Sweden).

Example 58 – Normo-/Hyper-glycemic clamp experiments in non-diabetic rats

This example describes the use of glucose clamps to maintain glucose levels in rats at a constant value to obtain the steady state serum insulin concentration as a function of glucose concentration. 0.300 ml (~ 0.6 ml/kg of body weight) of the material prepared in Example 56 is injected subcutaneously into each of four normal, double jugular vein catheterized, male Sprague Dawley (SD) rats (Charles River Laboratories, Wilmington, MA) weighing between 300 and 400 g. One catheter from each rat is connected to a variable rate syringe pump containing a concentrated glucose solution. Blood glucose values are measured via tail vein bleeding every five minutes using a Precision Xtra glucometer (Abbott Laboratories, Alameda, CA) and the syringe pump intravenous infusion rate is adjusted periodically for the first two hours post-injection to maintain the rats at 100 mg/dl. After the first two hours, the glucose infusion rate is increased to and maintained at 400 mg/dl for an additional two hours. Serum is collected at regular intervals for insulin concentration (Human Insulin ELISA, Mercodia, Uppsala, Sweden) and blood glucose values.

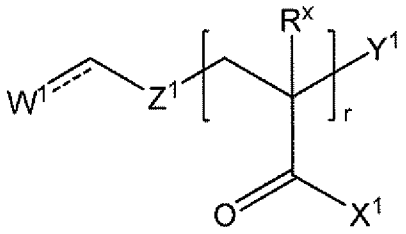
Example 59 – Normo-/Hyper-glycemic clamp experiments in non-diabetic pigs and correspondence to results obtained in rats

The following experiment may be performed to determine if similar glucose-responsive profiles could be obtained in pigs. 0.300 ml (~ 0.012 ml/kg of body weight) of the material prepared in Example 56 is injected subcutaneously into each of four normal, jugular vein catheterized, male Yucatan Miniature pigs (Sinclair Research, Columbia, MO) weighing 20-25 kg. The catheter from each pig is connected to a variable rate syringe pump containing a concentrated glucose solution. Blood glucose values are measured via intravenous catheter blood withdrawals every five minutes using a Precision Xtra glucometer (Abbott Laboratories, Alameda, CA) and the syringe pump intravenous infusion rate is adjusted periodically for the first two hours post-injection to maintain the pigs at 65 mg/dl. After the first two hours, the glucose infusion rate is increased to and maintained at 400 mg/dl for an additional two hours. Serum is collected at regular intervals for insulin concentration and blood glucose values. Because the insulin conjugate may cross-react with endogenous porcine insulin, a new assay

methodology was developed and may be implemented to detect the insulin in pigs. A radioimmunoassay (RIA) kit (Millipore, Billerica, MA) has been developed to detect both porcine and the exemplary insulin conjugate with roughly the same signal to noise. The signal on this kit due to endogenous porcine insulin is determined by running a particular blank pig serum sample on a c-Peptide RIA kit (Millipore, Billerica, MA) and on the insulin RIA kit. Once the resulting correlation is determined, any serum sample RIA insulin signal can be converted into a contribution from endogenous insulin and conjugated insulin.

Example 60 – Conjugates of formula (IV)

This example describes some exemplary conjugates of formula (IV):



Yet other embodiments of these conjugates as well as intermediates and methods of making these conjugates can be found in PCT application Serial No. PCT/US10/22251 filed January 27, 2010. The entire contents of this related application is incorporated herein by reference.

In certain embodiments, a conjugate of formula (IV) may include one or more of the following exemplary groups:

R^x

In certain embodiments, R^x is hydrogen. In certain embodiments, R^x is optionally substituted C_{1-6} alkyl. In certain embodiments, R^x is optionally substituted C_{1-3} alkyl. In certain embodiments, R^x is optionally substituted methyl. In certain embodiments, R^x is $-CH_3$.

Z^1

In certain embodiments, Z^1 is an optionally substituted bivalent C_{1-10}, C_{1-8}, C_{1-6}, C_{1-4}, or C_{1-2} hydrocarbon chain. In certain embodiments, Z^1 is $-(CH_2)-$, $-(CH_2CH_2)-$, $-(CH_2CH_2CH_2)-$, $-(CH_2CH_2CH_2CH_2)-$, $-(CH_2CH_2CH_2CH_2CH_2)-$, or $-(CH_2CH_2CH_2CH_2CH_2CH_2)-$. In certain embodiments, Z^1 is $-(CH_2)-$ or $-(CH_2CH_2)-$. In certain embodiments, Z^1 is $-(CH_2)-$. In certain

embodiments, Z^1 is $-(CH_2CH_2)-$. In certain embodiments, Z^1 is $-(CH_2CH_2CH_2)-$. In certain embodiments, Z^1 is $-(CH_2CH_2CH_2CH_2)-$.

In certain embodiments, Z^1 is an optionally substituted bivalent C_{1-10} hydrocarbon chain, wherein 1, 2 or 3 methylene units of Z^1 are optionally and independently replaced with one or more groups selected from $-S-$, $-O-$, $-NR^a-$, $-(C=NR^a)-$, $-(C=O)-$, $-(S=O)-$, $-S(=O)_2-$, $-(CR^b=CR^b)-$, $-(N=N)-$, an optionally substituted arylene moiety or an optionally substituted heteroarylene moiety. In certain embodiments, Z^1 is an optionally substituted bivalent C_{1-10} hydrocarbon chain, wherein 1, 2 or 3 methylene units of Z^1 are optionally and independently replaced with one or more groups selected from $-S-$, $-O-$, $-NR^a-$, $-(C=NR^a)-$, or $-(C=O)-$. In certain embodiments, Z^1 is $-CH_2CH_2NH(C=O)C(CH_3)_2-$, $-CH_2CH_2N(C=NH)(CH_2)_3S-$, $-CH(R^f)_2$, $-CH_2CH(R^f)_2$, $-CH_2CH_2CH(R^f)_2-$, $-CH_2S-$, or $-CH_2CH_2S-$, wherein R^f is optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, optionally substituted heteroaryl (e.g., in certain embodiments, R^f is optionally substituted aryl; in certain embodiments, R^f is phenyl). In certain embodiments, Z^1 is $-CH_2CH_2NH(C=O)C(CH_3)_2-$ or $-CH_2CH_2N(C=NH)(CH_2)_3S-$. In certain embodiments, Z^1 is $-CH_2CH_2NH(C=O)C(CH_3)_2-$. In certain embodiments, Z^1 is $-CH_2CH_2N(C=NH)(CH_2)_3S-$.

Y^1

In certain embodiments, Y^1 is a fragment of a free radical initiator. Such a fragment is encompassed by the definition of Y^1, as initiator fragments may include halogen, $-OR^e$, $-SR^e$, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, and optionally substituted heteroaryl moieties.

In certain embodiments, Y^1 is hydrogen, halogen, or an initiator fragment. In certain embodiments, Y^1 is hydrogen or halogen. In certain embodiments, Y^1 is hydrogen or bromine.

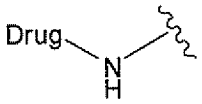
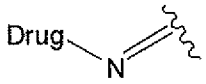
X^1

In certain embodiments, X^1 is $-OR^c$. In certain embodiments, X^1 is a mixture of $-OR^c$ and $-N(R^d)_2$. In certain embodiments, X^1 is $-N(R^d)_2$.

W^1 and ------

In certain embodiments, ------ is a single covalent bond.

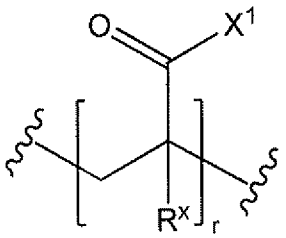
In certain embodiments, W^1 is covalently bound to the polymer *via* an amino group. In certain embodiments, W^1 is covalently bound to the polymer *via* a primary amino group.

For example, in certain embodiments, the group  corresponds to the group  or  wherein the group [Drug–NH-] or [Drug–N=] is the drug *directly* covalently conjugated *via* a primary amino group. In other embodiments, the drug may include a spacer group (e.g., an alkylene group, arylene group, heteroarylene group, ester linkage, amide linkage, and the like) which terminates with a pendant amino group. The latter embodiments enable greater separation between the active portion of the drug and the polymer.

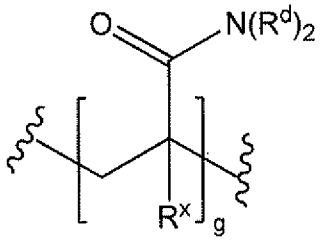
r

In certain embodiments, r is an integer between 10–25, inclusive. In certain embodiments, r is an integer between 15–25, inclusive. In certain embodiments, r is an integer between 20–25, inclusive. In certain embodiments, r is an integer between 5–20, inclusive. In certain embodiments, r is an integer between 10–20, inclusive. In certain embodiments, r is an integer between 15–20, inclusive. In certain embodiments, r is 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25. In certain embodiments r is 5. In certain embodiments r is 10. In certain embodiments r is 15. In certain embodiments r is 20. In certain embodiments r is 25.

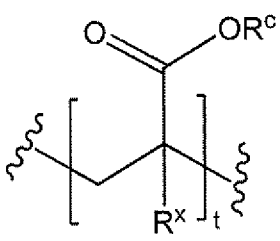
In certain embodiments, the group:



corresponds to a mixture of the groups:



and

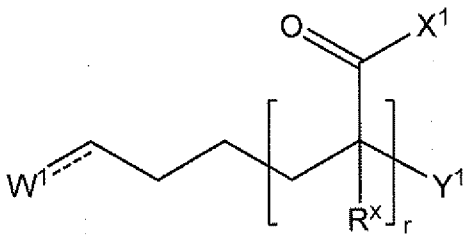


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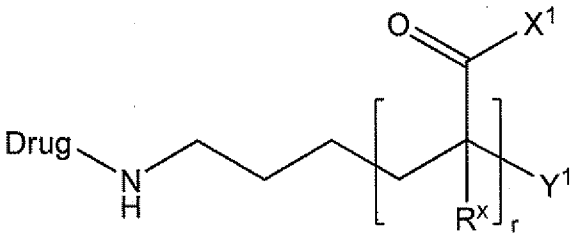
wherein the sum of (g+t) is equal to r. In certain embodiments, each instance of g and t is, independently, an integer between 1 and 24, inclusive, with the proviso that the sum of (g+t) is greater than or equal to 5 and less than or equal to 25. In certain embodiments, g and t are present in a ratio of about 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, or 1:1 (g to t). In certain embodiments, t and g are present in a ratio of about 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, or 1:2 (t to g).

Exemplary conjugates

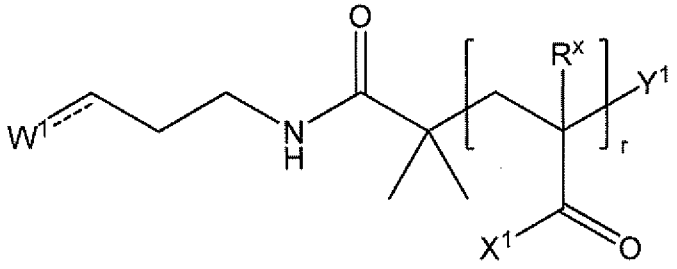
In certain embodiments, a conjugate of formula (IV-a1) may be used:



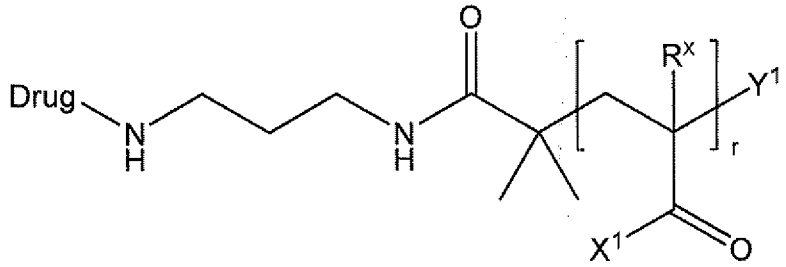
In certain embodiments, a conjugate of formula (IV-a2) may be used:



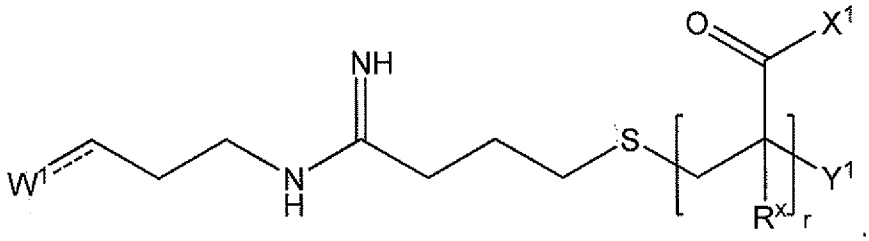
In certain embodiments, a conjugate of formula (IV-b1) may be used:



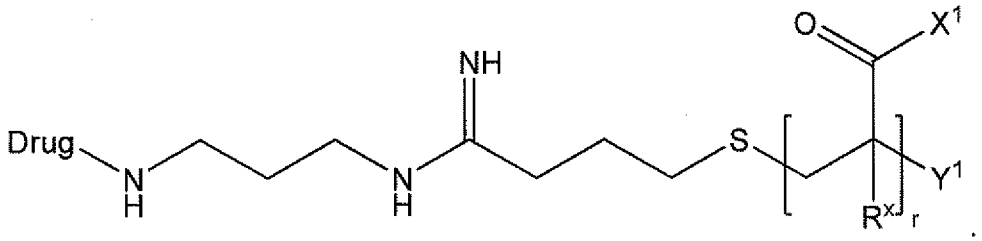
In certain embodiments, a conjugate of formula (IV-b2) may be used:



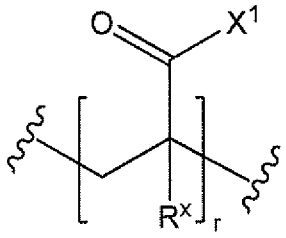
In certain embodiments, a conjugate of formula (IV-c1) may be used:



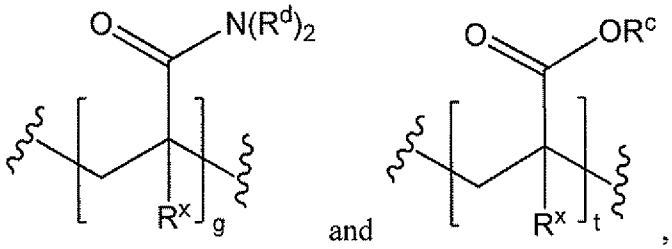
In certain embodiments, a conjugate of formula (IV-c2) may be used:



In any of these exemplary conjugates, the group:



may correspond to a mixture of the groups:



wherein the sum of (g+t) is equal to r, respectively. In certain embodiments, r is 10. In certain embodiments, r is 20.

Characterization of conjugates

The conjugates can be characterized by any analytical method including nuclear magnetic resonance (e.g., ^{1}H NMR); gel permeation chromatography (GPC) for molecular weight and polydispersity; and Fourier transform infrared spectroscopy (FTIR) or acid titration for determination of the number of acid groups per chain.

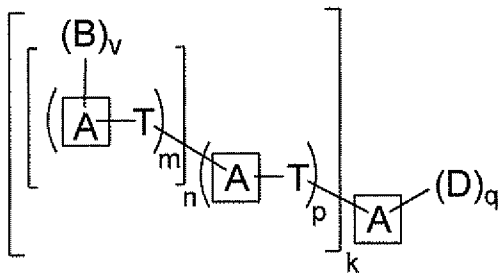
In certain embodiments the conjugate framework (i.e., without including the affinity ligands, drug or detectable label) has a molecular weight of less than 10,000 Da, e.g., in the range of about 100 to about 10,000 Da. In certain embodiments, the conjugate framework has a molecular weight in the range of about 300 to about 5,000 Da. In certain embodiments, the conjugate framework has a molecular weight in the range of about 500 to about 2,500 Da. In certain embodiments, the conjugate framework has a molecular weight in the range of about 1,000 to 2,000 Da. In certain embodiments, the conjugate framework has a molecular weight in the range of about 200 to 1,000 Da. In certain embodiments, the conjugate framework has a molecular weight in the range of about 300 to 800 Da.

In certain embodiments, a mixture of conjugates is generated. The conjugates in this mixture may have the same or different molecular weights. In one embodiment, the

polydispersity of the mixture is less than 1.5. In one embodiment, the polydispersity of the mixture is less than 1.25.

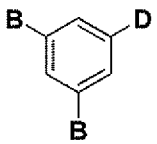
Example 61 – Conjugates of formula (V)

This example describes some exemplary conjugates of formula (V):

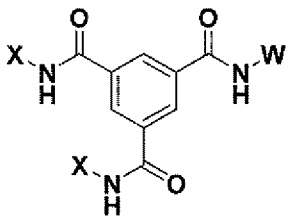


Yet other embodiments of these conjugates as well as intermediates and methods of making these conjugates can be found in PCT application serial No. PCT/US10/22268 filed on January 27, 2010. The entire contents of this related application is incorporated herein by reference.

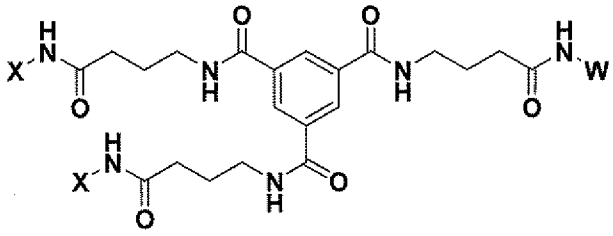
In some embodiments, the present disclosure provides conjugates of general formula (IX-a):



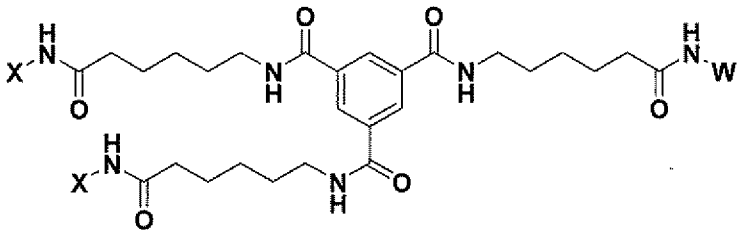
For example, in some embodiments, the present disclosure provides conjugates of formula:



;



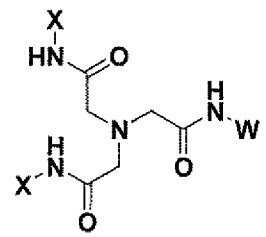
; or



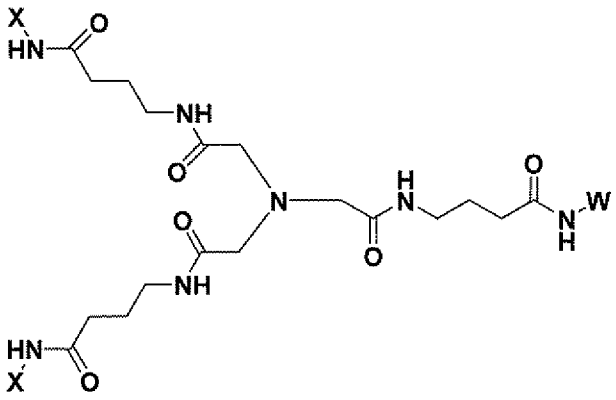
In some embodiments, the present disclosure provides conjugates of general formula (IX-a):



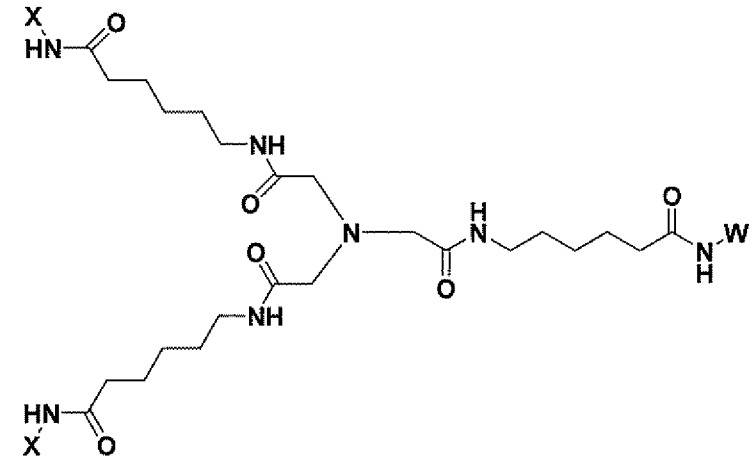
For example, in some embodiments, the present disclosure provides conjugates of formula:



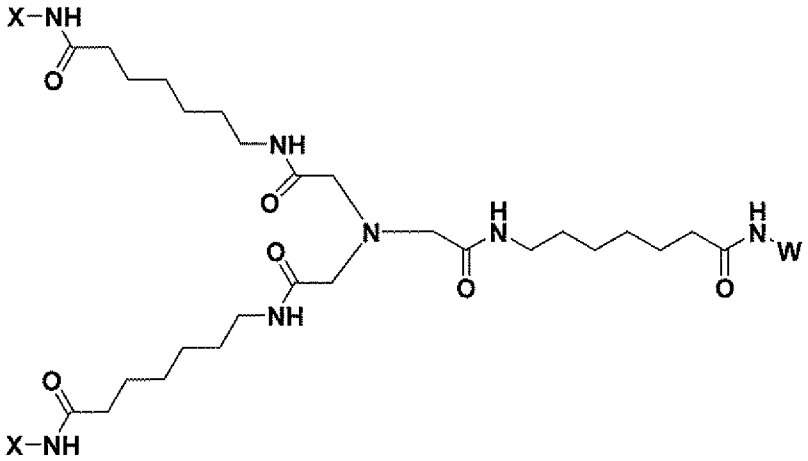
;



;



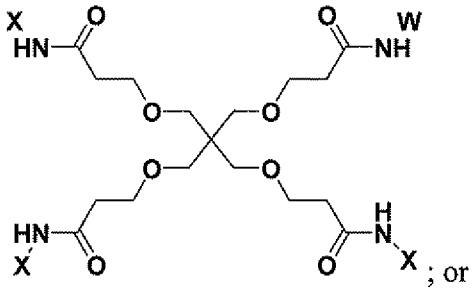
; or



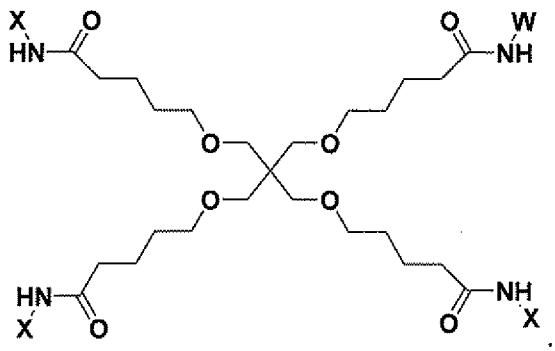
In some embodiments, the present disclosure provides conjugates of general formula (IX-a):



For example, in some embodiments, the present disclosure provides conjugates of formula:



; or



Characterization of conjugates

The conjugates can be characterized by any analytical method including nuclear magnetic resonance (e.g., ^{1}H NMR); gel permeation chromatography (GPC) for molecular weight and polydispersity; Fourier transform infrared spectroscopy (FTIR), etc.

In certain embodiments the conjugate framework (i.e., without including the affinity ligands, drug or detectable label) has a molecular weight of less than 10,000 Da, e.g., in the range of about 100 to about 10,000 Da. In certain embodiments, the conjugate framework has a molecular weight in the range of about 300 to about 5,000 Da. In certain embodiments, the conjugate framework has a molecular weight in the range of about 500 to about 2,500 Da. In certain embodiments, the conjugate framework has a molecular weight in the range of about 1,000 to 2,000 Da. In certain embodiments, the conjugate framework has a molecular weight in the range of about 200 to 1,000 Da. In certain embodiments, the conjugate framework has a molecular weight in the range of about 300 to 800 Da.

Example 62 – Preparation of fluorescently-labeled polysaccharides

This example describes a method for making fluorescent polysaccharides, specifically tetramethylrhodamine isothiocyanate (TRITC, Sigma Aldrich, St. Louis, MO) derived mannan which is sometimes used in FRET-based glucose sensors. The TRITC-mannan compound is the one used in the application of Example 63.

Briefly, in a Schlenk tube under nitrogen, 1 g of mannan (Sigma Aldrich, St. Louis, MO) is dissolved in 20 ml of dimethylsulfoxide (DMSO, Sigma Aldrich, St. Louis, MO) at 95 C until the solution is clear. Next two drops of pyridine (anhydrous, Sigma Aldrich, St. Louis, MO) are added to the mixture. 20 mg of TRITC powder is added directly to the heated solution, and then 10 ul of a dibutyltin dilaurate (Sigma Aldrich, St. Louis, MO) is added and the mixture is

allowed to react for 2 hours, after which time the flask is removed from the temperature bath and allowed to cool. The TRITC-mannan is purified by several precipitation cycles using 50:50 ethanol:diethyl ether mixtures, where the precipitate is centrifuged at 2000 x g for 10 min (Allegra 21R, Beckman Coulter, Fullerton, CA) and redissolved in the minimum amount of deionized water to redissolve the centrifuged particle cake between each precipitation step. This is repeated until no visible red or orange color was visibly seen in the supernatant after centrifuging the solution under the above conditions. The precipitate is redissolved in deionized water a final time and lyophilized to give the purified TRITC-mannan product.

Example 63 – Use of modified lectin compositions in FRET applications

This method describes an application of the inventive modified rCon A compositions as a glucose sensor based on fluorescence resonance energy transfer (FRET). FRET is based on the fact that when two different fluorophores are brought closely together this allow for energy transfer between the two fluorophores, resulting in a decrease in the fluorescence of one or both of the fluorophores, which is called fluorescence quenching (Ballerstadt et al., *Anal. Chim. Acta* 345:203-212, 1997).

In the absence of a saccharide inhibitor, a mixture of a fluorescent modified rCon A and a fluorescent polysaccharide will form a compact gel and the neighboring fluorophores will undergo FRET. In the presence of a saccharide inhibitor such as glucose, the average distance between the fluorescent modified rCon A and the fluorescent polysaccharide will increase causing the level of FRET to decrease and thereby leading to an increase in the individual fluorescence signals.

Because of their improved safety profile the inventive modified rCon A compositions may provide for a safe *in vivo* glucose sensor than those that use unmodified rCon A.

The following *in vitro* tests are performed using a modified rCon A of the present disclosure. A FITC-labeled modified rCon A can be made using fluorescein isothiocyanate (FITC, Sigma Aldrich, St. Louis, MO). The purified FITC-modified rCon A is then mixed with TRITC-mannan synthesized according to Example 62.

Three stock solutions are made as follows:

(i) FITC-modified rCon A – 60 mg of FITC-modified rCon A is dissolved in 2 ml of 100 mM BES, pH 7, 1.0 M NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$.

(ii) TRITC-mannan Stock – 60 mg of TRITC-mannan is dissolved in 2 ml of 100 mM BES, pH 7, 1.0 M NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$.

(iii) Glucose Stock – a 1200 mg/dl glucose solution is made by dissolving 1200 mg glucose in 100 ml of 100 mM BES, pH 7, 1.0 M NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$.

1:2 serial dilutions of the FITC-modifed rCon A and TRITC-mannan stock solutions are then performed in 100 mM BES, pH 7, 1.0 M NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$ separately so that the final concentrations of FITC-modified rCon A and TRITC-Mannan are 30, 3, 0.3, 0.03, 0.003, 0.0003, 0.00003, and 0.000003 mg/ml. The stock solutions are mixed together, e.g., on a 96-well microtiter plate (VWR Scientific, Bridgeport, NJ). The plate is designed so that the final concentrations of all components are decreased by a factor of 3x after mixing all solutions together.

After mixing the solutions together, the fluorescence of the plate is assayed by a fluorescence plate reader (*f*max, Molecular Devices, Sunnyvale, CA) using the 485/525 nm filter pair for FITC and 544/590 nm filter pair for measuring TRITC fluorescence.

After measuring with both sets of filter pairs using the 1200 mg/dl glucose buffer at room temperature, the plate is heated to 37 C using the plate reader incubator function. After 30 minutes of equilibration, the plate is read for both FITC and TRITC fluorescence a second time. After which the plate is allowed to recool to room temperature.

Rows 2, 4, 6, and 8 all receive another 50 ul of a 9600 mg/dl glucose solution, while Rows 1, 3, 5, and 7 all receive another 50 ul of buffer. The plate is read a third time at room temperature, and the process is repeated a final time using 0.1 M Methyl-α-mannopyrannoside.

Further optimization of the glucose sensor can be made by adjusting the affinity of the polymer, optimizing the fluorescence loading of the modified rCon A and TRITC-mannan, and rerunning the experiment on a fluorescence spectrophotometer to allow for the maximum FRET or FRET quenching compared to the plate reader/filter pair setup.

Example 64 – Viscosimetric glucose sensor

This example demonstrates how a modified rCon A composition can be used in a system that is capable of detecting glucose based on the changes in viscosity of a glucose-responsive solution. A modified rCon A composition is dissolved in a 20 mM BES buffer at pH 7 containing 1 mM $MnCl_2$ and $CaCl_2$ at a concentration of 100 mg rCon A equivalents/ml. Separately, yeast mannan (Sigma-Aldrich, St. Louis, MO) is dissolved in five solutions of 200

mM BES buffer at pH 7 at a concentration of 50 mg/ml with each solution containing 0, 100, 800, 1600, and 3200 mg/dl of glucose, respectively. 0.700 ml of the modified rCon A stock solution is mixed with each of the five mannan stock solutions containing the varying concentrations of glucose such that the five resulting solutions contain 0, 50, 400, 800, and 1600 mg/dl of glucose.

The viscosity of each solution is measured as a function of shear rate using a microviscometer set up in a cone-and-plate geometry. The cone measures 4 cm in diameter and has a 2 degree angle, requiring a sample volume of 0.58 ml. A solvent trap is used to reduce sample evaporation. Steady state flow viscosity is measured for a range of shear rates for each sample at both 22 C and 37 C.

When this liquid is contacted by a body fluid, the measured viscosity will directly correlate to the fluid's glucose concentration.

OTHER EMBODIMENTS

Other embodiments of the invention will be apparent to those skilled in the art from a consideration of the specification or practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with the true scope and spirit of the invention being indicated by the following claims.

CLAIMS

We claim:

1. A cross-linked material comprising:

multivalent lectins with at least two binding sites for glucose, wherein the lectins include at least one affinity ligand which is capable of competing with glucose for binding with at least one of said binding sites and is covalently linked to a cysteine residue of the lectins; and

conjugates that include two or more separate affinity ligands bound to a conjugate framework, wherein the two or more affinity ligands compete with glucose for binding with the lectins at said binding sites and wherein conjugates are cross-linked within the material as a result of non-covalent interactions between lectins and affinity ligands on different conjugates.

2. The material of claim 1, wherein the conjugates further comprise a drug bound to the conjugate framework.

3. The material of claim 1, wherein the conjugates further comprise a detectable label bound to the conjugate framework.

4. The material of claim 1, wherein the lectins include only one covalently linked affinity ligand per lectin.

5. The material of claim 1, wherein the lectins include one covalently linked affinity ligand per glucose binding site.

6. The material of claim 1, wherein the lectins are recombinant Con A lectins that contain at least one cysteine residue introduced by site directed mutagenesis.

7. The material of claim 6, wherein the at least one cysteine residue substitutes an amino acid in the wild-type Con A sequence that forms part of a binding site for glucose.

8. The material of claim 6, wherein the at least one cysteine residue is at a location within the wild-type Con A sequence that is within 1-5 amino acids (as measured in the primary amino acid sequence) of an amino acid that forms part of a binding site for glucose.

9. The material of claim 6, wherein the at least one cysteine residue is at a location within the wild-type Con A sequence that it is separated by at least 1, 2, 3, 4 or 5 amino acids (as measured in the primary amino acid sequence) from any amino acid that forms part of a binding site for glucose.

10. The material of any one of claims 7-9, wherein the wild-type Con A sequence is the amino acid sequence of SEQ ID NO:1.

11. The material of claim 10, wherein one or more of the amino acids at position 12, 14, 99, 100, 208 and 228 of SEQ ID NO:1 form part of the binding site for glucose.

12. The material of claim 10, wherein each of the amino acids at position 12, 14, 99, 100, 208 and 228 of SEQ ID NO:1 forms part of the binding site for glucose.

13. The material of claim 6, wherein lectins have an amino acid sequence that is at least 90%, identical to the amino acid sequence of SEQ ID NO:1.

14. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:2 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

15. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:3 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

16. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:4 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

17. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:5 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

18. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:6 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

19. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:7 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

20. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:8 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

21. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:9 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

22. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:10 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

23. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:11 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

24. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:12 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

25. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:13 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

26. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:14 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

27. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:15 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

28. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:16 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

29. The material of any one of claims 14-28, wherein each X that is not a cysteine is the corresponding amino acid from SEQ ID NO:1.

30. The material of any one of claims 14-28, wherein the lectins include a single cysteine.

31. The material of any one of claims 14-28, wherein one of the X positions is a cysteine and all other X positions are the corresponding amino acid from SEQ ID NO:1.

32. The material of claim 6, wherein lectins have an amino acid sequence that is at least 90%, identical to the amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 with the proviso that in each of these sequences each X is a codable amino acid and at least one X is a cysteine.

33. The material of claim 6, wherein lectins have an amino acid sequence that is at least 90%, identical to the amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 where just one of the X positions is a cysteine and all other X positions are codable amino acids.

34. The material of claim 6, wherein the lectins have an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO:17 with the proviso that at least one X is a cysteine.

35. The material of claim 34, wherein the lectins include a single cysteine.

36. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:17 with the proviso that at least one X is a cysteine.

37. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:17 where X at position 100 is a cysteine.

38. The material of claim 37, wherein X at position 101 is lysine.

39. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:17 where X at position 101 is cysteine.

40. The material of claim 39, wherein X at position 100 is tyrosine.

41. The material of claim 6, wherein the lectins have an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO:18 with the proviso that at least one X is a cysteine.

42. The material of claim 41, wherein the lectins include a single cysteine.

43. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:18 with the proviso that at least one X is a cysteine.

44. The material of claim 43, wherein X at position 117 is cysteine.

45. The material of claim 44, wherein X at position 120 is histidine.

46. The material of claim 43, wherein X at position 120 is cysteine.

47. The material of claim 46, wherein X at position 117 is asparagine.

48. The material of claim 43, wherein X at position 117 is cysteine and X at position 120 is cysteine.

49. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:19.

50. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:20.

51. The material of claim 6, wherein the lectins have an amino acid sequence of SEQ ID NO:21.

52. The material of any one of claims 6-51, wherein the lectins bind glucose with substantially the same affinity as wild-type Con A.

53. The material of any one of claims 6-51, wherein the lectins forms multimers under physiological conditions.

54. The material of any one of claims 6-51, wherein the lectins form a gel in the presence of glycogen.

55. The material of claim 1, wherein the affinity ligands that are covalently linked to the lectins include a saccharide.

56. The material of claim 55, wherein the saccharide is glucose.

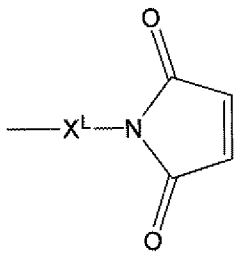
57. The material of claim 55, wherein the saccharide is mannose.

58. The material of claim 55, wherein the affinity ligands that are covalently linked to the lectins include a saccharide and a linker and the saccharide is covalently linked to the linker via an anomeric carbon.

59. The material of claim 58, wherein the anomeric carbon is an alpha anomer.

60. The material of claim 58, wherein the affinity ligands were covalently linked to the lectins using a reactive linker with a terminal maleimide group that reacts with the sulfhydryl group of the cysteine residue.

61. The material of claim 60, wherein the affinity ligands were covalently linked to the lectins using a reactive linker of the formula:



(II)

wherein:

X^L is a covalent bond or a bivalent, straight or branched, saturated or unsaturated, optionally substituted C_{1-100} hydrocarbon chain wherein one or more methylene units of X^L are optionally and independently replaced by -O-, -S-, -N(R')-, -C(O)-, -C(O)O-, -OC(O)-, -N(R')C(O)-, -C(O)N(R')-, -S(O)-, -$S(O)_2$-, -N(R')SO_2-, -SO_2N(R')-, a carbocyclic group, a heterocyclic group, an aryl group, or a heteroaryl group; and

each occurrence of R' is independently hydrogen, a suitable protecting group, or an acyl moiety, arylalkyl moiety, aliphatic moiety, aryl moiety, heteroaryl moiety, or heteroaliphatic moiety.

62. The material of claim 61, wherein 1-5 methylene units of X^L are optionally and independently replaced.

63. The material of claim 61, where X^L is constructed from a C_{1-20} hydrocarbon chain.

64. The material of claim 61, wherein one or more methylene units of X^L is replaced by a carbocyclic group.

65. The material of claim 61, wherein one or more methylene units of X^L is replaced by a cyclohexyl moiety.

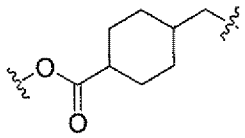
66. The material of claim 61, wherein one or one or more methylene units of X^L is replaced by a benzene ring.

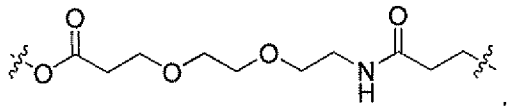
67. The material of claim 61, wherein one or more methylene units of X^L is replaced by -C(O)-.

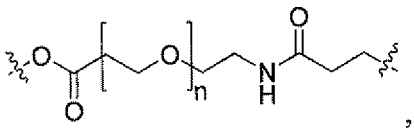
68. The material of claim 61, wherein one or more methylene units of X^L is replaced by -C(O)N(R')-.

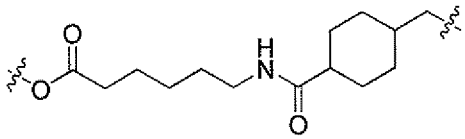
69. The material of claim 61, wherein one or more methylene units of X^L is replaced by -O-.

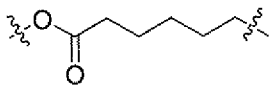
70. The material of claim 61, wherein X^L contains a PEG moiety.

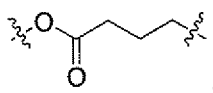
71. The material of claim 61, wherein X^L is .

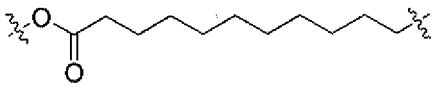
72. The material of claim 61, wherein X^L is .

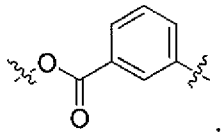
73. The material of claim 61, wherein X^L is , wherein n is 2, 4, 6, 8, 12, or 24.

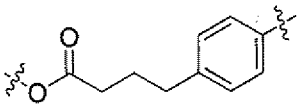
74. The material of claim 61, wherein X^L is .

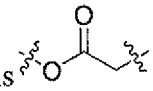
75. The material of claim 61, wherein X^L is .

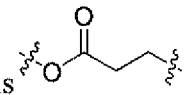
76. The material of claim 61, wherein X^L is .

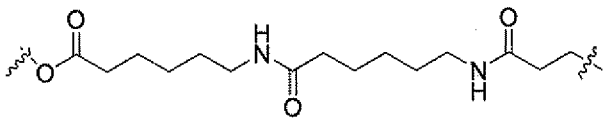
77. The material of claim 61, wherein X^L is .

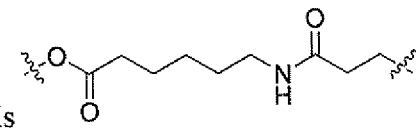
78. The material of claim 61, wherein X^L is .

79. The material of claim 61, wherein X^L is .

80. The material of claim 61, wherein X^L is 

81. The material of claim 61, wherein X^L is 

82. The material of claim 61, wherein X^L is 

83. The material of claim 64, wherein X^L is 

84. The material of claim 1, wherein the affinity ligands of the conjugates include a saccharide.

85. The material of claim 84, wherein the affinity ligands of the conjugates include a saccharide selected from glucose, mannose, glucosamine, mannosamine, methylglucose, methylmannose, ethylglucose, and ethylmannose.

86. The material of claim 84, wherein the affinity ligands of the conjugates include a bimmanose or trimannose.

87. The material of claim 84, wherein the affinity ligands of the conjugates include aminoethylglucose (AEG), aminoethylmannose (AEM), aminoethylbimannose (AEBM) or aminoethyltrimannose (AETM).

88. The material of claim 84, wherein the affinity ligands of the conjugates include a saccharide and a linker and the saccharide is covalently bound to the linker via an anomeric carbon.

89. The material of claim 88, wherein the anomeric carbon is an alpha anomer.

90. The material of claim 2, wherein the drug is an anti-diabetic drug.

91. The material of claim 2, wherein the drug is an insulin molecule.

92. The material of claim 2, wherein the drug is an insulin sensitizer.

93. The material of claim 2, wherein the drug is an insulin secretatogue.

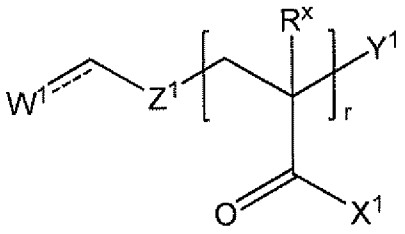
94. The material of claim 1, wherein the conjugate framework is polymeric.

95. The material of claim 1, wherein the conjugate framework is non-polymeric.

96. The material of claim 1, wherein the conjugate framework is branched or hyperbranched.

97. The material of claim 1, wherein the conjugate framework includes a polysaccharide.

98. The material of claim 1, wherein the conjugate has the general formula:



IV

wherein:

R^x is hydrogen or optionally substituted C_{1-6} alkyl;

Z^1 is an optionally substituted bivalent C_{1-10} hydrocarbon chain, wherein 1, 2, 3, 4 or 5 methylene units of Z^1 are optionally and independently replaced with one or more groups selected from –S–, –O–, –NR^a–, –(C=NR^a)–, –(C=O)–, –(S=O)–, –$S(=O)_2$–, –(CR^b=CR^b)–, –(N=N)–, an optionally substituted arylene moiety or an optionally substituted heteroarylene moiety, wherein R^a is hydrogen, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, optionally substituted heteroaryl, or a suitable amino protecting group; and R^b is hydrogen, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, or optionally substituted heteroaryl;

each occurrence of X^1 is independently –OR^c or –$N(R^d)_2$, wherein R^c is hydrogen, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, optionally substituted heteroaryl, a suitable hydroxyl protecting group, a cation group, or an affinity ligand, and each R^d is, independently, hydrogen, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, optionally substituted heteroaryl, a suitable amino protecting group, or an affinity ligand, with the proviso that at least two occurrences of X^1 include an affinity ligand;

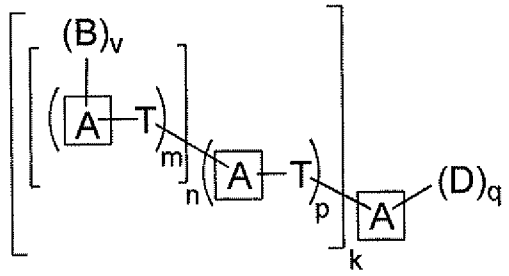
Y^1 is hydrogen, halogen, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, optionally substituted heteroaryl, –OR^e or –SR^e wherein R^e is hydrogen, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, or optionally substituted heteroaryl;

r is an integer between 5–25, inclusive;

W^1 is a drug or detectable label; and

====== corresponds to a single or double covalent bond.

99. The material of claim 1, wherein the conjugate has the general formula:



V

wherein:

each occurrence of $\left[\left(\boxed{A}-T\right)\right]$ represents a potential branch within the conjugate;

each occurrence of $\left(\boxed{A}-T\right)$ represents a potential repeat within a branch of the conjugate;

each occurrence of $\boxed{A}$ is independently a covalent bond, a carbon atom, a heteroatom, or an optionally substituted group selected from the group consisting of acyl, aliphatic, heteroaliphatic, aryl, heteroaryl, and heterocyclic;

each occurrence of T is independently a covalent bond or a bivalent, straight or branched, saturated or unsaturated, optionally substituted C_{1-30} hydrocarbon chain wherein one or more methylene units of T are optionally and independently replaced by -O-, -S-, -N(R)-, -C(O)-, -C(O)O-, -OC(O)-, -N(R)C(O)-, -C(O)N(R)-, -S(O)-, $-S(O)_2-$, $-N(R)SO_2-$, $-SO_2N(R)-$, a heterocyclic group, an aryl group, or a heteroaryl group;

each occurrence of R is independently hydrogen, a suitable protecting group, or an acyl moiety, arylalkyl moiety, aliphatic moiety, aryl moiety, heteroaryl moiety, or heteroaliphatic moiety;

–B is $-T-L^B-X$;

each occurrence of X is independently an affinity ligand;

each occurrence of L^B is independently a covalent bond or a group derived from the covalent conjugation of a T with an X;

–D is $-T-L^D-W$;

each occurrence of W is independently a drug or a detectable label;

each occurrence of L^D is independently a covalent bond or a group derived from the covalent conjugation of a T with a W;

k is an integer from 2 to 11, inclusive, defining at least two k-branches within the conjugate;

q is an integer from 1 to 4, inclusive;

k + q is an integer from 3 to 12, inclusive;

each occurrence of p is independently an integer from 1 to 5, inclusive; and

each occurrence of n is independently an integer from 0 to 5, inclusive; and

each occurrence of m is independently an integer from 1 to 5, inclusive; and

each occurrence of v is independently an integer from 0 to 5, inclusive, with the proviso that within each k-branch at least one occurrence of n is ≥ 1 and at least one occurrence of v is ≥ 1.

100. The material of claim 98 or 99, wherein the molecular weight of the conjugate absent each X and W is less than 10,000 Da.

101. The material of claim 100, wherein the molecular weight of the conjugate absent each X and W is in the range of about 300 to about 5,000 Da.

102. The material of claim 100, wherein the molecular weight of the conjugate absent each X and W is in the range of about 300 to about 800 Da.

103. The material of claim 100, wherein at least two occurrences of X include an affinity ligand that comprises a saccharide.

104. The material of claim 100, wherein at least two occurrences of X include an affinity ligand that comprises a saccharide selected from the group consisting of glucose, mannose, glucosamine, mannosamine, methylglucose, methylmannose, ethylglucose, and ethylmannose.

105. The material of claim 100, wherein at least two occurrences of X include an affinity ligand that comprises a bimmanose or a trimannose.

106. The material of claim 100, wherein at least two occurrences of X include an affinity ligand selected from aminoethylglucose (AEG), aminoethylmannose (AEM), aminoethylbimannose (AEBM) and aminoethyltrimannose (AETM).

107. The material of claim 100, wherein at least two occurrences of X include an affinity ligand which comprises a saccharide and a linker and the saccharide is covalently bound to the linker via an anomeric carbon.

108. The material of claim 107, wherein the anomeric carbon is an alpha anomer.

109. The material of claim 1, wherein the material is insoluble when placed at 37 C in pH 7, 25 mM HEPES buffer containing 150 mM NaCl and no glucose.

110. The material of claim 109, wherein conjugates are released from the material at a rate or to an extent that is dependent on the concentration of glucose.

111. The material of claim 110, wherein the material remains substantially insoluble when placed at 37 C in pH 7, 25 mM HEPES buffer containing 150 mM NaCl and 100 mg/dL glucose for six hours using USP dissolution test method II at 50 rpm.

112. The material of claim 110, wherein less than 10% of the material dissolves when placed at 37 C in pH 7, 25 mM HEPES buffer containing 150 mM NaCl and 100 mg/dL glucose for six hours using USP dissolution test method II at 50 rpm.

113. The material of claim 110, wherein at least 50% of the material dissolves when placed at 37 C in pH 7, 25 mM HEPES buffer containing 150 mM NaCl and 400 mg/dL glucose for six hours using USP dissolution test method II at 50 rpm.

114. The material of claim 110, wherein 100% of the material dissolves when placed at 37 C in pH 7, 25 mM HEPES buffer containing 150 mM NaCl and 400 mg/dL glucose for six hours using USP dissolution test method II at 50 rpm.

115. A method comprising administering a material of any one of claims 1-114 to a patient.

116. The method of claim 115, wherein the material is administered by subcutaneous injection.

117. The method of claim 115, wherein the conjugates comprise an insulin molecule bound to the framework.

118. The method of claim 117, wherein the patient is diabetic.

119. The method of claim 117, wherein the material is administered such that the average daily dose of the insulin molecule is in the range of 10 to 200 U.

120. The method of claim 119, wherein the material is administered daily.

121. The method of claim 119, wherein the material is administered weekly.

122. The method of claim 119, wherein the material is administered monthly.

123. The method of claim 119, wherein the patient is also receiving an insulin sensitizer.

124. The method of claim 119, wherein the patient is also receiving an insulin secretagogue.

125. A method comprising steps of:

(I) mixing:

(a) multivalent lectins with at least two binding sites for glucose, wherein the lectins include at least one affinity ligand which is capable of competing with glucose for binding with at least one of said binding sites and is covalently linked to a cysteine residue of the lectins and wherein the

lectins include a first label which generates a measurable response when in close proximity to a second label, and

(b) conjugates that comprise an affinity ligand and the second label;

(II) exposing a sample to the mixture of multivalent lectins and conjugates, wherein:

(a) if glucose is absent from the sample, the conjugates form a cross-linked material with the lectins through affinity binding to the multivalent lectins to produce a measurable response, and

(b) if glucose is present in the sample, the response is reduced because formation of cross-linked material is inhibited as a result of glucose from the sample competing with the conjugates for the binding sites on the multivalent lectins; and

(III) detecting and optionally measuring the response with a sensor to determine the presence and optionally the amount of glucose in the sample.

126. A method comprising steps of:

(I) mixing:

(a) multivalent lectins with at least two binding sites for glucose, wherein the lectins include at least one affinity ligand which is capable of competing with glucose for binding with at least one of said binding sites and is covalently linked to a cysteine residue of the lectins,

(b) a first group of molecules that comprise an affinity ligand and a first label which generates a measurable response when in close proximity to a second label, and

(c) a second group of molecules that comprise an affinity ligand and the second label;

(II) exposing a sample to the mixture of multivalent lectins, and the first and second groups of molecules, wherein:

(a) if glucose is absent from the sample, members of the first and second group of molecules are brought in close proximity through affinity binding to the multivalent lectins to produce a binding complex and a measurable response, and

(b) if glucose is present in the sample, the response is reduced because fewer of said binding complexes form as a result of glucose from the sample competing with the first and second molecules for the binding sites on the multivalent lectins; and

(III) detecting and optionally measuring the response with a sensor to determine the presence and optionally the amount of glucose in the sample.

127. A method comprising steps of:

(I) providing:

(a) conjugates that comprise a plurality of affinity ligands, and

(b) multivalent lectins with at least two binding sites for glucose, wherein the lectins include at least one affinity ligand which is capable of competing with glucose for binding with at least one of said binding sites and is covalently linked to a cysteine residue of the lectins;

(II) mixing the conjugates and multivalent lectins, wherein the viscosity of the resulting mixture is due to the binding between the conjugates and lectins;

(III) contacting the mixture with a sample containing glucose which displaces conjugates from the lectins and causes a concentration dependent reduction in viscosity; and

(IV) detecting and optionally measuring the resulting change in viscosity to determine the presence and optionally the amount of glucose in the sample.

128. A recombinant Con A polypeptide that includes a cysteine that substitutes an amino acid in the wild-type Con A sequence that forms part of a binding site for glucose.

129. A recombinant Con A polypeptide that includes a cysteine at a location within the wild-type Con A sequence that is within 1-5 amino acids (as measured in the primary amino acid sequence) of an amino acid that forms part of a binding site for glucose.

130. The recombinant Con A polypeptide of claim 128 or 129, wherein the wild-type Con A sequence is the amino acid sequence of SEQ ID NO:1.

131. The recombinant Con A polypeptide of claim 130, wherein one or more of the amino acids at position 12, 14, 99, 100, 208 and 228 of SEQ ID NO:1 form part of the binding site for glucose.

132. The recombinant Con A polypeptide of claim 130, wherein each of the amino acids at position 12, 14, 99, 100, 208 and 228 of SEQ ID NO:1 forms part of the binding site for glucose.

133. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:2 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

134. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:3 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

135. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:4 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

136. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:5 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

137. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:6 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

138. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:7 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

139. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:8 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

140. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:9 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

141. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:10 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

142. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:11 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

143. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:12 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

144. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:13 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

145. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:14 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

146. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:15 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

147. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:16 with the proviso that each X is a codable amino acid and at least one X is a cysteine.

148. The recombinant Con A polypeptide of any one of claims 133-147, wherein each X that is not a cysteine is the corresponding amino acid from SEQ ID NO:1.

149. The recombinant Con A polypeptide of any one of claims 133-147, wherein the lectins include a single cysteine.

150. The recombinant Con A polypeptide of any one of claims 133-147, wherein one of the X positions is a cysteine and all other X positions are the corresponding amino acid from SEQ ID NO:1.

151. A recombinant Con A polypeptide that has an amino acid sequence that is at least 90%, identical to the amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 with the proviso that in each of these sequences each X is a codable amino acid and at least one X is a cysteine.

152. A recombinant Con A polypeptide that has an amino acid sequence that is at least 90%, identical to the amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 where just one of the X positions is a cysteine and all other X positions are codable amino acids.

153. A recombinant Con A polypeptide that has an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO:17 with the proviso that at least one X is a cysteine.

154. The recombinant Con A polypeptide of claim 153, wherein the recombinant Con A polypeptide includes a single cysteine.

155. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:17 with the proviso that at least one X is a cysteine.

156. The recombinant Con A polypeptide of claim 155, wherein the recombinant Con A polypeptide has the amino acid sequence of SEQ ID NO:17 where X at position 100 is cysteine.

157. The recombinant Con A polypeptide of claim 156, wherein X at position 101 is lysine.

158. The recombinant Con A polypeptide of claim 155, wherein the recombinant Con A polypeptide has the amino acid sequence of SEQ ID NO:17 where X at position 101 is cysteine.

159. The recombinant Con A polypeptide of claim 158, wherein X at position 100 is tyrosine.

160. A recombinant Con A polypeptide that has an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO:18 with the proviso that at least one X is a cysteine.

161. The recombinant Con A polypeptide of claim 160, wherein the recombinant Con A polypeptide includes a single cysteine.

162. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:18 with the proviso that at least one X is a cysteine.

163. The recombinant Con A polypeptide of claim 162, wherein X at position 117 is cysteine.

164. The recombinant Con A polypeptide of claim 163, wherein X at position 120 is histidine.

165. The recombinant Con A polypeptide of claim 162, wherein X at position 120 is cysteine.

166. The recombinant Con A polypeptide of claim 165, wherein X at position 117 is asparagine.

167. The recombinant Con A polypeptide of claim 162, wherein X at position 117 is cysteine and X at position 120 is cysteine.

168. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:19.

169. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:20.

170. A recombinant Con A polypeptide that has the amino acid sequence of SEQ ID NO:21.

171. The recombinant Con A polypeptide of any one of claims 128-170 wherein at least one affinity ligand which is capable of competing with glucose for binding with the glucose binding site of Con A is covalently linked to the cysteine.

172. The recombinant Con A polypeptide of claim 171, wherein the affinity ligand includes a saccharide.

173. The recombinant Con A polypeptide of claim 172, wherein the saccharide is glucose.

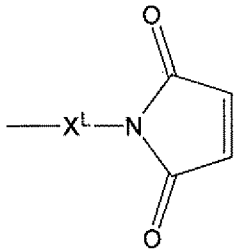
174. The recombinant Con A polypeptide of claim 172, wherein the saccharide is mannose.

175. The recombinant Con A polypeptide of claim 172, wherein the affinity ligand includes a saccharide and a linker and the saccharide is covalently linked to the linker via an anomeric carbon.

176. The recombinant Con A polypeptide of claim 175, wherein the anomeric carbon is an alpha anomer.

177. The recombinant Con A polypeptide of claim 175, wherein the affinity ligand was covalently linked to the recombinant Con A polypeptide using a reactive linker with a terminal maleimide group that reacts with the sulfhydryl group of the cysteine.

178. The recombinant Con A polypeptide of claim 177, wherein the affinity ligand was covalently linked to the recombinant Con A polypeptide using a reactive linker of the formula:



(II)

wherein:

X^L is a covalent bond or a bivalent, straight or branched, saturated or unsaturated, optionally substituted C_{1-100} hydrocarbon chain wherein one or more methylene units of X^L are optionally and independently replaced by -O-, -S-, -N(R')-, -C(O)-, -C(O)O-, -OC(O)-, -N(R')C(O)-, -C(O)N(R')-, -S(O)-, $-S(O)_2-$, $-N(R')SO_2-$, $-SO_2N(R')-$, a carbocyclic group, a heterocyclic group, an aryl group, or a heteroaryl group; and

each occurrence of R' is independently hydrogen, a suitable protecting group, or an acyl moiety, arylalkyl moiety, aliphatic moiety, aryl moiety, heteroaryl moiety, or heteroaliphatic moiety.

179. The recombinant Con A polypeptide of claim 178, wherein 1-5 methylene units of X^L are optionally and independently replaced.

180. The recombinant Con A polypeptide of claim 178, where X^L is constructed from a C_{1-20} hydrocarbon chain.

181. The recombinant Con A polypeptide of claim 178, wherein one or more methylene units of X^L is replaced by a carbocyclic group.

182. The recombinant Con A polypeptide of claim 178, wherein one or more methylene units of X^L is replaced by a cyclohexyl moiety.

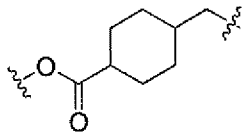
183. The recombinant Con A polypeptide of claim 178, wherein one or one or more methylene units of X^L is replaced by a benzene ring.

184. The recombinant Con A polypeptide of claim 178, wherein one or more methylene units of X^L is replaced by -C(O)-.

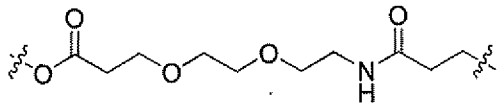
185. The recombinant Con A polypeptide of claim 178, wherein one or more methylene units of X^L is replaced by -C(O)N(R')-.

186. The recombinant Con A polypeptide of claim 178, wherein one or more methylene units of X^L is replaced by -O-.

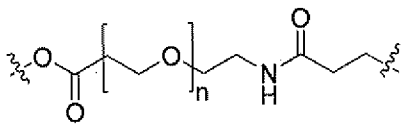
187. The recombinant Con A polypeptide of claim 178, wherein X^L contains a PEG moiety.

188. The recombinant Con A polypeptide of claim 178, wherein X^L is .

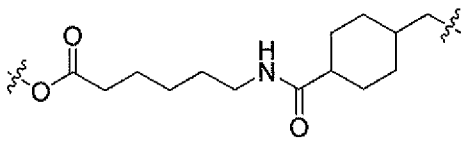
189. The recombinant Con A polypeptide of claim 178, wherein X^L is

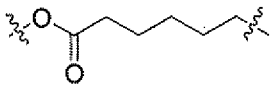
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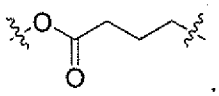
190. The recombinant Con A polypeptide of claim 178, wherein X^L is

, wherein n is 2, 4, 6, 8, 12, or 24.

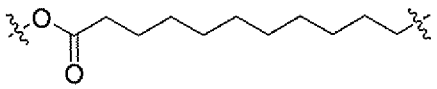
191. The recombinant Con A polypeptide of claim 178, wherein X^L is

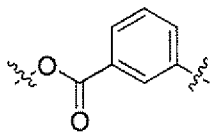


192. The recombinant Con A polypeptide of claim 178, wherein X^L is .

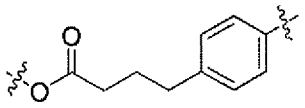
193. The recombinant Con A polypeptide of claim 178, wherein X^L is .

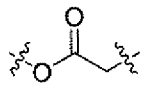
194. The recombinant Con A polypeptide of claim 178, wherein X^L is

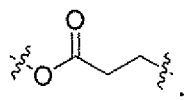


195. The recombinant Con A polypeptide of claim 178, wherein X^L is .

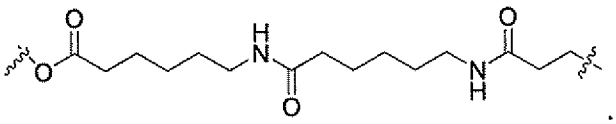
196. The recombinant Con A polypeptide of claim 178, wherein X^L is



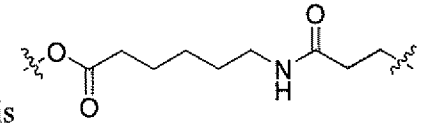
197. The recombinant Con A polypeptide of claim 178, wherein X^L is .

198. The recombinant Con A polypeptide of claim 178, wherein X^L is .

199. The recombinant Con A polypeptide of claim 178, wherein X^L is



200. The recombinant Con A polypeptide of claim 178, wherein X^L is



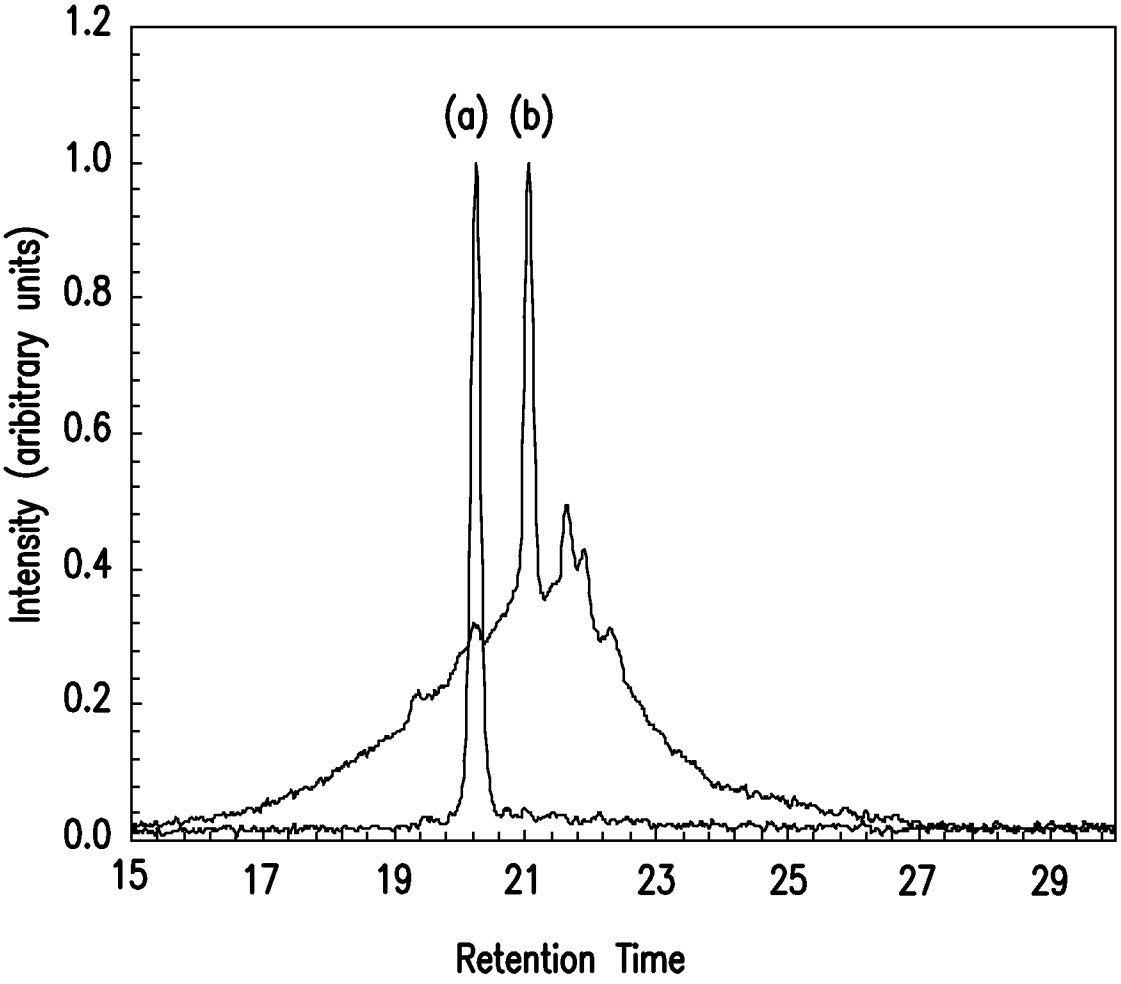

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Intensity (aribitrary units)
(a) (b)
15
17
19
21
23
25
27
29
Retention Time

FIG. 1

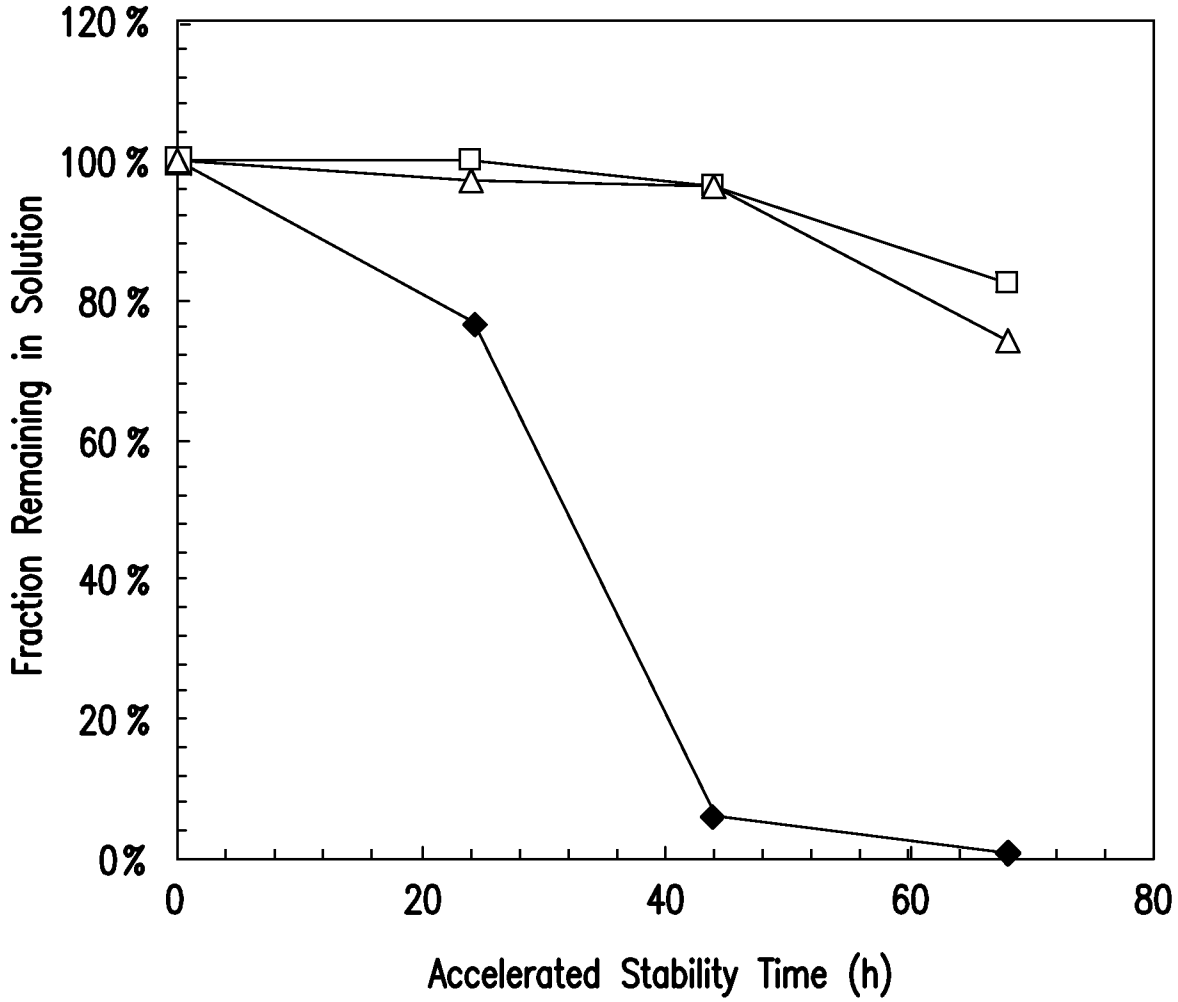

120 %
100 %
80 %
60 %
40 %
20 %
0%
Fraction Remaining in Solution
0
20
40
60
80
Accelerated Stability Time (h)

FIG.2

IPC Peak Retention Times, %Free Insulin, and %Desamido Insulin

Sample	Peak 1 RT[1] (min)	Peak 2 RT(min)	Peak 3 RT(min)	% Purity IPC	% Free Insulin	% Desamido Insulin
HS-1-60-1 (fresh)	20.62	–	–	>99	<0.1	<0.1
HS-1-60-1 (AST, PBS)	20.61	19.19	–	85	<0.1	<0.1
HS-1-60-1 (AST, HEPES)	20.65	–	–	>99	<0.1	<0.1
TL-13-85 (fresh)	20.46	–	–	>99	<0.1	<0.1
TL-13-85 (AST, PBS)	20.40	19.84	18.91	30	<0.1	<0.1
TL-13-85 (AST, HEPES)	20.46	–	–	>99	<0.1	<0.1

[1] C8 reverse phase column, water:acetonitrile (0.1% TFA) elution gradient, RT = Retention Time

FIG.3A

Mass Spectroscopy Data on IPC's and IPC Breakdown Products

Sample	Peak 1 MW (Da)	Peak 2 MW (Da)	Peak 3 MW (Da)
HS-1-60-1 (fresh)	6730[2]	–	–
HS-1-60-1 (AST, PBS)	6730	5947	–
HS-1-60-1 (AST, HEPES)	6730	–	–
TL-13-85 (fresh)	6829[2]	–	–
TL-13-85 (AST, PBS)	6829	5944	7928
TL-13-85 (AST, HEPES)	6829	–	–

[2]The two IPC's shown here have different structures and, therefore, different MW's

FIG.3B

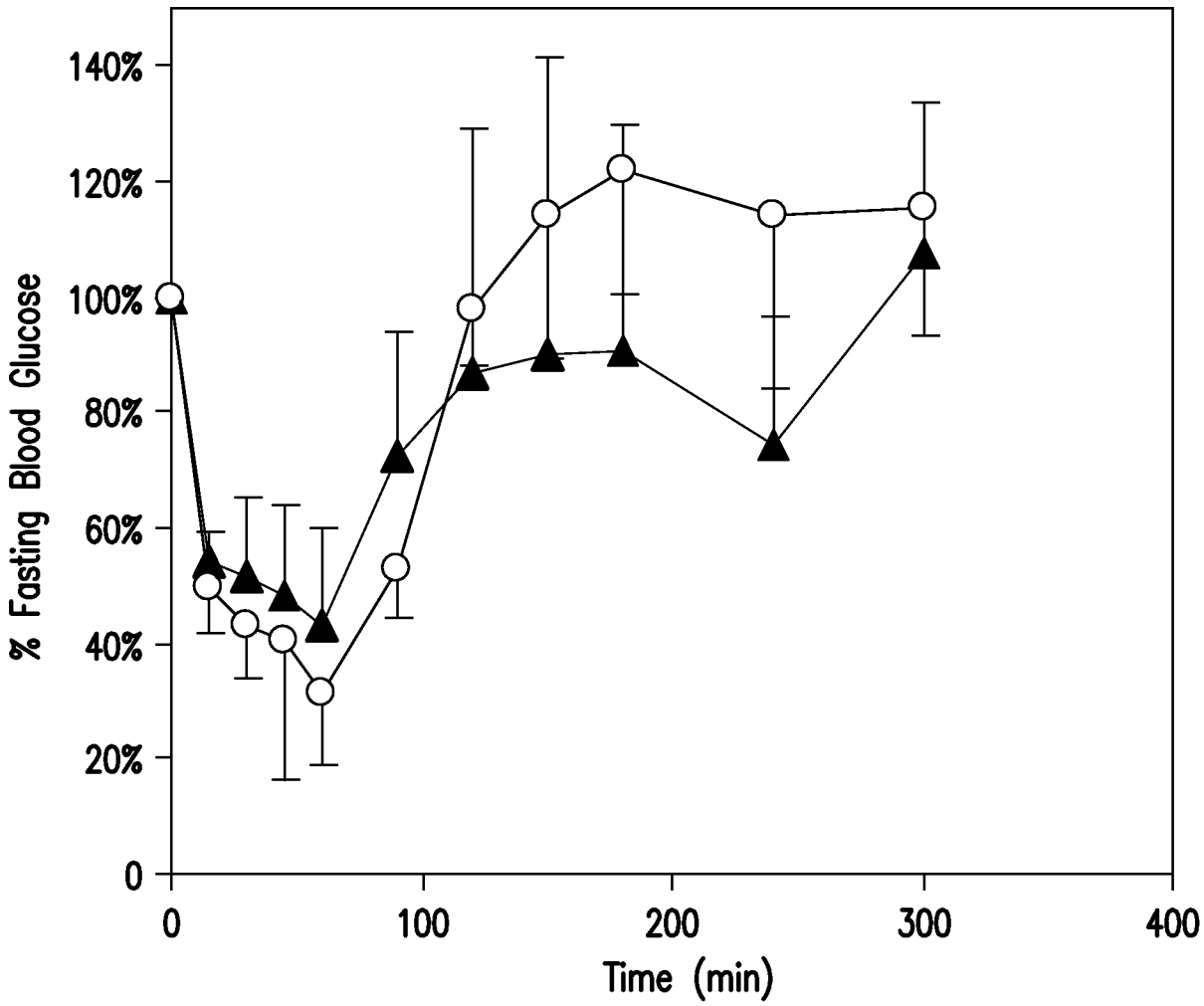

140%
120%
100%
80%
60%
40%
20%
0
% Fasting Blood Glucose
0
100
200
300
400
Time (min)

FIG.4

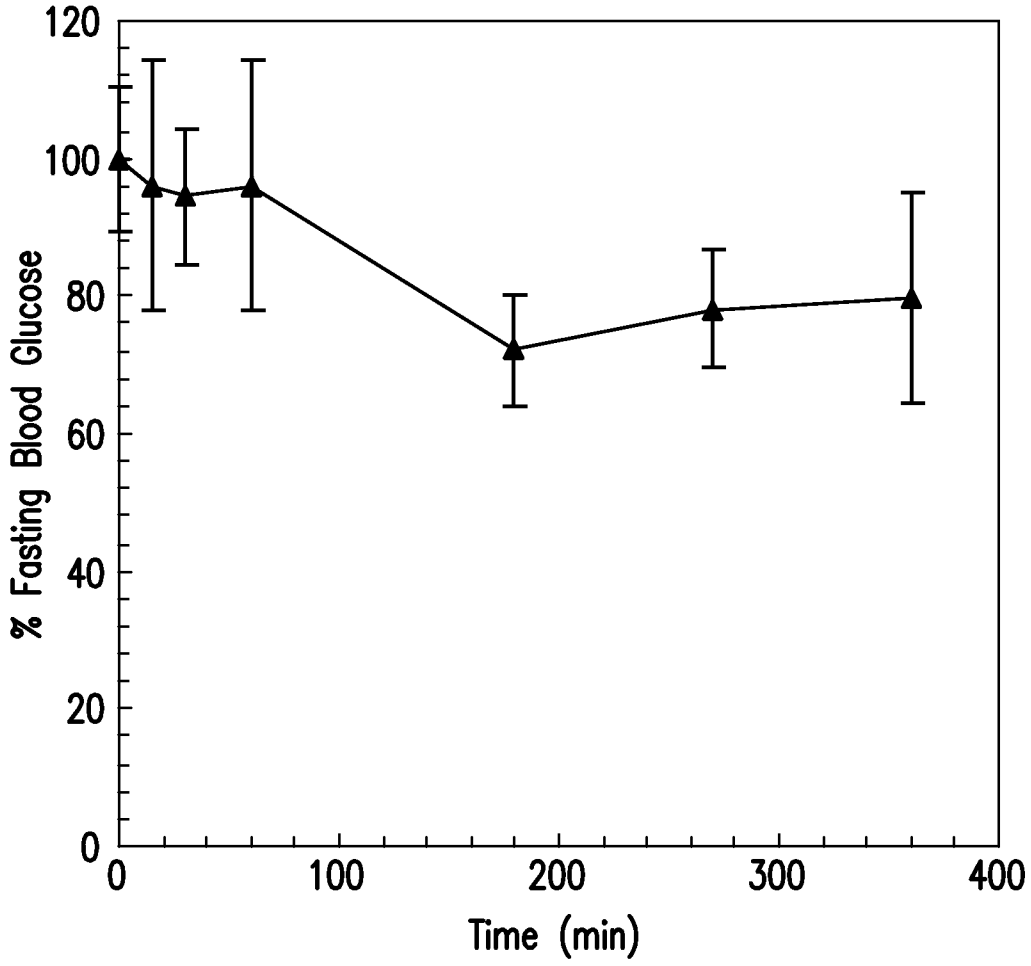

120
100
80
60
40
20
0
% Fasting Blood Glucose
0
100
200
300
400
Time (min)

FIG.5

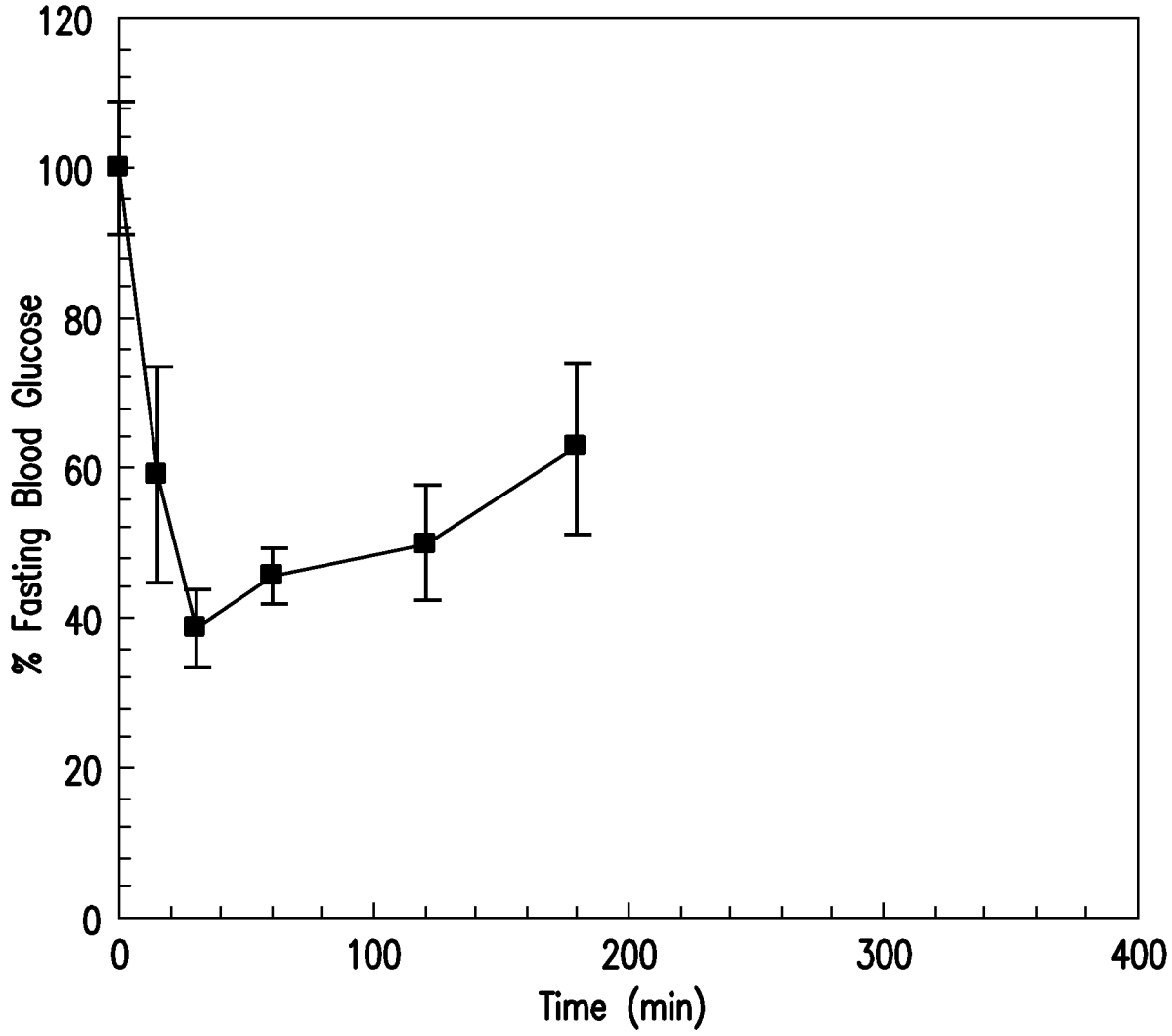

120
100
80
60
40
20
0
% Fasting Blood Glucose
0
100
200
300
400
Time (min)

FIG.6

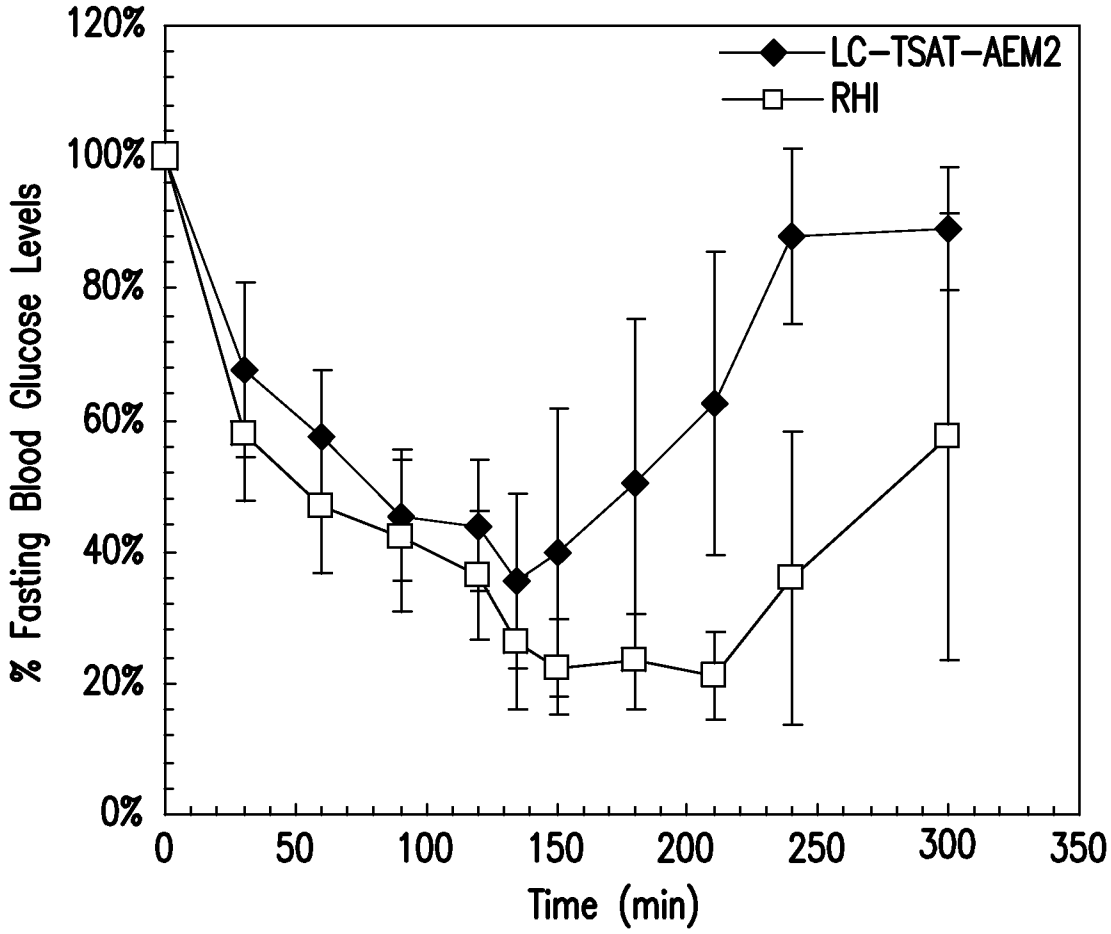

120%
100%
80%
60%
40%
20%
0%
% Fasting Blood Glucose Levels
0
50
100
150
200
250
300
350
Time (min)
LC-TSAT-AEM2
RHI

FIG.7

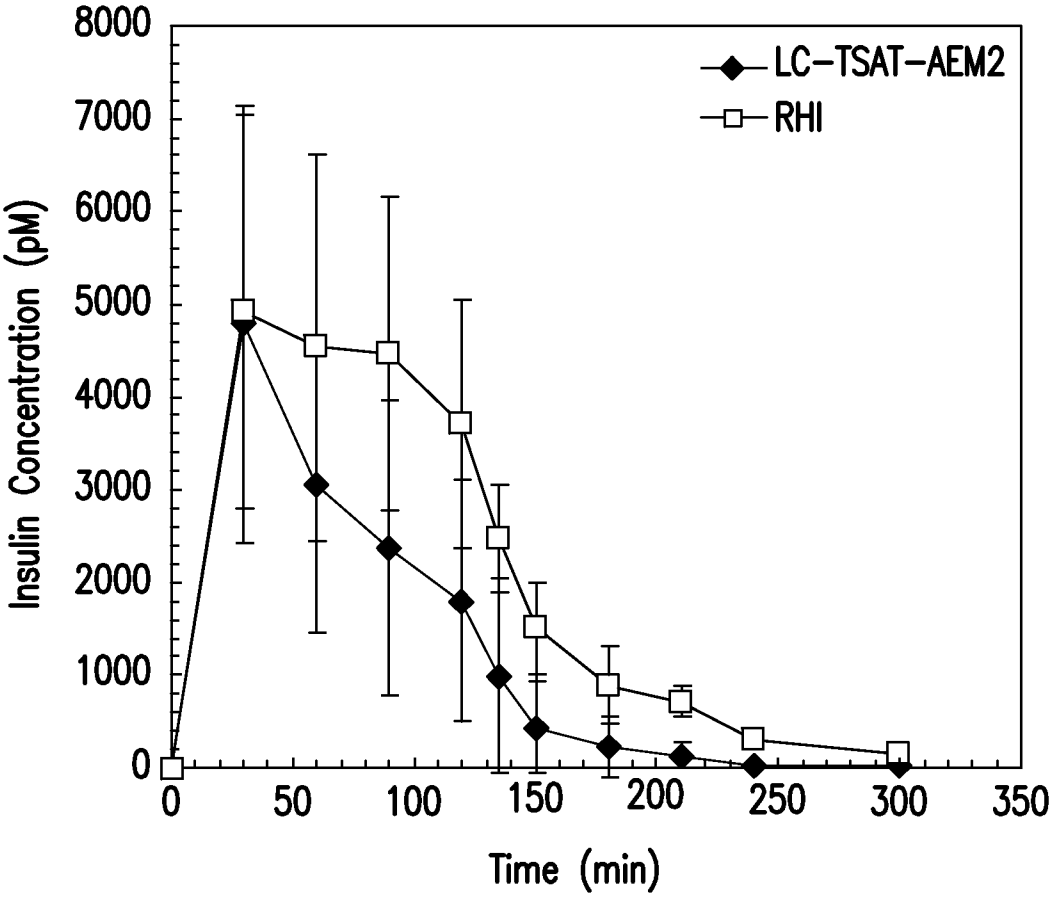

8000
7000
6000
5000
4000
3000
2000
1000
0
Insulin Concentration (pM)
0
50
100
150
200
250
300
350
Time (min)
LC-TSAT-AEM2
RHI

FIG.8

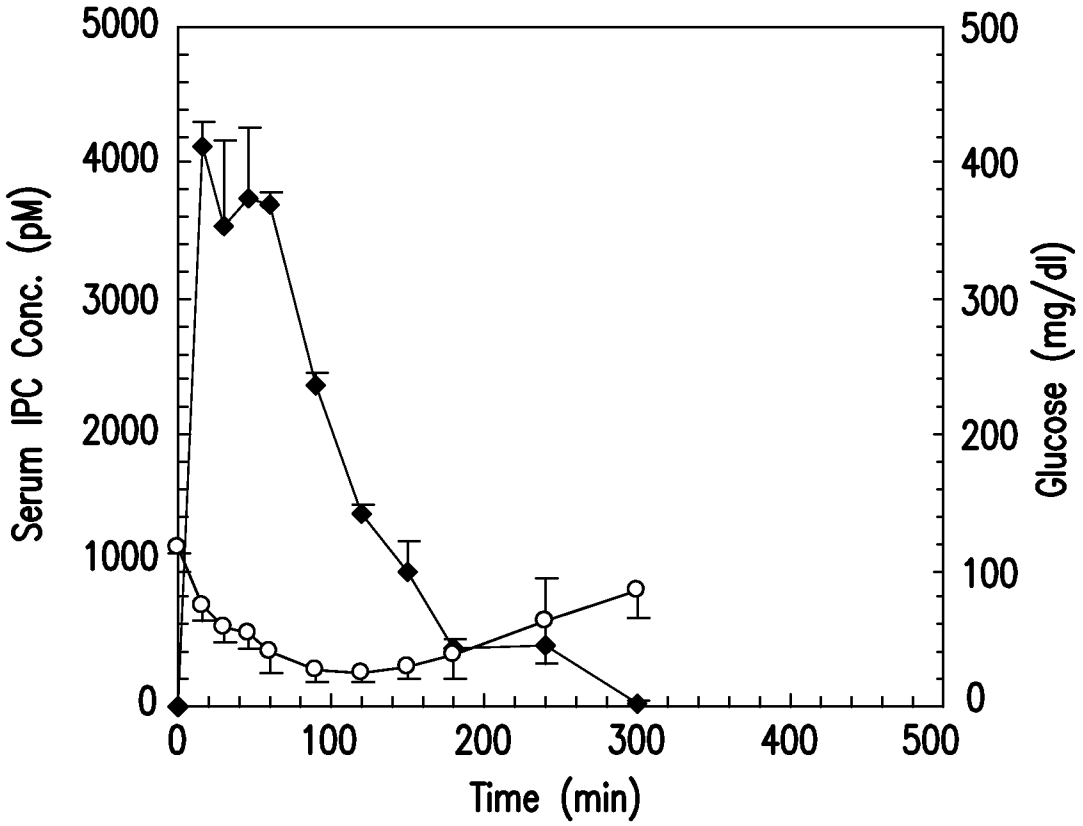

5000
4000
3000
2000
1000
0
Serum IPC Conc. (pM)
500
400
300
200
100
0
Glucose (mg/dl)
0
100
200
300
400
500
Time (min)

FIG.9

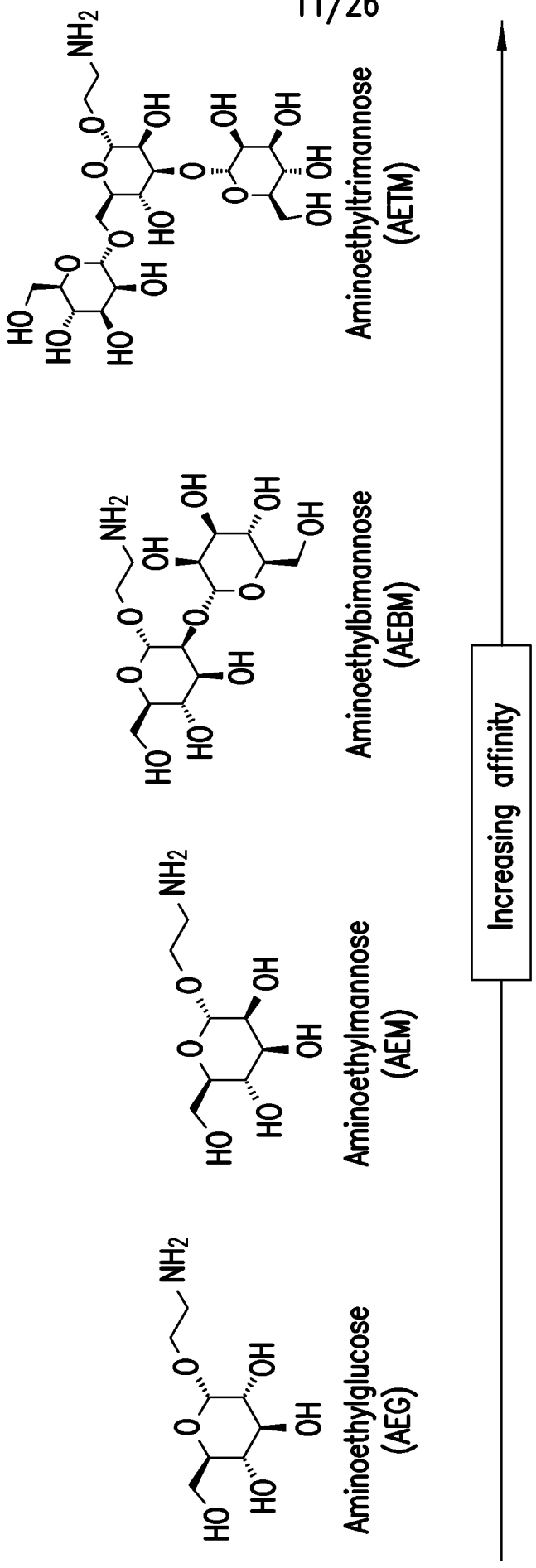

Aminoethylglucose
(AEG)
Aminoethylmannose
(AEM)
Aminoethylbimannose
(AEBM)
Aminoethyltrimannose
(AETM)
Increasing affinity

FIG. 10

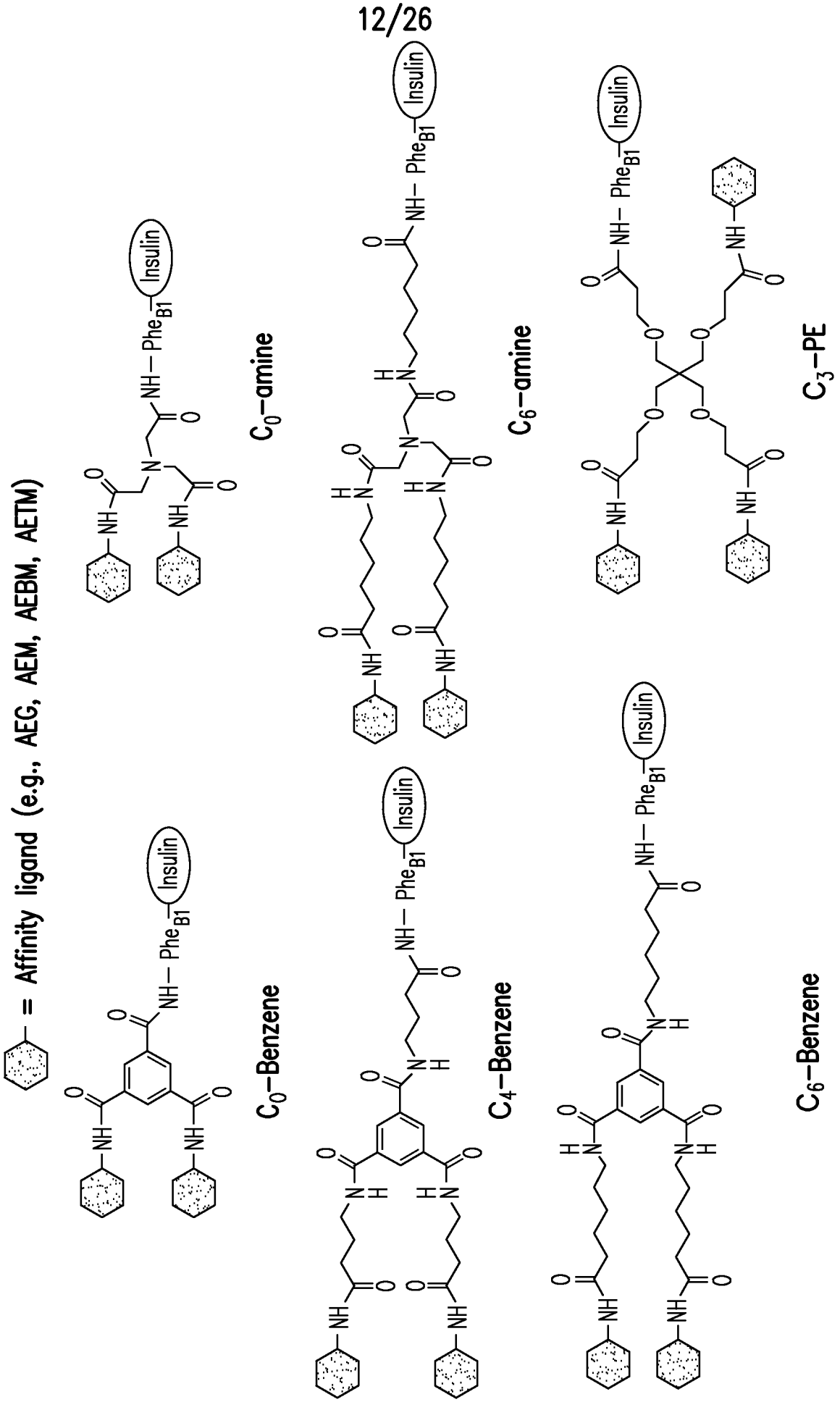

= Affinity ligand (e.g., AEG, AEM, AEBM, AETM)
Insulin
NH– PheB1
C0–amine
C0–Benzene
C6–amine
C4–Benzene
C3–PE
C6–Benzene

FIG.11

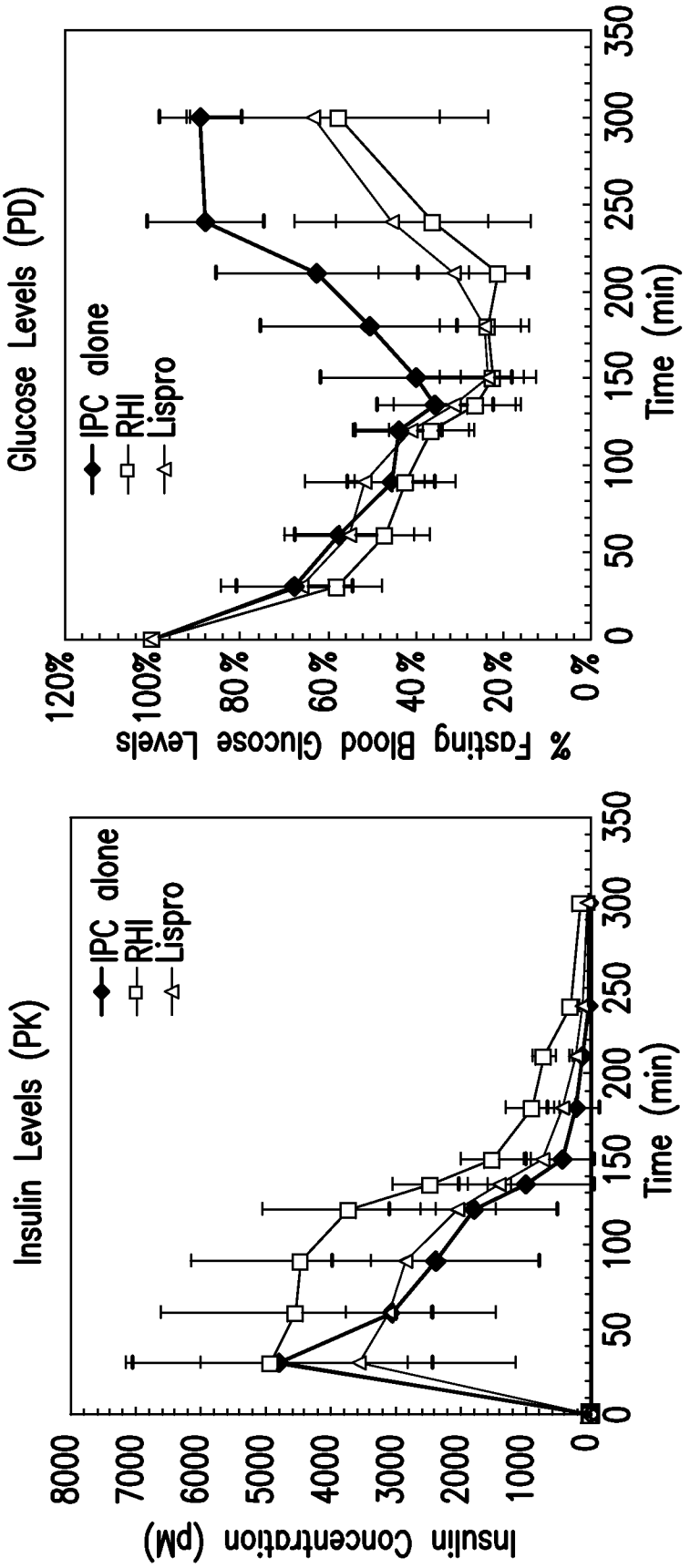

Insulin Levels (PK)
Insulin Concentration (pM)
8000
7000
6000
5000
4000
3000
2000
1000
0
0
50
100
150
200
250
300
350
Time (min)
IPC alone
RHI
Lispro
Glucose Levels (PD)
% Fasting Blood Glucose Levels
120%
100%
80%
60%
40%
20%
0%
0
50
100
150
200
250
300
350
Time (min)
IPC alone
RHI
Lispro

FIG.12

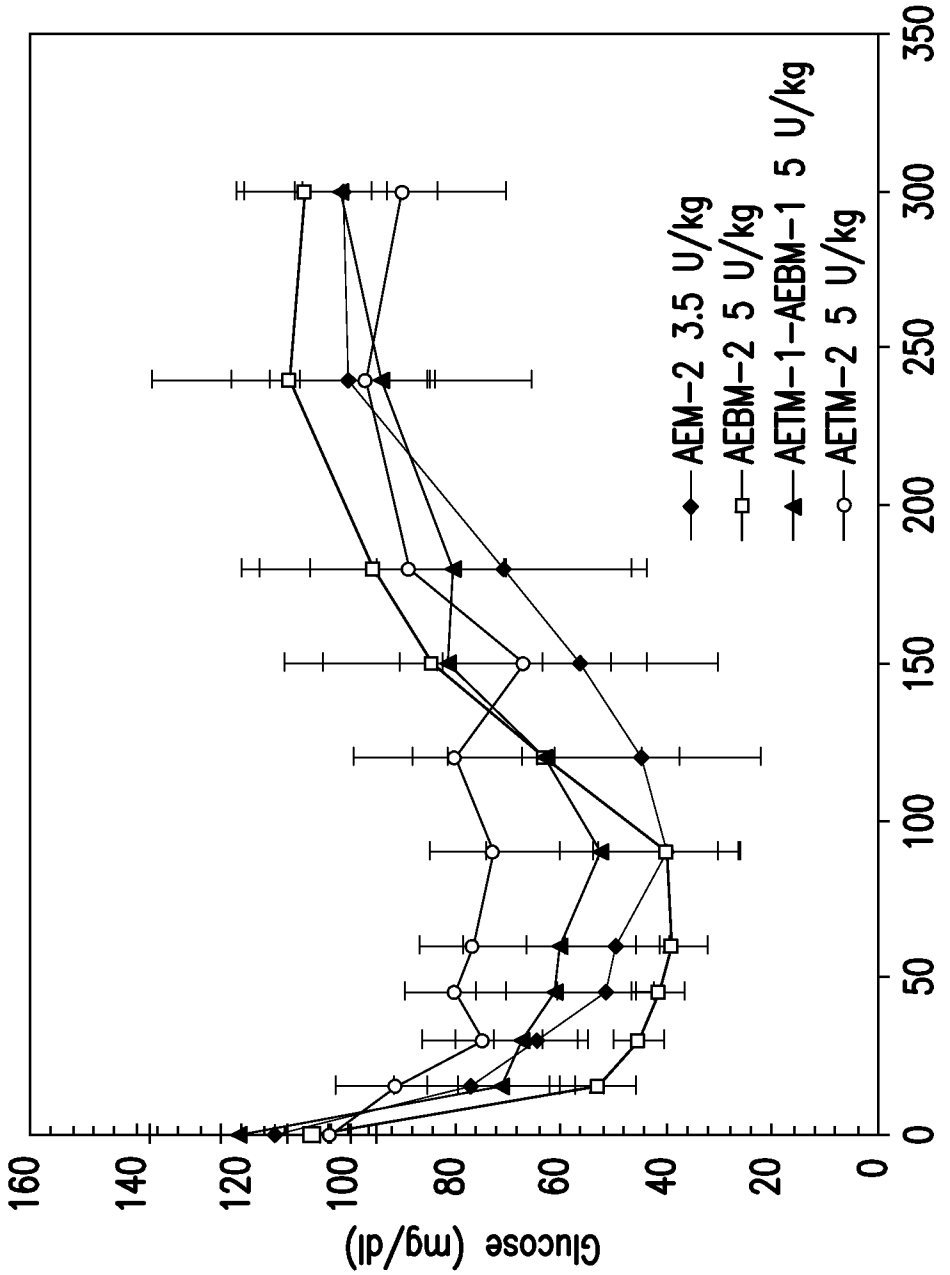

Glucose (mg/dl)
160
140
120
100
80
60
40
20
0
0
50
100
150
200
250
300
350
Time (min)
AEM-2 3.5 U/kg
AEBM-2 5 U/kg
AETM-1-AEBM-1 5 U/kg
AETM-2 5 U/kg

FIG.13

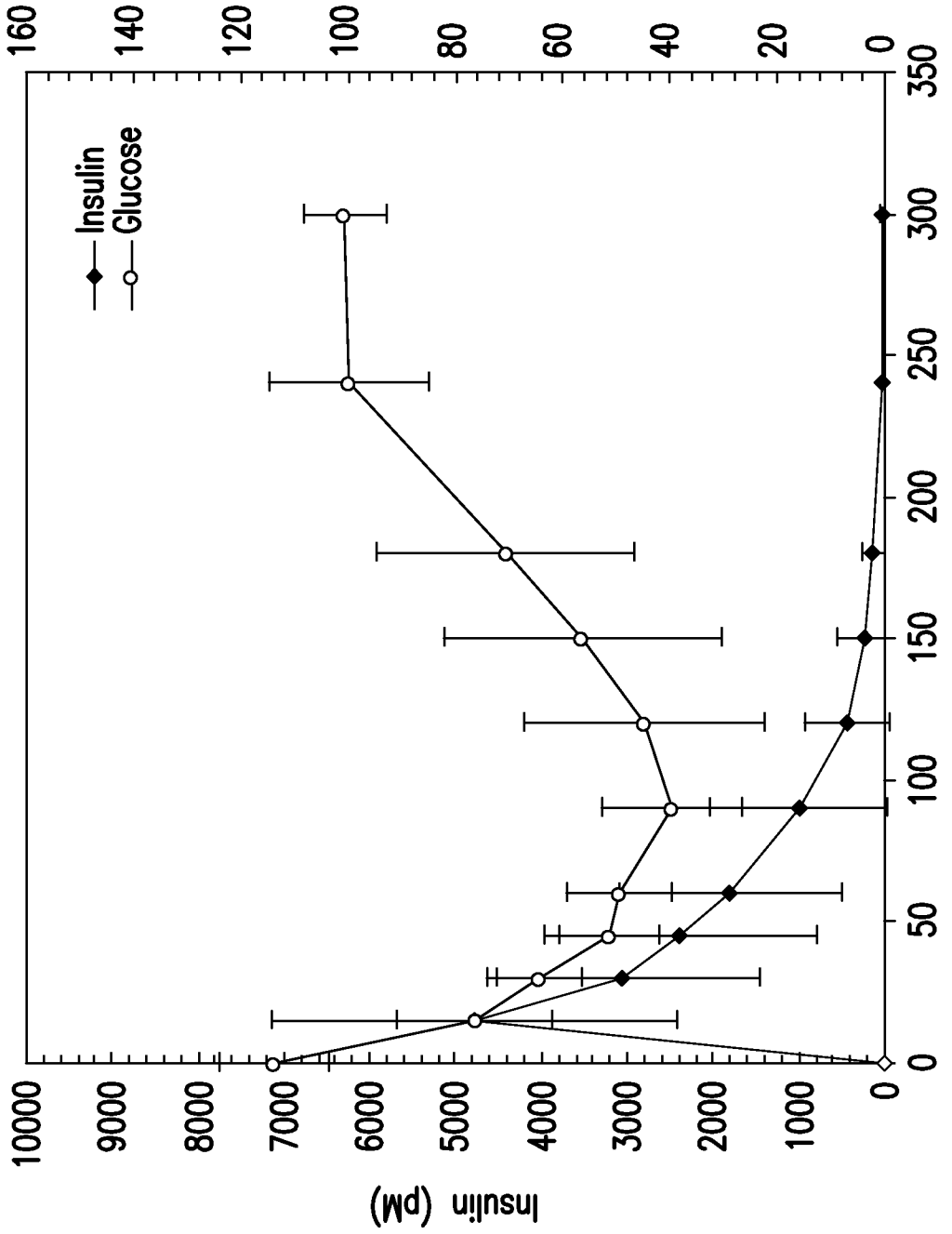

Insulin
Glucose
Insulin (pM)
Time (min)
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
0
20
40
60
80
100
120
140
160
0
50
100
150
200
250
300
350

FIG.14

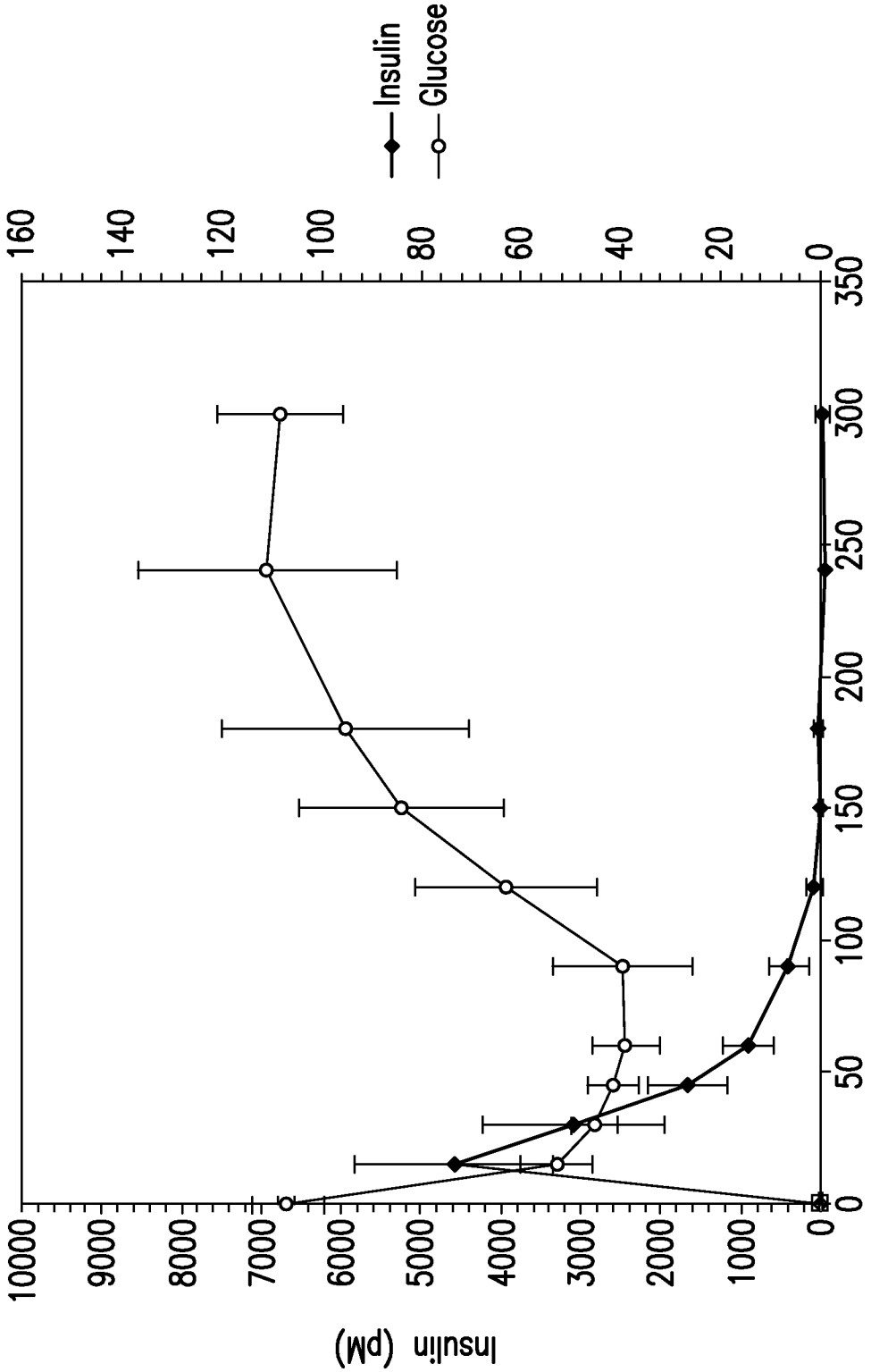

Insulin
Glucose
160
140
120
100
80
60
40
20
0
350
300
250
200
150
100
50
0
Time (min)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Insulin (pM)

FIG.15

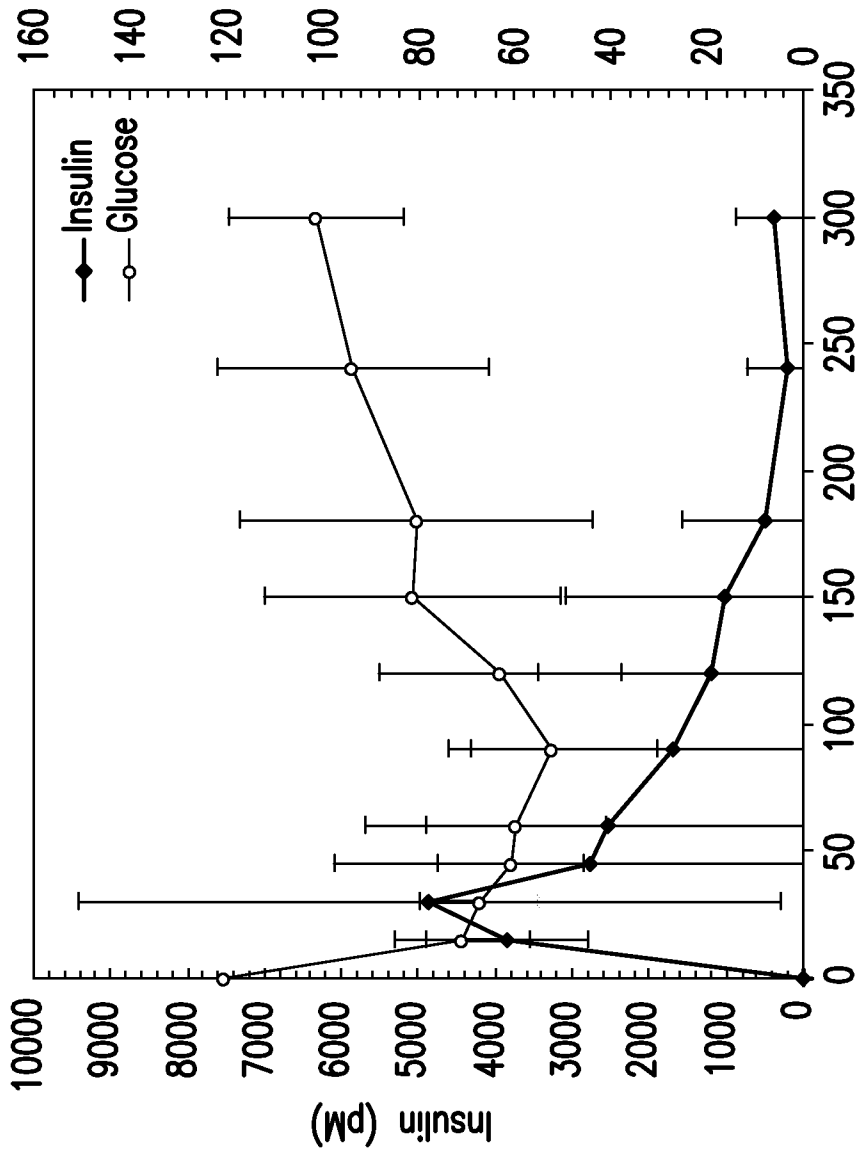

Insulin
Glucose
Insulin (pM)
Time (min)
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
0
20
40
60
80
100
120
140
160
0
50
100
150
200
250
300
350

FIG.16

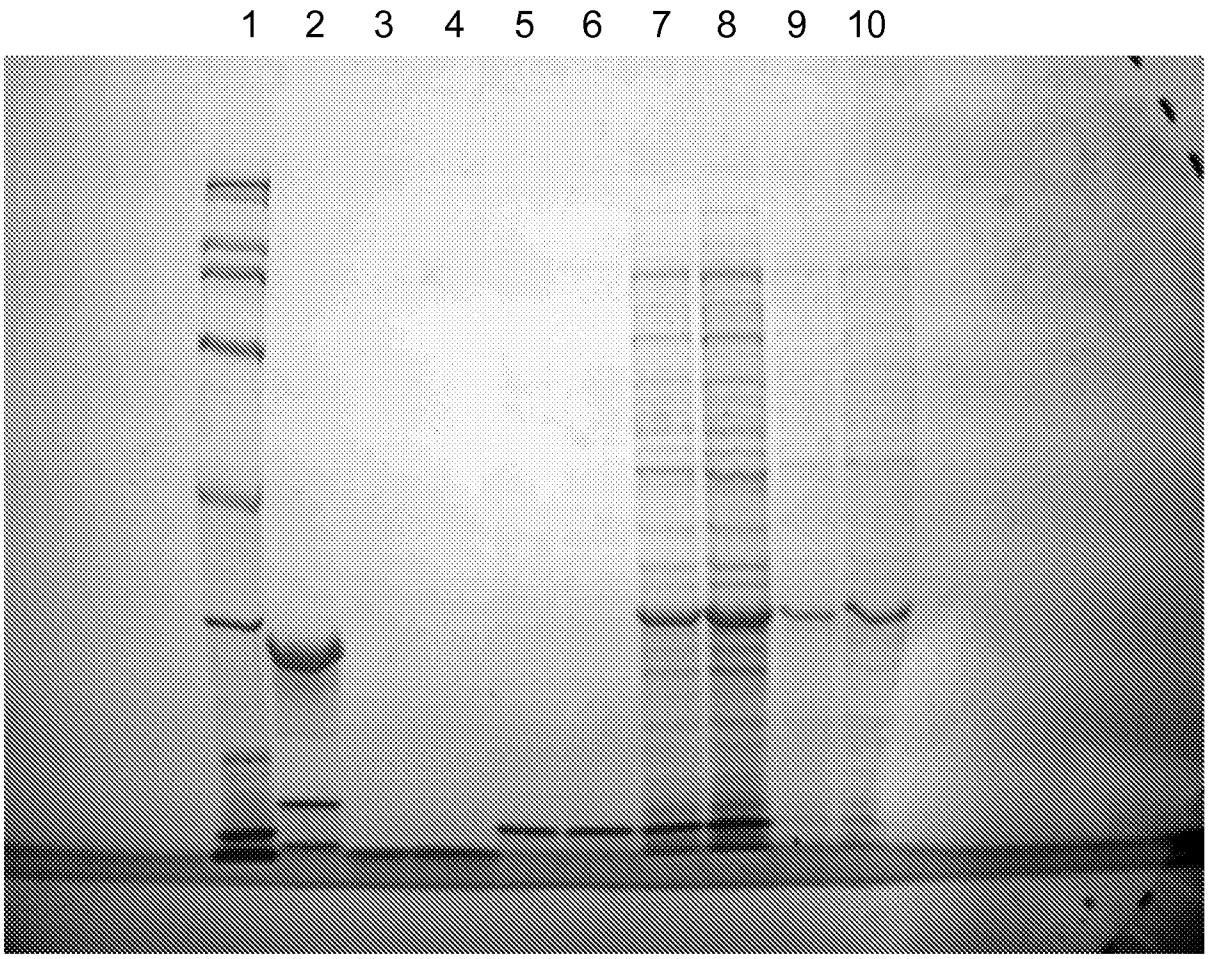


FIG.17

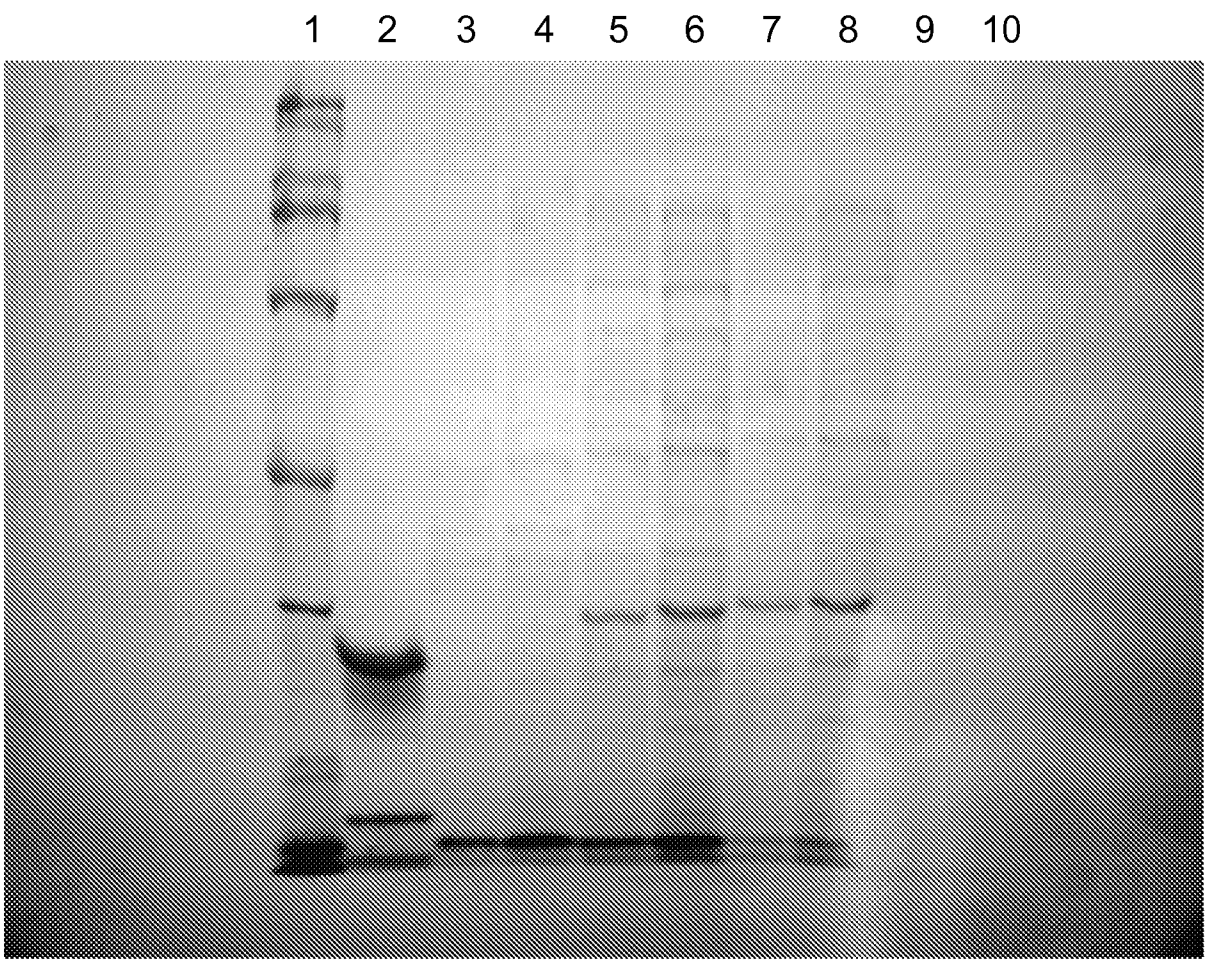


FIG.18

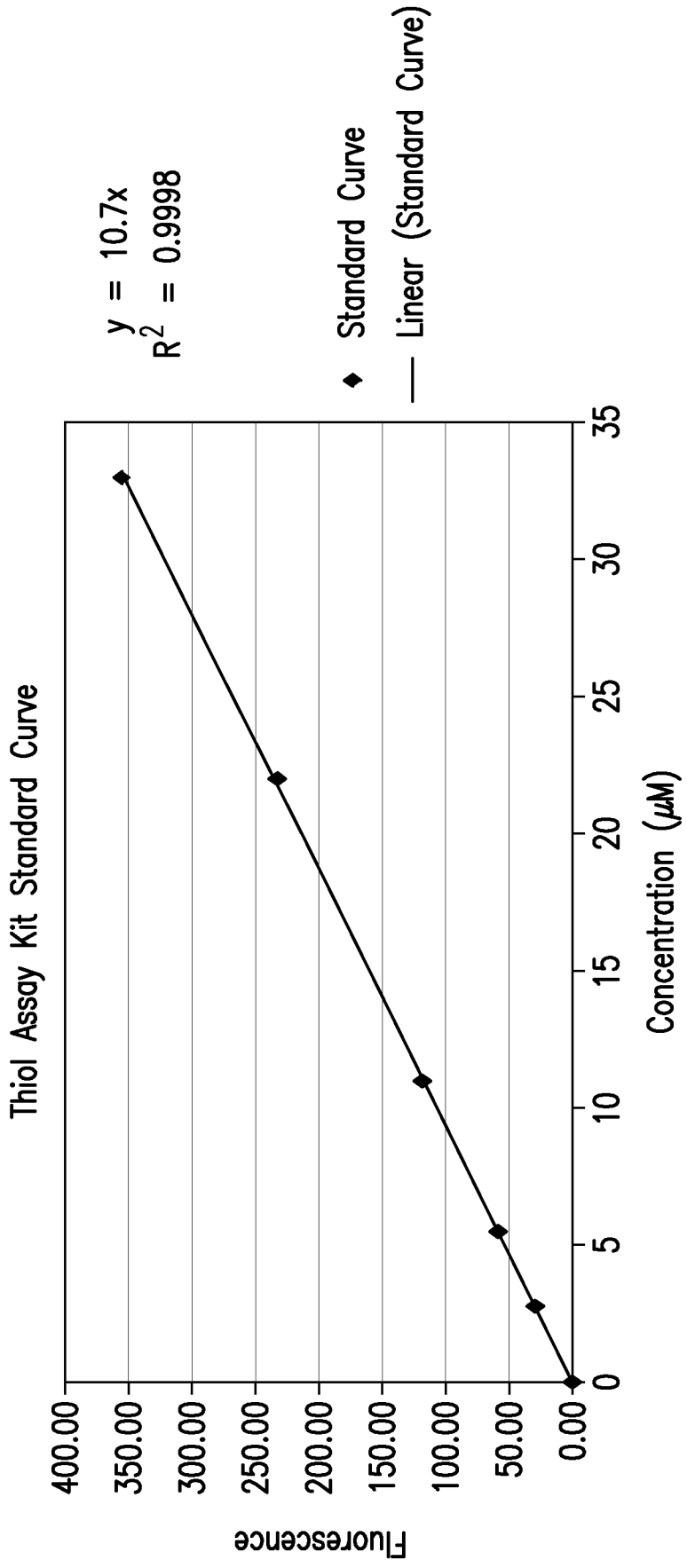

Thiol Assay Kit Standard Curve
y = 10.7x
R2 = 0.9998
Standard Curve
Linear (Standard Curve)
Fluorescence
Concentration (μM)
0.00
50.00
100.00
150.00
200.00
250.00
300.00
350.00
400.00
0
5
10
15
20
25
30
35

FIG.19

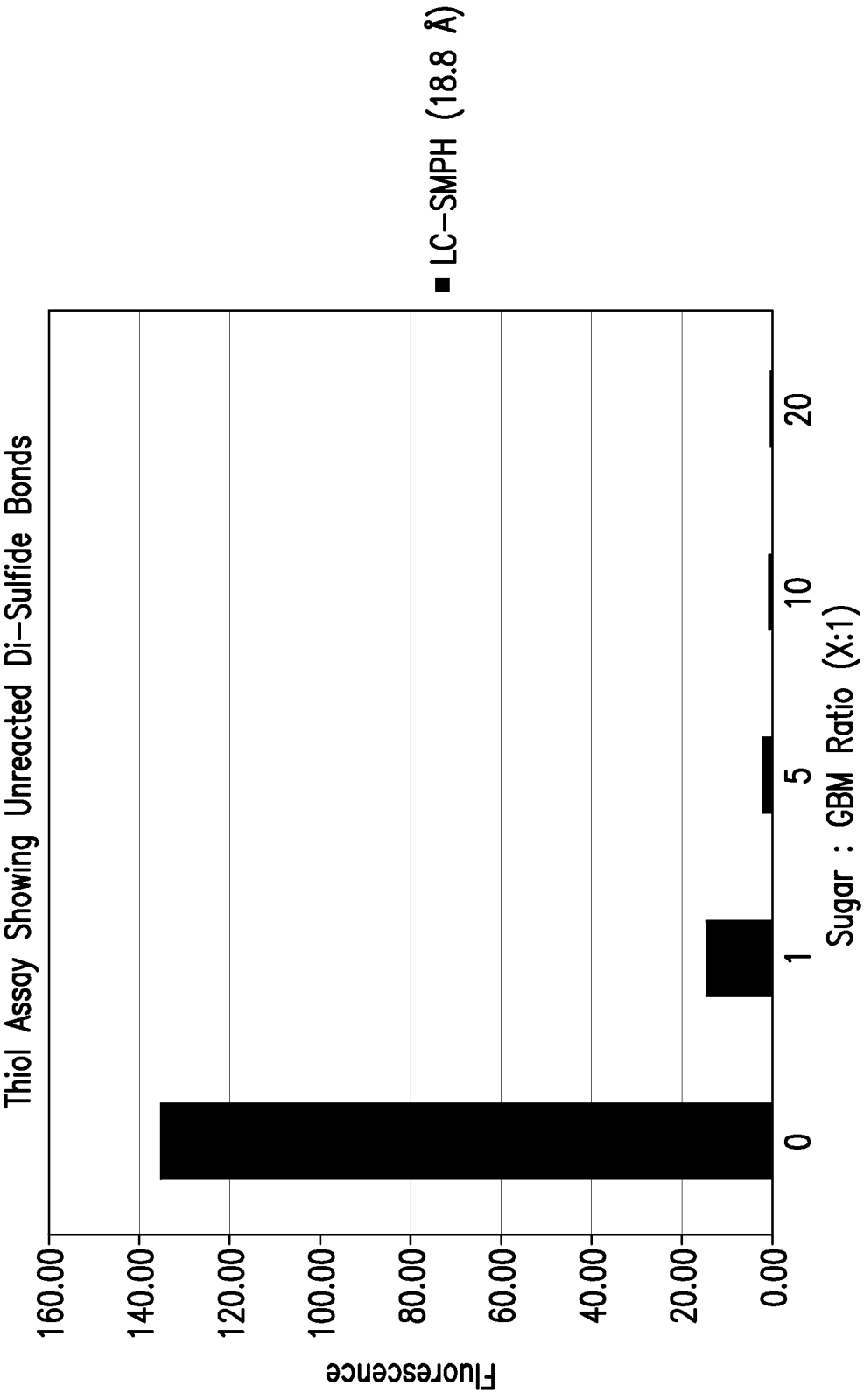

Thiol Assay Showing Unreacted Di-Sulfide Bonds
■ LC-SMPH (18.8 Å)
Fluorescence
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
0
1
5
10
20
Sugar : GBM Ratio (X:1)

FIG.20

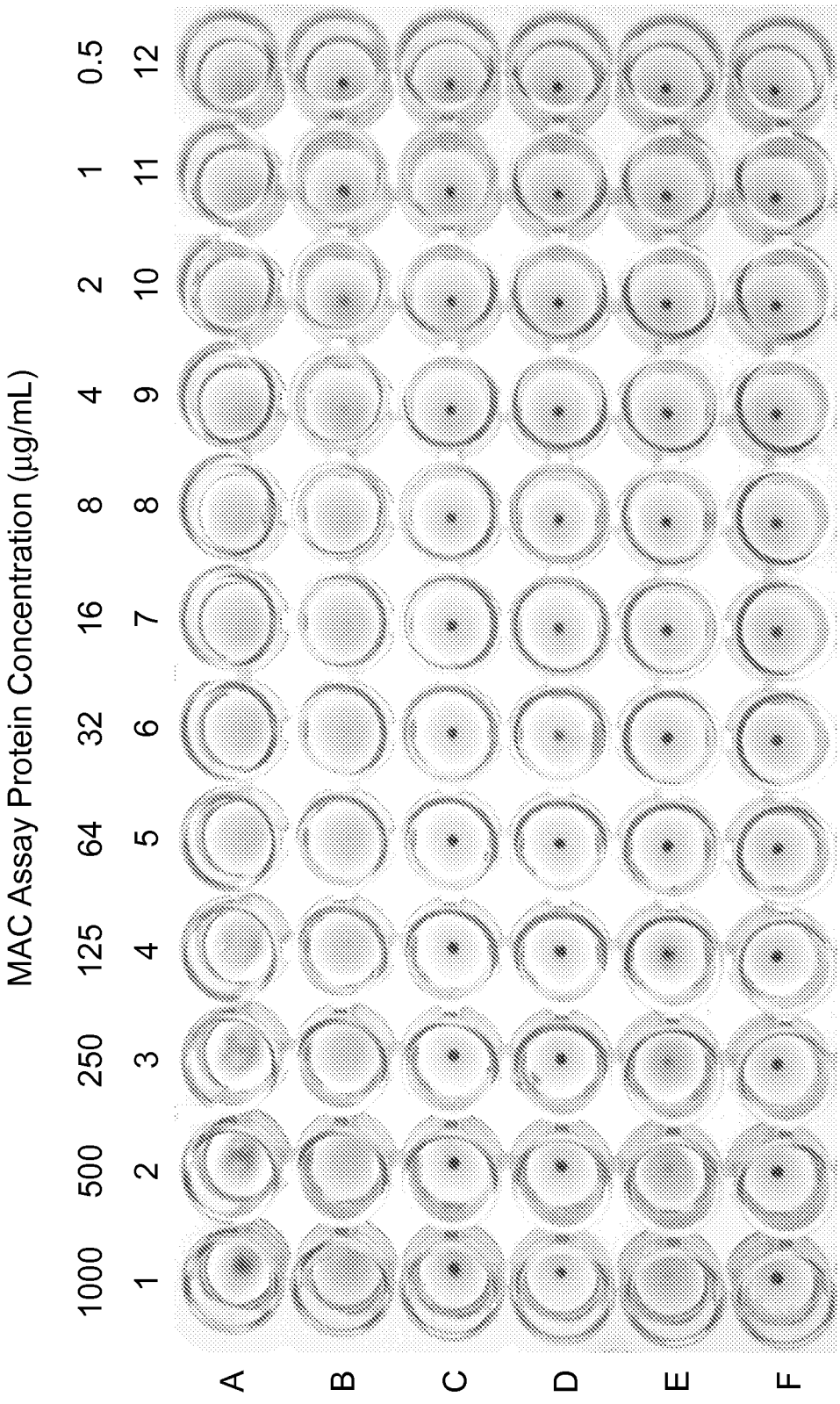

MAC Assay Protein Concentration (µg/mL)
1000 500 250 125 64 32 16 8 4 2 1 0.5
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F

FIG.21

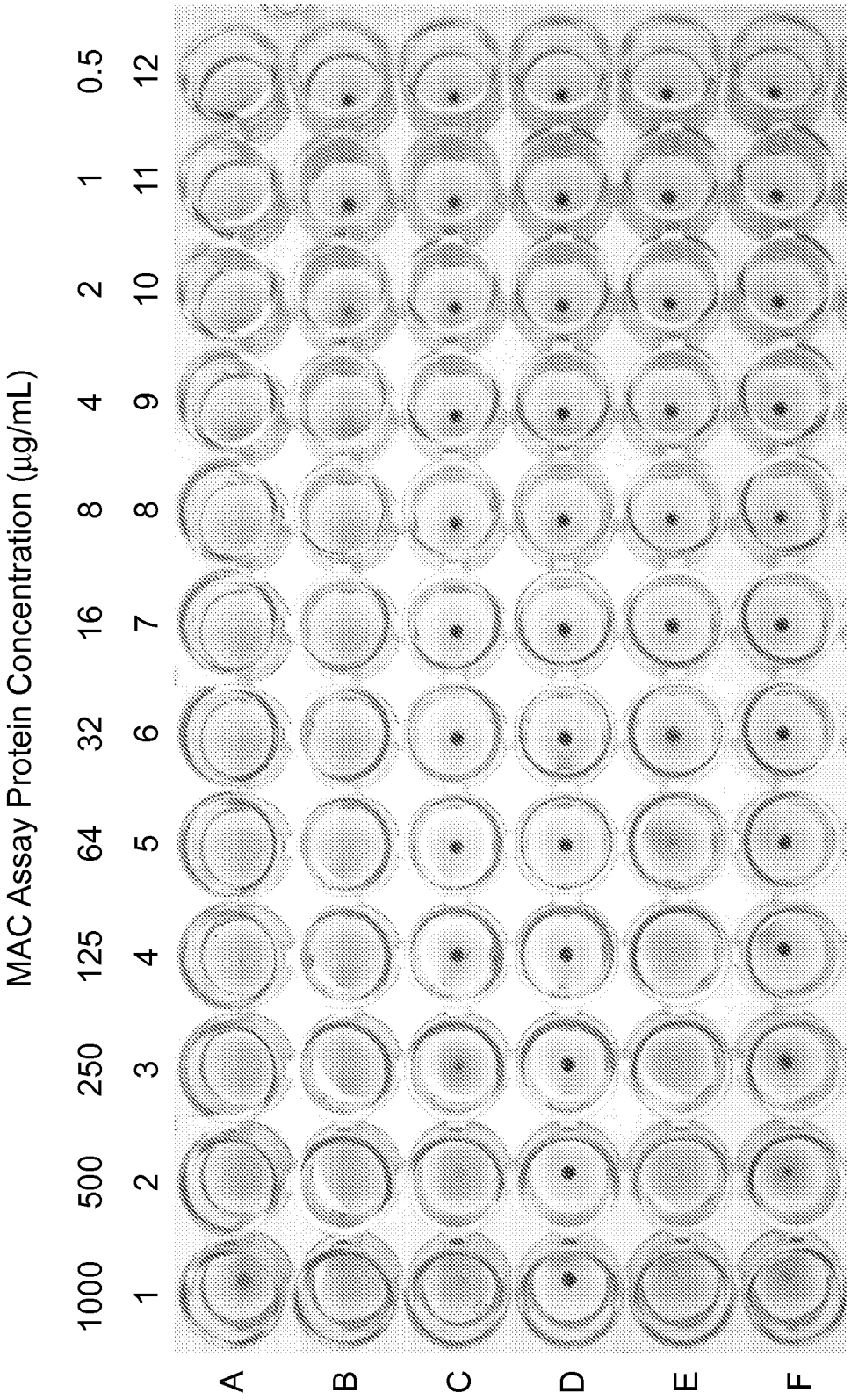

MAC Assay Protein Concentration (µg/mL)
1000 500 250 125 64 32 16 8 4 2 1 0.5
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F

FIG.22

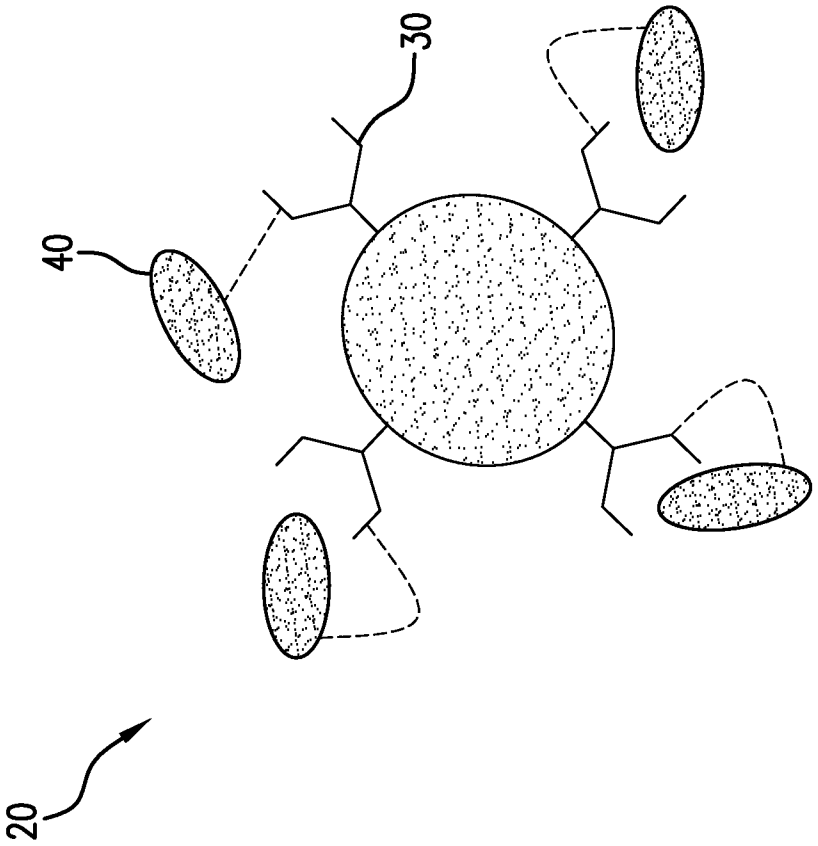


FIG.23

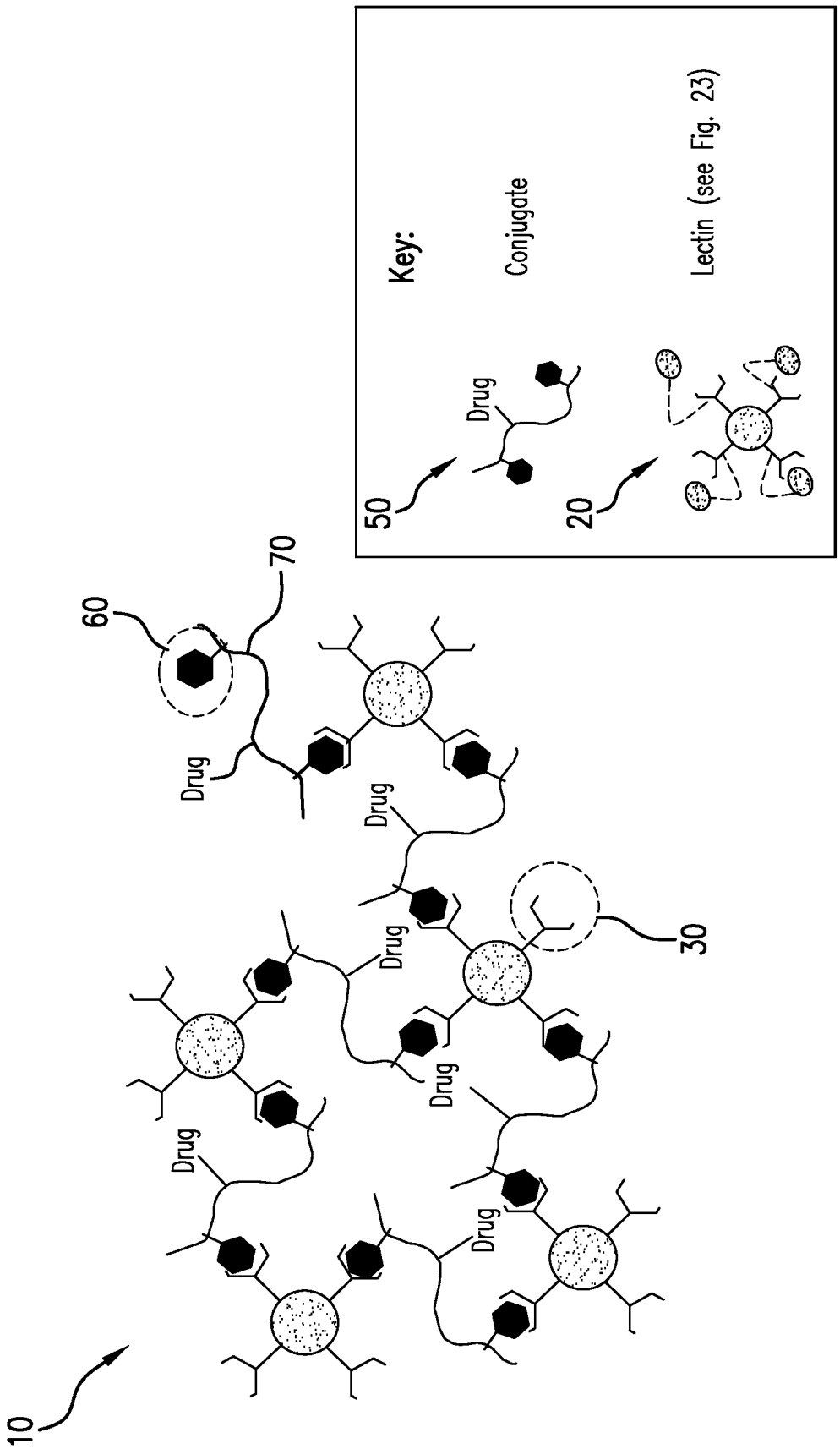


FIG.24

A-Peptide (SEQ ID NO:22)
Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
B-Peptide (SEQ ID NO:23)
Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Phe-Phe-Tyr-Thr-Pro-Lys-Thr
24 25 26 27 28 29 30

FIG.25