ANTIBODY COMPRISING A GLUTAMINE-CONTAINING LIGHT CHAIN C-TERMINAL EXTENSION, CONJUGATES THEREOF, AND METHODS AND USES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. §119(e) of US Provisional Application Ser. No. 62/773,708, filed November 30, 2018; the disclosure of which is incorporated herein by reference.

SEQUENCE LISTING

[0002] Incorporated herein by reference in its entirety is a Sequence Listing named "191017_SEQT_13142WOPCT_YC.txt," comprising SEQ ID NO:1 through SEQ ID NO:40, which include nucleic acid and/or amino acid sequences disclosed herein. The Sequence Listing has been submitted herewith in ASCII text format via EFS-Web, and thus constitutes both the paper and computer readable form thereof. The Sequence Listing was first created using PatentIn 3.5 on Oct. 26, 2018, and is approximately 14 KB in size.

BACKGROUND OF THE DISCLOSURE

[0003] This disclosure relates to modified antibodies conjugatable by the enzyme transglutaminase and conjugates made from such antibodies.

[0004] A type of biologic that has attracted intense current interest is one in which an antibody is covalently linked to a partner molecule ("conjugate" or "immunoconjugate"). Thus, a conjugate comprises three components: (1) the antibody, (2) the partner molecule, and (3) a linker covalently joining the first two components.

[0005] The partner molecule can be a therapeutic agent such as an anti-cancer drug, an adjuvant, another protein, or a radioisotope. The antibody is one whose antigen is expressed by a target cell or tissue. The antibody, through its binding to the antigen, serves to deliver the conjugate to the target. Once there, cleavage of the covalent link or degradation of the antibody results in the release of the therapeutic agent at the target location. Conversely, while the conjugate is circulating in the blood system, the therapeutic agent is held inactive because of its covalent linkage to the antibody, reducing the risk of side effects. For a review on conjugates in anti-cancer treatment, see Gerber *et al.* 2013.

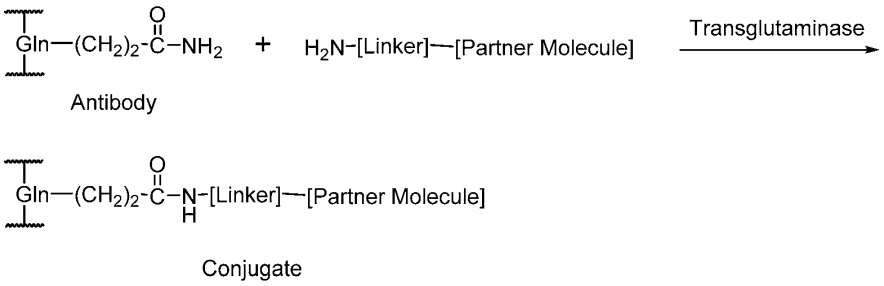
[0006] Alternatively to a therapeutic agent, the partner molecule can be an assay agent for diagnosing, locating a disease site, or monitoring of a medical condition. In such instance, the assay agent can be, for example, biotin, a fluorescent label, a radioactive label, or a deuterated polymer. Smith *et al.* 2019 discloses a conjugate comprising a deuterated polymer for MRI imaging. In such instance, cleavage of the linker at the target site is not necessary and may in fact be undesirable. For such use, the linker can be designed to be of the non-cleavable type.

[0007] A key step in the preparation of a conjugate is the covalent joining step, also referred to as the conjugation step. Many methods having been disclosed for effecting conjugation. One that has attracted substantial recent interest is conjugation mediated by the enzyme transglutaminase (EC 2.3.2.13).

[0008] Many transglutaminase variants are known, either produced naturally by different organisms or made by bioengineering. One commonly used in the food industry for texturing proteins is *Streptomyces mobaraensis* transglutaminase, obtained by fermentation or recombinant expression. Herein, the term "transglutaminase" is used generically unless a specific type or source is indicated.

[0009] Transglutaminase forms an amide bond between the carboxamide side chain of a glutamine (the amine acceptor, or, reciprocally, the acyl donor) and the ε-amino group of a lysine (the amine donor, or, reciprocally, the acyl acceptor). Specificity-wise, transglutaminase is selective regarding the glutamine residue, requiring it to be located in a flexible part of a protein loop and flanked by certain amino acids, but is promiscuous regarding the lysine residue, for example readily accepting the amino group of an alkyleneamino compound as a lysine ε-amino surrogate. See Fontana *et al.* 2008.

[0010] In a typical transglutaminase-mediated conjugation, the glutamine residue is located on the antibody, while the amino group is located on the linker-partner molecule moiety, as shown below:



[0011] The location of a glutamine residue on a polypeptide chain has a large effect on its availability as an amine acceptor. Normally, none of the glutamine residues on an antibody are available and some modification of the antibody is necessary to make them available. Typically, an antibody is glycosylated at asparagine 297 (N297) of the heavy chain (N-linked glycosylation). Jeger *et al.* 2010 discovered that deglycosylation of the antibody, either by eliminating the glycosylation site through an N297A substitution or post-translation enzymatic deglycosylation, renders nearby glutamine 295 (Q295) available for transamidation by *S. mobaraensis* transglutaminase. They further showed that an N297Q substitution not only eliminates glycosylation, but also introduces a second glutamine residue (at position 297) that too is an amine acceptor. Thus, simple deglycosylation generates two transglutaminase reactive glutamine residues per antibody (one per heavy chain, at Q295), while an antibody with an N297Q substitution generates four such glutamine residues (two per heavy chain, at positions Q295 and Q297).

[0012] In addition to the N297A and N297Q substitutions disclosed by Jeger *et al.* 2010, there have been other disclosures on modifying an antibody or another protein to make it a substrate for transglutaminase.

(a) Strop *et al.* 2017 and Farias *et al.* 2016 disclose antibody Fc regions engineered with glutamine-containing tags such as LLQGG, LSLSQG, GGGLLQGG, GLLQG, etc., where the glutamine in the tag can act as an amine acceptor and be positioned at various places of an antibody heavy or light chain, including the carboxy termini thereof.

(b) Chen *et al.* 2005 discloses the modification of a protein with the tag QSKVX, where X is L or I, which protein can then be conjugated with transglutaminase.

(c) Fischer *et al.* 2015 discloses the incorporation into an antibody fragment lacking an Fc domain a glutamine (Q) containing tag of the formula

$$(Q)\text{-}NH\text{-}(C)\text{-}X\text{-}L\text{-}(V\text{-}(Y\text{-}(M \text{ or } Z)_z)_q)_r \quad .$$

(d) Rao-Naik *et al.* 2018 discloses appending glutamine-containing heavy chain C-terminal extensions to an antibody to render it transglutaminase-reactive.

[0013] In an approach complementary to modifying an antibody to make it transglutaminase-reactive, Rao-Naik *et al.* 2017 discloses modifying transglutaminase to make it capable of conjugating to a wild-type antibody.

[0014] There also have been studies on the substrate specificity of transglutaminase using small peptide-containing molecules: Ando *et al.* 1989, Kamiya *et al.* 2011, Ohtsuka *et al.* 2000.

[0015] Other disclosures relating to conjugation of antibodies or other proteins using transglutaminase are: Bregeon 2016, Bregeon *et al.* 2016, Bregeon *et al.* 2017, Dennler *et al.* 2014, Innate Pharma 2013, Lin *et al.* 2006, Mero *et al.* 2009, Mindt *et al.* 2008, Sato 2002, Sato *et al.* 2001, Schibli *et al.* 2007, and Sugimura *et al.* 2007.

[0016] It is also known to attach cysteine-containing terminal extensions to an antibody for the purpose of effecting conjugation via Michael addition to a maleimide group. Liu *et al.* 2014 disclose attaching such extensions to the C-terminus of a heavy chain. Babcook *et al.* 2017 disclose attaching such extensions to the C-terminus of a light chain.

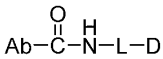
[0017] Full citations for the documents cited herein by first author or inventor and year are listed at the end of this specification.

BRIEF SUMMARY OF THE DISCLOSURE

[0018] The location of the conjugation site can affect the stability and pharmacokinetics of a conjugate. Strop *et al.* 2013. Thus, it is desirable to provided alternative conjugation sites and conjugate structures to diversify the options available for the development of biologics.

[0019] This specification discloses antibodies having C-terminal glutamine-containing extensions on a light chain for conjugation with transglutaminase. In one embodiment, there is provided a full length antibody having on the C-terminus (carboxy terminus) of a light chain thereof a glutamine-containing extension comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5, NO:6, NO:7, NO:8, NO:9, NO:10, NO:11, NO:12, NO:13, NO:14, NO:15, NO:16, NO:17, NO:18, NO:19, NO:20, NO:21, NO:22, NO:23, NO:24, NO:25, NO:26, NO:29, NO:30, NO:31, NO:32, NO:33, NO:34, NO:35, NO:36, NO:37, NO:38, NO:39, and NO:40.

[0020] In another aspect, this specification provides a conjugate of the formula (IV)



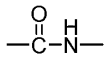
(IV)

wherein

Ab is a full length antibody having on the C-terminus (carboxy terminus) of a light chain thereof a glutamine-containing extension comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5,

NO:6, NO:7, NO:8, NO:9, NO:10, NO:11, NO:12, NO:13, NO:14, NO:15, NO:16, NO:17, NO:18, NO:19, NO:20, NO:21, NO:22, NO:23, NO:24, NO:25, NO:26, NO:29, NO:30, NO:31, NO:32, NO:33, NO:34, NO:35, NO:36, NO:37, NO:38, NO:39, and NO:40;

L is a linker moiety bonded to Ab via the amide bond



to a glutamine in a glutamine containing extension; and

D is selected from the group consisting of a protein, a radioisotope, an assay agent, and a therapeutic agent.

[0021] In another aspect, this specification provides a method of making an antibody conjugate, comprising the steps of

(a) mixing a full length antibody having on the C-terminus (carboxy terminus) of a light chain thereof a glutamine-containing extension comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5, NO:6, NO:7, NO:8, NO:9, NO:10, NO:11, NO:12, NO:13, NO:14, NO:15, NO:16, NO:17, NO:18, NO:19, NO:20, NO:21, NO:22, NO:23, NO:24, NO:25, NO:26, NO:29, NO:30, NO:31, NO:32, NO:33, NO:34, NO:35, NO:36, NO:37, NO:38, NO:39, and NO:40 with an amine donor compound comprising a primary amine and a moiety selected from the group consisting of a protein, a radioisotope, an assay agent, and a therapeutic agent, in the presence of a transglutaminase; and

(b) allowing the transglutaminase to catalyze the formation of an amide bond between the side chain carboxamide of a glutamine of the glutamine-containing extension and the primary amine of the amine donor compound, thereby making the antibody conjugate.

[0022] In another aspect, this specification provides a method of making an antibody conjugate, comprising the steps of

(a) mixing a full length antibody having on the C-terminus (carboxy terminus) of a light chain thereof a glutamine-containing extension comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5, NO:6, NO:7, NO:8, NO:9, NO:10, NO:11, NO:12, NO:13, NO:14, NO:15, NO:16, NO:17, NO:18, NO:19, NO:20, NO:21, NO:22, NO:23, NO:24,

NO:25, NO:26, NO:29, NO:30, NO:31, NO:32, NO:33, NO:34, NO:35, NO:36, NO:37, NO:38, NO:39, and NO:40 with a first compound, which first compound is an amine donor compound having a primary amine and a first reactive functional group, in the presence of a transglutaminase;

(b) allowing the transglutaminase to catalyze the formation of an amide bond between the side chain carboxamide of a glutamine of the glutamine-containing extension and the primary amine of the first compound, to make an adduct of the antibody and the first compound;

(c) contacting the adduct with a second compound having a second reactive functional group and a moiety selected from the group consisting of a protein, a radioisotope, an assay agent, and a therapeutic agent; the second reactive functional group being capable of reacting with the first reactive functional group to form a covalent bond therebetween; and

(d) allowing the first and second reactive functional groups to react and form a covalent bond therebetween, thereby making the antibody conjugate.

[0023] Where moiety (in the first compound or second compound, as the case may be) is a protein, the resultant conjugate is a fusion protein. Where the moiety is a radioisotope, the resultant conjugate can be used for radiation therapy or radioimaging. The moiety can be an assay agent such as a fluorescent label, a deuterated polymer, or a ligand like biotin, in which case the conjugate can be used for diagnosing a medical condition, monitoring of treatment, or analytical applications. Preferably, the moiety is a therapeutic agent (in which case the product is also referred to as an antibody-drug conjugate or ADC), which can be used in medical treatments, especially the treatment of cancer.

BRIEF DESCRIPTION OF THE DRAWING(S)

[0024] **FIG. 1** shows schematically an antibody light chain having a C-terminus extension (SEQ ID NO:1) as disclosed herein.

[0025] **FIG. 2** compares the one- and two-step methods for making conjugates using transglutaminase (BTG).

DETAILED DESCRIPTION OF THE DISCLOSURE

DEFINITIONS

[0026] "Antibody" means whole antibodies and any antigen binding fragment (*i.e.*, "antigen-binding portion") or single chain variants thereof. A whole antibody is a protein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain comprises a heavy chain variable region (V_H) and a heavy chain constant region comprising three domains, C_{H1}, C_{H2} and C_{H3}. Each light chain comprises a light chain variable region (V_L or V_k) and a light chain constant region comprising one single domain, C_L. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with more conserved framework regions (FRs). Each V_H and V_L comprises three CDRs and four FRs, arranged from amino- to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. The variable regions contain a binding domain that interacts with an antigen. The constant regions may mediate the binding of the antibody to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system. An antibody is said to "specifically bind" to an antigen X if the antibody binds to antigen X with a K_D of 5×10^{-8} M or less, more preferably 1×10^{-8} M or less, more preferably 6×10^{-9} M or less, more preferably 3×10^{-9} M or less, even more preferably 2×10^{-9} M or less. The antibody can be chimeric, humanized, or, preferably, human. The heavy chain constant region can be engineered to affect glycosylation type or extent, to extend antibody half-life, to enhance or reduce interactions with effector cells or the complement system, or to modulate some other property. The engineering can be accomplished by replacement, addition, or deletion of one or more amino acids or by replacement of a domain with a domain from another immunoglobulin type, or a combination of the foregoing.

[0027] "Antigen binding fragment" and "antigen binding portion" of an antibody (or simply "antibody portion" or "antibody fragment") mean one or more fragments of an antibody that retain the ability to specifically bind to an antigen. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody, such as (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fab' fragment, which is essentially an Fab with part of the hinge region (see, for example, Abbas *et al.*, *Cellular and Molecular Immunology*, 6th Ed., Saunders

Elsevier 2007); (iv) a Fd fragment consisting of the V_H and C_{H1} domains; (v) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (vi) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a V_H domain; (vii) an isolated complementarity determining region (CDR); and (viii) a nanobody, a heavy chain variable region containing a single variable domain and two constant domains. Preferred antigen binding fragments are Fab, F(ab')$_2$, Fab', Fv, and Fd fragments. Furthermore, although the two domains of the Fv fragment, V_L and V_H, are encoded by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv, or scFv); *see, e.g.,* Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883*)*. Such single chain antibodies are also encompassed within the term "antigen-binding portion" of an antibody.

[0028] Unless indicated otherwise – for example by reference to the linear numbering in a SEQ ID NO: listing – references to the numbering of amino acid positions in an antibody heavy or light chain variable region (V_H or V_L) are according to the Kabat system (Kabat et al., "Sequences of proteins of immunological interest, 5th ed., Pub. No. 91-3242, U.S. Dept. Health & Human Services, NIH, Bethesda, Md., 1991, hereinafter "Kabat") and references to the numbering of amino acid positions in an antibody heavy or light chain constant region (C_{H1}, C_{H2}, C_{H3}, or C_L) are according to the EU index as set forth in Kabat. See Lazar *et al.*, US 2008/0248028 A1, the disclosure of which is incorporated herein by reference, for examples of such usage. Further, the ImMunoGeneTics Information System (IMGT) provides at its website a table entitled "IMGT Scientific Chart: Correspondence between C Numberings" showing the correspondence between its numbering system, EU numbering, and Kabat numbering for the heavy chain constant region.

[0029] An "isolated antibody" means an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds antigen X is substantially free of antibodies that specifically bind antigens other than antigen X). An isolated antibody that specifically binds antigen X may, however, have cross-reactivity to other antigens, such as antigen X molecules from other species. In certain embodiments, an isolated antibody specifically binds to human antigen X and does not cross-react with other (non-human) antigen X antigens. Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

[0030] "Monoclonal antibody" or "monoclonal antibody composition" means a preparation of antibody molecules of single molecular composition, which displays a single binding specificity and affinity for a particular epitope.

[0031] "Human antibody" means an antibody having variable regions in which both the framework and CDR regions (and the constant region, if present) are derived from human germline immunoglobulin sequences. Human antibodies may include later modifications, including natural or synthetic modifications. Human antibodies may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, "human antibody" does not include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

[0032] "Human monoclonal antibody" means an antibody displaying a single binding specificity, which has variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. In one embodiment, human monoclonal antibodies are produced by a hybridoma that includes a B cell obtained from a transgenic nonhuman animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

EMBODIMENTS

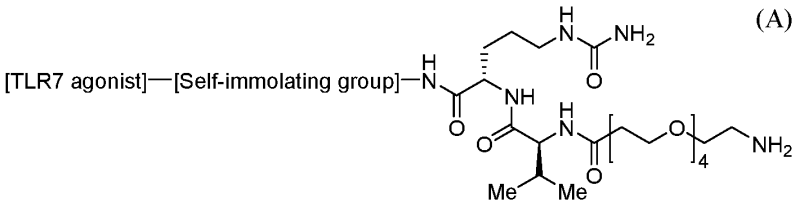
[0033] Generally, transglutaminase-mediated preparation of an antibody conjugate can be by a one-step process or a two-step process, as illustrated schematically in **FIG. 2**. In the one-step process, transglutaminase couples a glutamine carboxamide on the extension, acting as the amine acceptor, and an amine donor compound H_2N-L-D, where L is a linker moiety and partner molecule D is a protein, a radioisotope, an assay agent, or a therapeutic agent, to form the conjugate directly. In the two-step process, transglutaminase catalyzes the formation of an initial transamidation adduct between a glutamine carboxamide on the extension, acting as the amine receptor, and first compound (H_2N-L'-R'), which is an amine donor compound, where L' is a first linker moiety and R' is a first reactive functional group. Subsequently, the adduct is reacted with a second compound (R"-L"-D), where R" is a second reactive functional group capable of reacting with R', L" is a second linker moiety, and D is as defined above. Sometimes, the one-step process is referred to as the enzymatic process, and the two-step process as the chemo-enzymatic process because it entails both a chemical and an enzymatic step. Each of L, L', and

L" can be an alkyl chain $-(CH_2)_m-$ where m is an integer from 2 to 10, inclusive, or can be, especially in the case of L and L", a more complex structure, as discussed below.

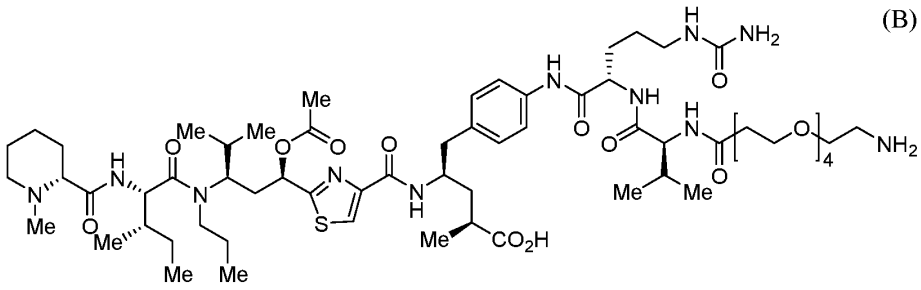
[0034] The amine donor, whether H_2N-L-D or H_2N-L'-R', is often used in large excess to suppress undesired transamidation between the glutamine carboxamide and an ε-amino group of an antibody lysine. If the moiety D is expensive or difficult to obtain, the use of a large excess may be impractical. In such instances, the two-step process may be preferable, even though it requires an additional step.

[0035] As a demonstration, we conjugated an anti-mesothelin antibody having the same heavy and light chain CDRs as antibody 6A4 of Terrett *et al.*, US 8,268,970 B2 (2012). Its heavy and light chain sequences are provided as SEQ ID NO:27 and SEQ ID NO:28, respectively. Light chain C-terminal extensions having the sequences disclosed below were attached and the antibodies so modified were then conjugated to either compound (A) or compound (B) as the amine donor, using the one-step process.

(A)



(B)



[0036] In Compound (A) the therapeutic agent is a Toll-like Receptor 7 (TLR7) agonist, which can be used as an adjuvant for vaccines and immunotherapy agents in treating a variety of conditions. Examples of TLR7 agonists are disclosed in US Applications Ser. Nos. 16/103210, 16/103511, 16/103581, 16/013601, and 16/103619; each filed Aug. 14, 2018; the disclosures of which are incorporated herein by reference. In Compound (B) the therapeutic agent is a tubulysin analog, a cytotoxin that can be used in anti-cancer treatment. The preparation of

Compound (B) is disclosed in US Provisional Application Ser. No. 62/688737, filed Jun. 22, 2018; the disclosure of which is incorporated herein by reference.

[0037] In one embodiment, light chain C-terminal extensions disclosed herein have one glutamine. Examples are listed in Table A below, along with the drug-antibody ratio (DAR) of conjugates prepared therefrom. As there are two light chains per antibody, each bearing an extension, the theoretical maximum DAR is 2.0. **FIG. 1** shows schematically an antibody light chain having the C-terminal extension of SEQ ID NO:1.

Table A – Extensions with One Glutamine

SEQ ID NO:	Extension Sequence	DAR (Cpd. A)	DAR (Cpd. B)
1	GGVLQRAS	1.99	2.0
2	GGVLQGAS	1.84	1.6
3	GGVLQRPS	1.96	2.0
4	GGVLQGPS	1.97	2.0
5	GGVLQSPS	1.94	1.77
6	GGVLQYAS	1.87	1.7
7	GGGGVLQRAS	1.99	2.0
8	GGGGVLQGAS	1.95	2.0
9	GGGGVLQRPS	1.99	2.0
10	GGGGVLQGPS	1.94	2.0
11	GGGGVLQSPS	1.99	2.0
12	GGGGVLQYAS	2	2.0
13	VLQYAS	0.95	0.97

[0038] In another embodiment, the light chain C-terminal extension have two glutamines. Examples are listed in Table B below, along with the drug-antibody ratio (DAR) of conjugates prepared therefrom. As there are two light chains per antibody, each bearing an extension with two glutamines, the theoretical maximum DAR is 4.0.

Table B – Extensions with Two Glutamines

SEQ ID NO:	Extension Sequence	DAR (Cpd. A)	DAR (Cpd. B)
14	GGVLQRQS	2.37	2.05
15	GGVLQGQS	2.04	2.04

Table B – Extensions with Two Glutamines			
SEQ ID NO:	Extension Sequence	DAR (Cpd. A)	DAR (Cpd. B)
16	GGVLQRQRPS	2.23	2.01
17	GGVLQGQGPS	3.27	3.19
18	GGVLQSQSPS	1.86	2.01
19	GGVLQYQS	2.90	2.60
20	GGGGVLQRQS	2.95	2.88
21	GGGGVLQGQS	2.60	2.89
22	GGGGVLQRQRPS	2.96	2.88
23	GGGGVLQGQGPS	3.72	3.71
24	GGGGVLQSQSPS	2.37	2.84
25	GGGGVLQYQS	3.32	3.58
26	VLQYQS	1.95	2.67
29	GGVLQVLQS		3.0
30	GGVLQGVLQS		3.8
31	GGVLQGGVLQS		3.4
32	GGVLQVLQGPS		2.5
33	GGVLQGGQGPS		3.0
34	GGVLQGGGQGPS		3.1
35	GGGGVLQVLQS		3.2
36	GGGGVLQGVLQS		4.0
37	GGGGVLQGGVLQS		4.0
38	GGGGVLQVLQGPS		3.2
39	GGGGVLQGGQGPS		3.5
40	GGGGVLQGGGQGPS		3.3

[0039] It may be that, in C-terminal extensions having two glutamines, it is beneficial to separate the glutamines from each other further than they are in Table B, by interposing between two and four amino acids between them.

[0040] As the C-terminus of a light chain is somewhat buried, it is desirable to provide spacers to make the glutamine in the extension more protruding and accessible to transglutaminase. Such effect can be achieved by using plural glycines (two to four) at the N-

terminus of the extension. Referring to Table A, it can be seen that the extension with no glycines (SEQ ID NO:13) leads to a lower DAR than those having two or four glycines.

[0041] In one embodiment, the antibody has a light chain C-terminal extension comprising two spacer glycines and one glutamine. Such extensions are exemplified by SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5, and NO:6.

[0042] In another embodiment, the antibody has a light chain C-terminal extension comprising four spacer glycines and one glutamine. Such extensions are exemplified by SEQ ID NO:7, NO:8, NO:9, NO:10, NO:11, and NO:12.

[0043] In another embodiment, the antibody has a light chain C-terminal extension comprising two spacer glycines and two glutamines. Such extensions are exemplified by SEQ ID NO:14, NO:15, NO:16, NO:17, NO:18, NO:19, NO:29, NO:30, NO:31, NO:32, NO:33, and NO:34.

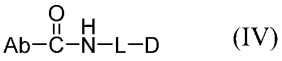
[0044] In another embodiment, the antibody has a light chain C-terminal extension comprising four spacer glycines and two glutamines. Such extensions are exemplified by SEQ ID NO:20, NO:21, NO:22, NO:23, NO:24, NO:25, NO:35, NO:36, NO:37, NO:38, NO:39 and NO:40.

[0045] Advantageously, the C-terminal light chain extensions contain a valine-leucine dipeptide (VL) on the N-terminal side of a glutamine.

[0046] Antibodies that can be modified and conjugated by the methods of this disclosure include those recognizing the following antigens: mesothelin, prostate specific membrane antigen (PSMA), CD19, CD22, CD30, CD70, B7H3, B7H4 (also known as O8E), protein tyrosine kinase 7 (PTK7), glypican-3, RG1, fucosyl-GM1, CTLA-4, and CD44. The antibody can be animal (*e.g.*, murine), chimeric, humanized, or, preferably, human. The antibody preferably is monoclonal, especially a monoclonal human antibody. The preparation of human monoclonal antibodies against some of the aforementioned antigens is disclosed in Korman *et al.*, US 8,609,816 B2 (2013; B7H4, also known as 08E; in particular antibodies 2A7, 1G11, and 2F9); Rao-Naik *et al.*, 8,097,703 B2 (2012; CD19; in particular antibodies 5G7, 13F1, 46E8, 21D4, 21D4a, 47G4, 27F3, and 3C10); King *et al.*, US 8,481,683 B2 (2013; CD22; in particular antibodies 12C5, 19A3, 16F7, and 23C6); Keler *et al.*, US 7,387,776 B2 (2008; CD30; in particular antibodies 5F11, 2H9, and 17G1); Terrett *et al.*, US 8,124,738 B2 (2012; CD70; in particular antibodies 2H5, 10B4, 8B5, 18E7, and 69A7); Korman *et al.*, US 6,984,720 B1 (2006;

CTLA-4; in particular antibodies 10D1, 4B6, and 1E2); Vistica *et al.*, US 8,383,118 B2 (2013, fucosyl-GM1, in particular antibodies 5B1, 5B1a, 7D4, 7E4, 13B8, and 18D5) Korman *et al.*, US 8,008,449 B2 (2011; PD-1; in particular antibodies 17D8, 2D3, 4H1, 5C4, 4A11, 7D3, and 5F4); Huang *et al.*, US 2009/0297438 A1 (2009; PSMA. in particular antibodies 1C3, 2A10, 2F5, 2C6); Cardarelli *et al.*, US 7,875,278 B2 (2011; PSMA; in particular antibodies 4A3, 7F12, 8C12, 8A11, 16F9, 2A10, 2C6, 2F5, and 1C3); Terrett *et al.*, US 8,222,375 B2 (2012; PTK7; in particular antibodies 3G8, 4D5, 12C6, 12C6a, and 7C8); Terrett *et al.*, US 8,680,247 B2 (2014; glypican-3; in particular antibodies 4A6, 11E7, and 16D10); Harkins *et al.*, US 7,335,748 B2(2008; RG1; in particular antibodies A, B, C, and D); Terrett *et al.*, US 8,268,970 B2 (2012; mesothelin; in particular antibodies 3C10, 6A4, and 7B1); Xu *et al.*, US 2010/0092484 A1 (2010; CD44; in particular antibodies 14G9.B8.B4, 2D1.A3.D12, and 1A9.A6.B9); Deshpande *et al.*, US 8,258,266 B2 (2012; IP10; in particular antibodies 1D4, 1E1, 2G1, 3C4, 6A5, 6A8, 7C10, 8F6, 10A12, 10A12S, and 13C4); Kuhne *et al.*, US 8,450,464 B2 (2013; CXCR4; in particular antibodies F7, F9, D1, and E2); and Korman *et al.*, US 7,943,743 B2 (2011; PD-L1; in particular antibodies 3G10, 12A4, 10A5, 5F8, 10H10, 1B12, 7H1, 11E6, 12B7, and 13G4); the disclosures of which are incorporated herein by reference.

[0047] In respect of conjugates of formula (IV)



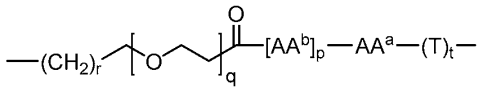
(IV)

(a) D preferably is, in one embodiment, cytotoxic drug.

(b) In another preferred embodiment, D is a TLR7, STING, NRLP3, or RIG-1 agonist.

(c) In a preferred embodiment, L is $-(CH_2)_{2\text{-}6}$-.

(d) In another preferred embodiment, L is



(Ia')

wherein

T is a self-immolating group;

t is 0 or 1;

AA^a and each AA^b are independently selected from the group consisting of alanine, β-alanine, γ-aminobutyric acid, arginine, asparagine, aspartic acid, γ-carboxyglutamic acid, citrulline, cysteine, glutamic

acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, norleucine, norvaline, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine;

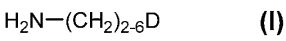
p is 1, 2, 3, or 4;

q is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and

r is 1, 2, 3, 4, or 5.

[0048] The one-step and two-step processes for conjugation are now discussed in greater detail.

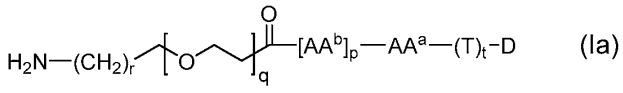
[0049] In a preferred embodiment, amine donor compound in a one-step process is represented by formula (I):



$$H_2N-(CH_2)_{2-6}D \qquad \textbf{(I)}$$

where D is a protein, a radioisotope, an assay agent, or a therapeutic agent.

[0050] In another preferred embodiment, the amine donor compound for the one-step process has a structure represented by formula (Ia):



$$H_2N-(CH_2)_r-[O-]_q-C(=O)-[AA^b]_p-AA^a-(T)_t-D \qquad \text{(Ia)}$$

wherein

D is a a protein, a radioisotope, an assay agent, or a therapeutic agent;

T is a self-immolating group;

t is 0 or 1;

AA^a and each AA^b are independently selected from the group consisting of alanine, β-alanine, γ-aminobutyric acid, arginine, asparagine, aspartic acid, γ-carboxyglutamic acid, citrulline, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, norleucine, norvaline, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine;

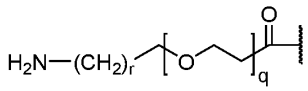
p is 1, 2, 3, or 4;

q is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and

r is 1, 2, 3, 4, or 5.

[0051] In formulae (Ia), (Ia') and (III) (below), $-AA^a-[AA^b]_p-$ represents a polypeptide whose length is determined by the value of p (dipeptide if p is 1, tetrapeptide if p is 3, etc.). AA^a is at

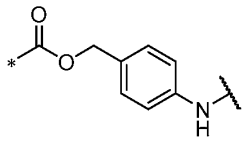
the carboxy terminus of the polypeptide and its carboxyl group forms a peptide (amide) bond with an amine nitrogen of D (or T, if present). Conversely, the last AA^b is at the amino terminus of the polypeptide and its α-amino group forms a peptide bond with



Preferred polypeptides $-AA^a-[AA^b]_p-$ are Val-Cit, Val-Lys, Lys-Val-Ala, Asp-Val-Ala, Val-Ala, Lys-Val-Cit, Ala-Val-Cit, Val-Gly, Val-Gln, and Asp-Val-Cit, written in the conventional N-to-C direction, as in H_2N-Val-Cit-CO_2H). More preferably, the polypeptide is Val-Cit, Val-Lys, or Val-Ala. Preferably, a polypeptide $-AA^a-[AA^b]_p-$ is cleavable by an enzyme found inside the target cell, for example a cathepsin and especially cathepsin B, or an enzyme in the environs of the target organ or tissue.

[0052] If the subscript q is other than 0, compound (Ia) contains a poly(ethylene glycol) (PEG) group, which can advantageously improve the solubility of compound (Ia), facilitating conjugation to the antibody – a step that is performed in aqueous media. Also, a PEG group can serve as a spacer between the antibody and the peptide $-AA^a-[AA^b]_p-$, so that the bulk of the antibody does not sterically interfere with action of a peptide-cleaving enzyme.

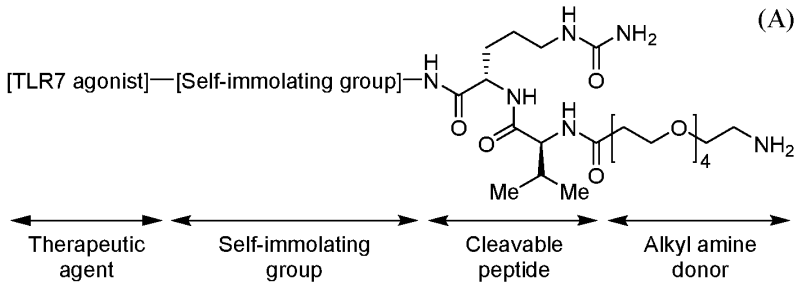
[0053] As indicated by the subscript t equals 0 or 1, a self-immolating group T is optionally present. A self-immolating group is one such that cleavage from AA^a or AA^b, as the case may be, initiates a reaction sequence resulting in the self-immolating group disbonding itself from D and freeing the latter to exert its therapeutic function. When present, the self-immolating group T preferably is a *p*-aminobenzyl oxycarbonyl (PABC) group, whose structure is shown below, with an asterisk (*) denoting the end of the PABC bonded to an amine nitrogen of drug D and a wavy line (~~~~) denoting the end bonded to the polypeptide $-AA^a-[AA^b]_p-$. The PABC group can be substituted, as disclosed in US Provisional Application Ser. No. 62/677307, filed May 29, 2018.

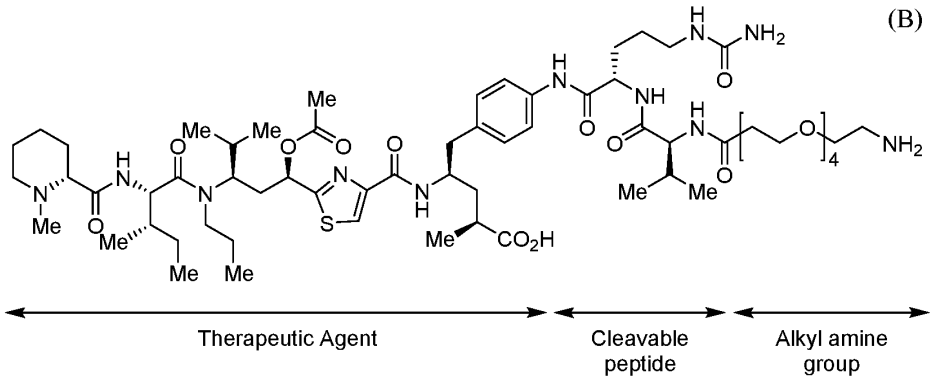


PABC

[0054] Another self-immolating group that can be used is a substituted thiazole, as disclosed in Feng, US 7,375,078 B2 (2008).

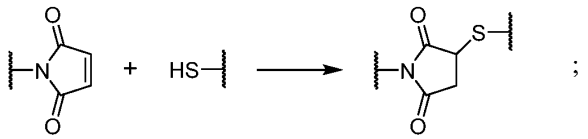
[0055] Compounds (A) and (B) illustrate the design of amine donor compounds according to formula (Ia), with the arrangement of their various elements as shown. Compound (A), but not compound (B), comprises a self-immolating group.





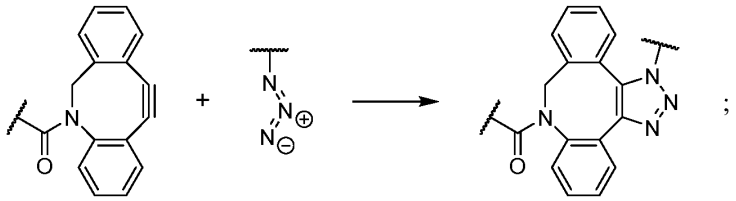
[0056] In a two-step conjugation, many combinations of groups R' and R" can be used. Suitable combinations of R' and R" (or, vice-versa, R" and R') include:

(a) a maleimide group and a sulfhydryl group, to form a Michael addition adduct, as in



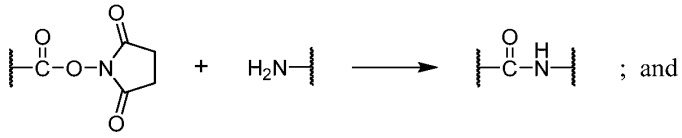
;

(b) a dibenzocyclooctyne group and an azide group, to form a cycloaddition product via "click" chemistry, as in



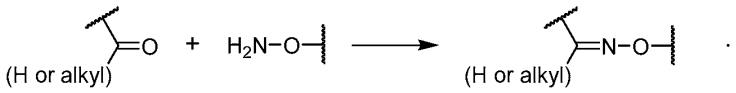
;

(c) an N-hydroxysuccinimide ester and an amine, to form an amide, as in

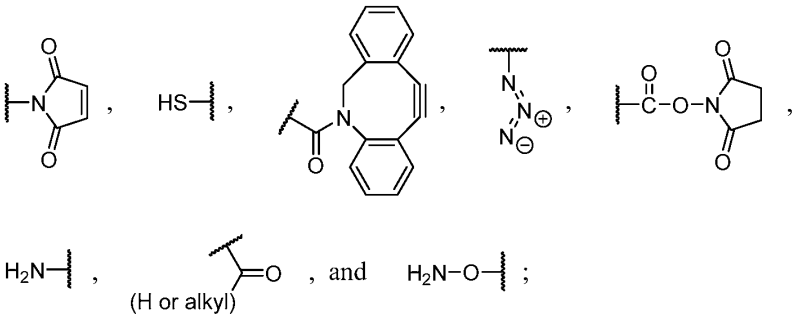


; and

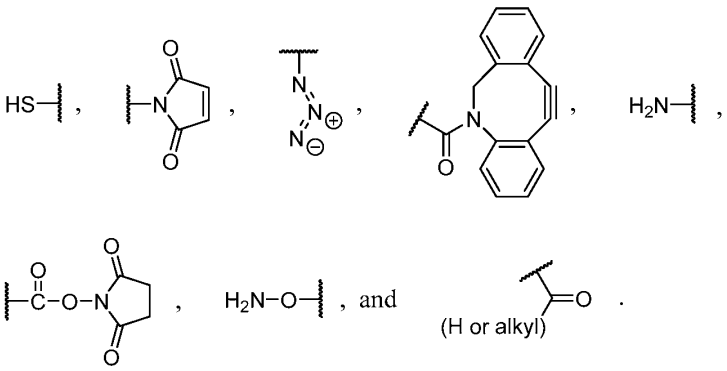
(d) an aldehyde or ketone (where "alkyl" preferably is C_{1-3} alkyl) and a hydroxylamine, to form an oxime, as in



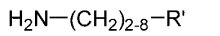
[0057] Thus, R' can be selected from



while, reciprocally, R" can be selected from

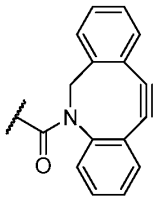
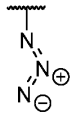


[0058] A suitable amine donor first compound for the two-step process is depicted in formula (II)

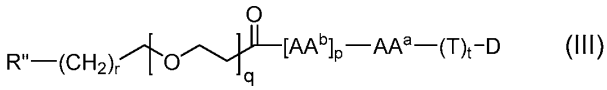


(II)

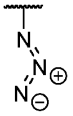
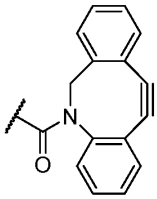
where R' is as defined above and preferably is

 or  .

[0059] A corresponding suitable compound R"-L"-D is shown in formula (III)

 (III)

where R" is as defined above and preferably is

 or  ,

and r, q, AA^b, p, AA^a, T, t, and D are as defined above in respect of formula (Ia).

[0060] In the instance where the conjugate is intended for use in cancer treatment, the therapeutic agent can be a cytotoxic drug that causes death of the targeted cancer cell. Cytotoxic drugs that can be used in conjugates include the following types of compounds and their analogs and derivatives:

(a) enediynes such as calicheamicin (see, *e.g.*, Lee *et al.*, *J. Am. Chem. Soc.* **1987**, 109, 3464 and 3466) and uncialamycin (see, *e.g.*, Davies *et al.*, WO 2007/038868 A2 (2007); Chowdari *et al.*, US 8,709,431 B2 (2012); and Nicolaou *et al.*, WO 2015/023879 A1 (2015));

(b) tubulysins (see, *e.g.*, Domling *et al.*, US 7,778,814 B2 (2010); Cheng *et al.*, US 8,394,922 B2 (2013); and Cong *et al.*, US 8,980,824 B2 (2015));

(c) DNA alkylators such as analogs of CC-1065 and duocarmycin (see, *e.g.*, Yang *et al.*, US 2018/0051031 A1 (2018); Boger, US 6,5458,530 B1 (2003); Sufi *et al.*, US 8,461,117 B2 (2013); and Zhang *et al.*, US 8,852,599 B2 (2014));

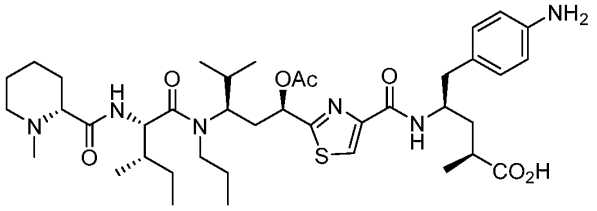
(d) epothilones (see, *e.g.*, Vite *et al.*, US 2007/0275904 A1 (2007) and US RE42930 E (2011));

(e) auristatins (see, *e.g.*, Senter *et al.*, US 6,844,869 B2 (2005) and Doronina *et al.*, US 7,498,298 B2 (2009));

(f) benzodiazepine dimers (see, *e.g.*, Zhang *et al.*, US 9,527,871 B2 (2016); Zhang *et al.*, US 9,688,694 B2 (2017); McDonald *et al.*, US 9,526,801 B2 (2016); Howard *et al.*, US 2013/0059800 A1(2013); US 2013/0028919 A1 (2013); and WO 2013/041606 A1 (2013)); and

(g) maytansinoids such as DM1 and DM4 (see, *e.g.*, Chari *et al.*, US 5,208,020 (1993) and Amphlett *et al.*, US 7,374,762 B2 (2008)).

[0061] In one embodiment, the cytotoxic drug is a DNA alkylator, a tubulysin, an auristatin, a benzodiazepine dimer, an enediyne, or a maytansinoid. Specific examples are, by way of illustration and not of limitation,



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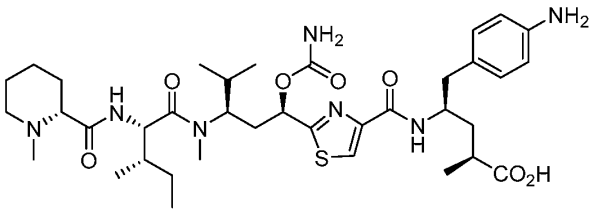

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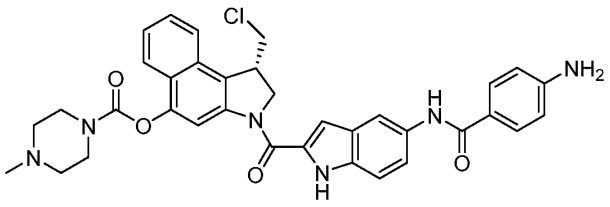

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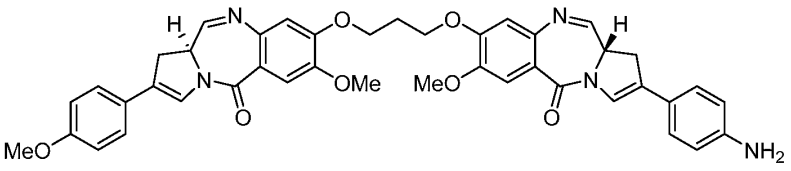

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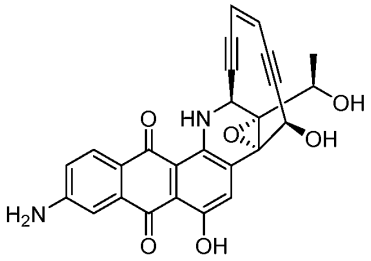

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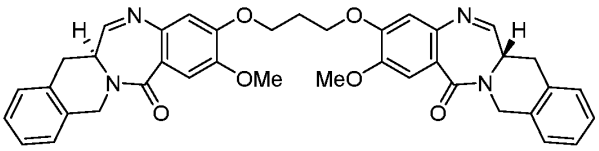

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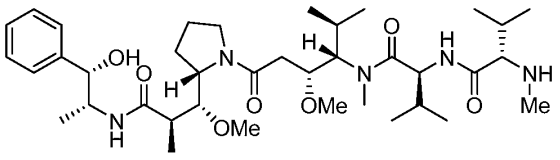

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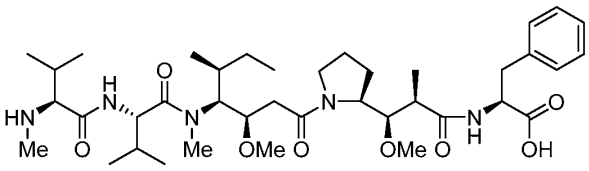

.

[0062] The immune system has receptors whose natural ligands are pathogen-associated molecular patterns (PAMPs). The binding of a PAMP to its cognate receptor activates the immune system to defend against an infection by the associated pathogen. Additionally, these receptors also can be activated by synthetic agonists that have an adjuvant effect on the action of vaccines and immunotherapy agents in treating a variety of conditions other than actual pathogen infection. Immuno-oncology agents such as ipilimumab, nivolumab, and pembrolizumab in particular can benefit from this adjuvant effect. Receptors that can be activated by synthetic

agonists include TLR3, TLR7, TLR9 (Toll-like receptor-3, -7, and -9, respectively), STING (STimulator of INterferon Genes; also known as MPYS, TMEM173, MITA or ERIS), NLRP3 (NOD-like receptor protein 3), and RIG-I (retinoic acid inducible gene I). Thus, in an alternative embodiment, the therapeutic agent is a TLR3, TLR7, TLR9, STING, NLRP3, or RIG-I agonist. In particular, the therapeutic agent can be a TLR7 agonist as disclosed in Poudel *et al.*, US 2019/0055243 A1 (2019); Young *et al.*, US 2019/0055244 A1 (2019); Poudel *et al.*, US 2019/0055245 A1 (2019); He *et al.*, US 2019/0055246 A1 (2019); He *et al.*, US 2019/0055247 A1 (2019); and Purandare *et al.*, PCT Application PCT/US2019/028697, filed Apr. 23, 2019.

[0063] The aforementioned therapeutic agents can be used in conjugates made by either the one-step or two-step process.

EXAMPLES

[0064] The practice of this invention can be further understood by reference to the following examples, which are provided by way of illustration and not of limitation.

Example 1 – Preparation of modified antibodies

[0065] ExpiCHO cells were used to express the antibodies. The ExpiCHO cells were grown in ExpiCHO medium at 37 °C, 8% CO_2 atmosphere in plastic flasks. The expression vector for the heavy chain and light chain were mixed in OptiMEM, added PEI solution in OptiMEM, and resulting PEI/DNA complex was incubated at room temperature for 30 min before adding to the cells. 24 hrs after transfection, Feed B and VPA were added to the cells and the cells were kept at 37 °C with agitation. After 7 days, the culture supernatant was harvested by centrifugation and filtration. The supernatant was purified with MabSelect SuRe LX resin. Briefly, cell supernatant was incubated with resin overnight at 4 °C, washed with 10 CV of PBS, followed by elution with 4 CV of 0.1 M citrate pH 3.5, immediately neutralized with 1/10 volume of 1 M tris pH 8.

Example 2 – Conjugation of modified antibodies

[0066] Conjugation of an antibody modified as described herein with an amine donor using transglutaminase was performed by the protocol listed below. We used dispase activate BTGase with V65I and Y75F point mutations. It was dialyzed it into 50 mM sodium acetate pH5.5 from formulation (buffer 20 mM acetate, 10% glycerol pH4) before use.

[0067] The antibody, at ~2 mg/mL, in 50 mM Tris-HCl, pH 8.0, or 20 mM Histidine, 50 mM Imidizaole, 10% sucrose, pH ~7.8 was reacted with 10-fold molar per site excess of the amine

donor in the presence of 0.2 molar excess of transglutaminase per antibody. The reaction was allowed to proceed overnight at 37 °C with continuous gentle mixing.

[0068] The antibody drug conjugate was 0.2 µm filtered and purified using a mAb Select SuRe™ column (GE Healthcare). The conjugate was loaded onto the column pre-equilibrated with 50 mM Tris-HCl, pH 8.0 and washed with 10 CV (column volumes) of equilibrating buffer followed by 10 CV of 50 mM Tris-HCl, 17% acetonitrile, pH 8.0 to remove unreacted amine donor. The column was re-equilibrated with 50 mM Tris-HCl, pH 8.0 before elution with 0.1 M citrate, pH 3.5 in 1 mL fractions and neutralized with 1/10 of elution volume with 1 M Tris, pH 8.0. The desired fractions are dialyzed in formulation buffer 20 mM Histidine, 10% Sucrose, pH 6.0 and analyzed by LC-MS (ESI-QTOF), RP-HPLC and SDS-PAGE for purity and Drug to Antibody Ratio (DAR).

Example 3 – Analysis of conjugated modified antibodies

[0069] The antibody at 5 mg/mL in 50 mM imidazole, 10% sucrose, pH 8 was reacted with 10-fold molar per site of the amine donor in the presence of 0.2 molar excess of recombinant bacterial transglutaminase per antibody. After overnight incubation at 37 °C with continuous gentle mixing, the reaction mixture was analyzed by LC-MS (ESI-QTOF) for DAR evaluation.

[0070] The foregoing detailed description of the invention includes passages that are chiefly or exclusively concerned with particular parts or aspects of the invention. It is to be understood that this is for clarity and convenience, that a particular feature may be relevant in more than just the passage in which it is disclosed, and that the disclosure herein includes all the appropriate combinations of information found in the different passages. Similarly, although the various figures and descriptions herein relate to specific embodiments of the invention, it is to be understood that where a specific feature is disclosed in the context of a particular figure or embodiment, such feature can also be used, to the extent appropriate, in the context of another figure or embodiment, in combination with another feature, or in the invention in general.

[0071] Further, while the present invention has been particularly described in terms of certain preferred embodiments, the invention is not limited to such preferred embodiments. Rather, the scope of the invention is defined by the appended claims.

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[0072] Full citations for the following references cited in abbreviated fashion by first author (or inventor) and date earlier in this specification are provided below. Each of these references is incorporated herein by reference for all purposes.

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CLAIMS

1. A full length antibody having on the C-terminus of a light chain thereof a glutamine-containing extension comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5, NO:6, NO:7, NO:8, NO:9, NO:10, NO:11, NO:12, NO:13, NO:14, NO:15, NO:16, NO:17, NO:18, NO:19, NO:20, NO:21, NO:22, NO:23, NO:24, NO:25, NO:26, NO:29, NO:30, NO:31, NO:32, NO:33, NO:34, NO:35, NO:36, NO:37, NO:38, NO:39, and NO:40.

2. A full length antibody according to claim 1, wherein the extension has an amino acid sequence selected from the group consisting of SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5, and NO:6.

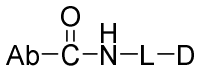
3. A full length antibody according to claim 1, wherein the extension has an amino acid sequence selected from the group consisting of SEQ ID NO:7, NO:8, NO:9, NO:10, NO:11, and NO:12.

4. A full length antibody according to claim 1, wherein the extension has an amino acid sequence selected from the group consisting of SEQ ID NO:14, NO:15, NO:16, NO:17, NO:18 and NO:19.

5. A full length antibody according to claim 1, wherein the extension has an amino acid sequence selected from the group consisting of SEQ ID NO:20, NO:21, NO:22, NO:23, NO:24, and NO:25.

6. A full-length antibody according to any one of claims 1-5, wherein the glutamine-containing extension has a valine-leucine (VL) on the N-terminal side of a glutamine.

7. A conjugate of the formula (IV)



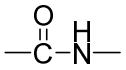
(IV)

wherein

Ab is a full length antibody having on the C-terminus (carboxy terminus) of a light chain thereof a glutamine-containing extension comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5, NO:6, NO:7, NO:8, NO:9, NO:10, NO:11, NO:12, NO:13, NO:14, NO:15, NO:16, NO:17, NO:18, NO:19, NO:20, NO:21, NO:22, NO:23, NO:24, NO:25, NO:26, NO:29, NO:30, NO:31, NO:32, NO:33, NO:34, NO:35, NO:36, NO:37,

NO:38, NO:39, and NO:40;

L is a linker moiety bonded to Ab via the amide bond



to a glutamine in a glutamine containing extension; and

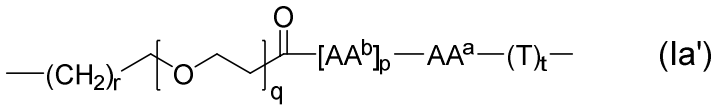
D is selected from the group consisting of a protein, a radioisotope, an assay agent, and a therapeutic agent.

8. A conjugate according to claim 7, wherein D is a cytotoxic drug.

9. A conjugate according to claim 7, wherein D is a TLR3, TLR7, TLR9, STING, NLRP3, or RIG-I agonist t.

10. A conjugate according to claim 7, wherein L is $–(CH_2)_{2\text{-}6}$-.

11. A conjugate according to claim 7, wherein L is



(Ia')

wherein

T is a self-immolating group;

t is 0 or 1;

AA^a and each AA^b are independently selected from the group consisting of alanine, β-alanine, γ-aminobutyric acid, arginine, asparagine, aspartic acid, γ-carboxyglutamic acid, citrulline, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, norleucine, norvaline, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine;

p is 1, 2, 3, or 4;

q is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and

r is 1, 2, 3, 4, or 5.

12. A method of making an antibody conjugate, comprising the steps of

(a) mixing a full length antibody having on the C-terminus (carboxy terminus) of a light chain thereof a glutamine-containing extension comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5, NO:6, NO:7, NO:8, NO:9, NO:10, NO:11, NO:12, NO:13, NO:14, NO:15, NO:16, NO:17, NO:18, NO:19, NO:20, NO:21, NO:22, NO:23, NO:24, NO:25, NO:26, NO:29, NO:30, NO:31, NO:32, NO:33, NO:34, NO:35, NO:36,

NO:37, NO:38, NO:39, and NO:40 with an amine donor compound comprising a primary amine and a moiety selected from the group consisting of a protein, a radioisotope, an assay agent, and a therapeutic agent, in the presence of a transglutaminase; and

(b) allowing the transglutaminase to catalyze the formation of an amide bond between the side chain carboxamide of a glutamine of the glutamine-containing extension and the primary amine of the amine donor compound, thereby making the antibody conjugate.

13. A method according to claim 12, wherein the amine donor compound has the structure

H_2N-L-D

wherein L is a linker moiety and D is a protein, a radioisotope, an assay agent, or a therapeutic agent.

14. A method according to claim 13, wherein the amine donor compound has the structure



(I)

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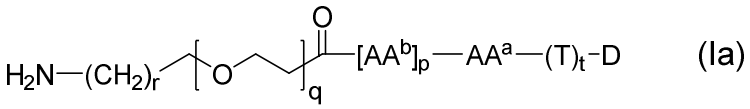
15. A method according to claim 12, wherein the amine donor compound has a structure represented by formula (Ia)



(Ia)

wherein

D is a protein, a radioisotope, an assay agent, or a therapeutic agent;

T is a self-immolating group;

t is 0 or 1;

AA^a and each AA^b are independently selected from the group consisting of alanine, β-alanine, γ-aminobutyric acid, arginine, asparagine, aspartic acid, γ-carboxyglutamic acid, citrulline, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, norleucine, norvaline, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine;

p is 1, 2, 3, or 4;

q is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and

r is 1, 2, 3, 4, or 5.

16. A method according to claim 12, wherein the moiety is a therapeutic agent.

17. A method according to claim 16, wherein the therapeutic agent is a cytotoxic drug.

18. A conjugate according to claim 16, wherein therapeutic agent is a TLR3, TLR7, TLR9, STING, NLRP3, or RIG-I agonist.

19. A method of making an antibody conjugate, comprising the steps of

(a) mixing a full length antibody having on the C-terminus (carboxy terminus) of a light chain thereof a glutamine-containing extension comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5, NO:6, NO:7, NO:8, NO:9, NO:10, NO:11, NO:12, NO:13, NO:14, NO:15, NO:16, NO:17, NO:18, NO:19, NO:20, NO:21, NO:22, NO:23, NO:24, NO:25, NO:26, NO:29, NO:30, NO:31, NO:32, NO:33, NO:34, NO:35, NO:36, NO:37, NO:38, NO:39, and NO:40 with a first compound, which first compound is an amine donor compound having a primary amine and a first reactive functional group, in the presence of a transglutaminase;

(b) allowing the transglutaminase to catalyze the formation of an amide bond between the side chain carboxamide of a glutamine of the glutamine-containing extension and the primary amine of the first compound, to make an adduct of the antibody and the first compound;

(c) contacting the adduct with a second compound having a second reactive functional group and a moiety selected from the group consisting of a protein, a radioisotope, an assay agent, and a therapeutic agent; the second reactive functional group being capable of reacting with the first reactive functional group to form a covalent bond therebetween; and

(d) allowing the first and second reactive functional groups to react and form a covalent bond therebetween, thereby making the antibody conjugate.

20. A method according to claim 19, wherein the first compound has the structure

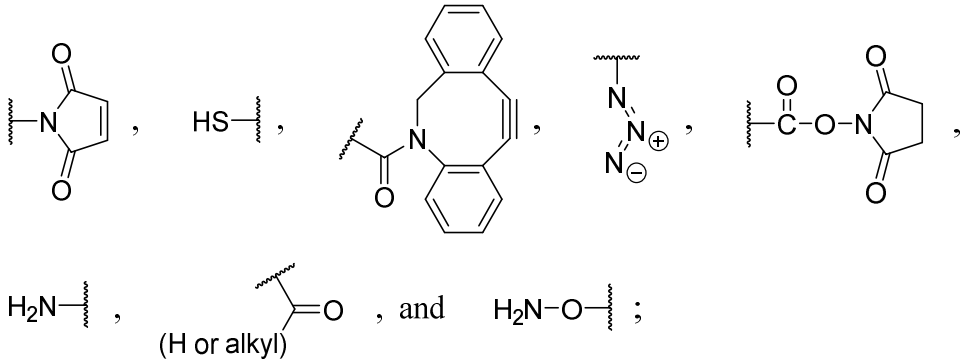
$$H_2N\text{-}L'\text{-}R'$$

wherein L’ is a first linker moiety and R’ is a first reactive functional group and the second compound has the structure

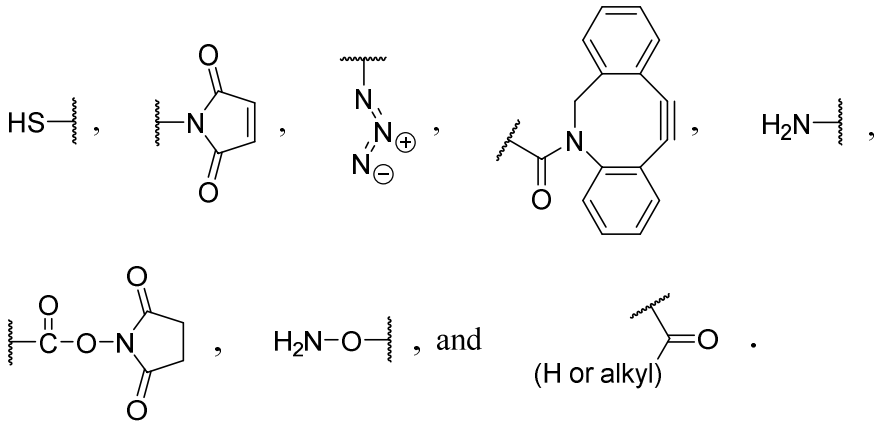
$$R''\text{-}L''\text{-}D$$

wherein R” is a second reactive functional group capable of reacting with R’, L” is a second linker moiety, and D is protein, a radioisotope, an assay agent, or a therapeutic agent.

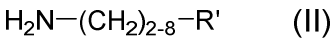
21. A method according to claim 20, wherein R' is selected from



and, reciprocally, R" is selected from

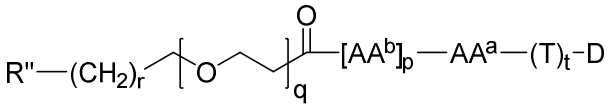


22. A method according to claim 20, wherein the first compound has a structure represented by formula (II)



(II)

and the second compound has a structure represented by formula (III)



(III)

wherein

R' is a first reactive functional group;

R" is a second reactive functional group capable of reacting with R';

D is a protein, a radioisotope, an assay agent, or a therapeutic agent;

T is a self-immolating group;

t is 0 or 1;

AA^a and each AA^b are independently selected from the group consisting of alanine, β-alanine, γ-aminobutyric acid, arginine, asparagine, aspartic acid, γ-carboxyglutamic acid, citrulline, cysteine, glutamic acid, glutamine, glycine,

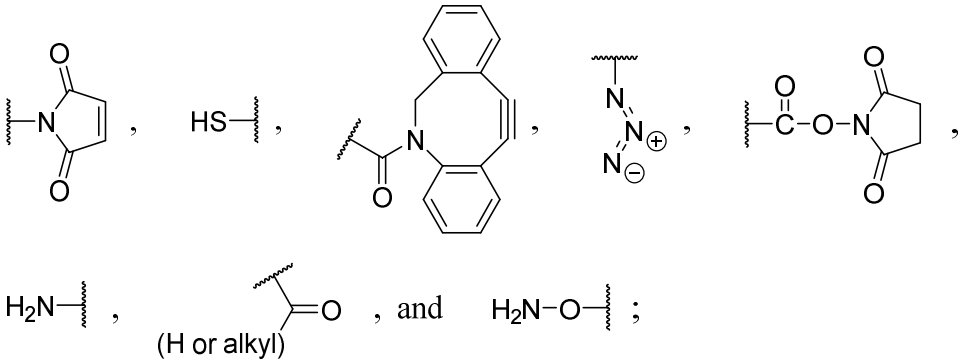
histidine, isoleucine, leucine, lysine, methionine, norleucine, norvaline, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine;

p is 1, 2, 3, or 4;

q is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and

r is 1, 2, 3, 4, or 5.

23. A method according to claim 22, wherein R' is selected from



and, reciprocally, R" is selected from

