SUSTAINED DELIVERY OF ANGIOPOETIN-LIKE 3 POLYPEPTIDES

The present application claims the benefit of U.S. Provisional Application Serial No. 62/740,609, filed on October 3, 2018, which is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

Described herein are drug delivery systems for delivering biologically active agents comprising primary or secondary amines, or a ring nitrogen atom of an azaheteroaryl ring, pharmaceutically acceptable salts thereof, drug delivery reagents related thereto, pharmaceutical compositions comprising the drug delivery systems, and the use of the drug delivery systems as sustained release therapeutics.

REFERENCE TO SEQUENCE LISTING

This application is filed with a Computer Readable Form of a Sequence Listing in accordance with 37 C.F.R. § 1.821(c). The text file submitted, "PAT058317-WO-PCT_SL.txt" was created on October 31, 2019, has a file size of 99,303 bytes, and is hereby incorporated by reference in its entirety.

BACKGROUND

Modulation of the physicochemical or pharmacokinetic properties of a drug in vivo may be affected by conjugation of the drug with a carrier. In particular, conjugation of a drug with a carrier is frequently used as a means to increase the therapeutic duration of action, reduce the maximum concentration of the drug after administration or localize delivery of the drug to a desired tissue or compartment or a combination of these purposes. Typically, carriers in drug delivery systems are either (a) used in a non-covalent fashion with the drug physicochemically formulated into a solvent-carrier mixture or (b) linked by covalent attachment of a carrier reagent to a functional group present in the drug.

Non-covalent approaches require a highly efficient drug encapsulation to prevent uncontrolled burst release of the drug that may occur either at initial administration of the carrier-drug system or during degradation of the carrier after administration to a subject. Restraining the diffusion of an unbound, water-soluble drug molecule requires strong van der Waals contacts,

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frequently mediated through hydrophobic moieties, hydrogen-bonding, or electrostatic binding mediated through charged moieties. Many conformationally sensitive drugs, such as proteins, peptides, or antibodies are rendered dysfunctional during the encapsulation process and/or during subsequent storage of the encapsulated drug.

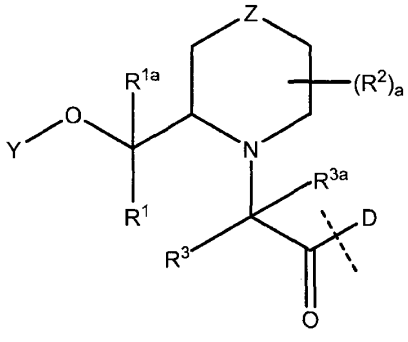
Alternatively, a drug may be covalently conjugated to a carrier via a stable linker or via a reversible linker moiety from which the drug is released. If the drug is stably connected to the carrier, such a conjugate needs to exhibit sufficient residual activity to have a pharmaceutical effect and the conjugate is constantly in an active form.

If the drug is conjugated to the carrier through a cleavable linker, such conjugates are typically referred to as carrier-linked drugs. This approach can be applied to various classes and sizes of biologically active molecules, from low molecular weight organic molecules, natural products, antibodies and analogs thereof, proteins, peptides, and the like. An important consideration for carrier-linked drugs is the mechanism for releasing the drug from the carrier. The release mechanism may be enzymatic, pH-dependent, or via autonomous hydrolysis. Typically, the drug release is not easily controllable and difficult to sustain over long time periods.

There continues to be a need for new drug delivery systems suitable for the sustained release of biologically active moieties in therapeutic applications. Described herein are drug delivery systems that provide sustained release of biologically active moieties for therapeutically relevant applications.

SUMMARY

One embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (I), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:



(I),

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where the dashed line indicates attachment to the primary amine; R^1 is hydrogen or C_1-C_4alkyl; R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl; R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination with the carbon atom(s) to which they are attached form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group; a is 0, 1, 2, 3 or 4; R^3 is hydrogen or C_1-C_4alkyl; R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl; Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$; R^{12} is hydrogen or C_1-C_4alkyl; R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl; R^{13} is C_1-C_4alkyl; or R^{12} and R^{13}, taken in combination with $C(R^{12a})$ and O form a 5, 6, or 7-member cyclic ether; R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy; R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl; R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups; b is an integer of from 1 to 10; Z is CH-L-A, CH-A, N-L-A, or N-A; L is an optionally substituted bivalent linker; A is hydrogen, C_1-C_8alkyl, $C(O)C_1$-C_8alkyl, $C(O)N(H)C_1$-C_8alkyl, $C(O)OC_1$-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10}; R^{10} is a reactive functional group suitable for coupling Formula (I) to a carrier; and R^{11} is a carrier. In one aspect, D comprises an ANGPTL3 polypeptide having at least 95% identity to any one of SEQ ID NO: 1 or 3–45. In another aspect, D comprises any one of SEQ ID NO:1 or 3–45. In another aspect, D comprises an ANGPTL3 polypeptide having a K423Q substitution or a K423 deletion. In another aspect, D comprises an ANGPTL3 polypeptide comprising amino acid residues 201–460; 207–460; 225–455; 225–455; 225–460; 225–460; 226–455; 226–455; 226–460; 226–460; 228–455; 228–455; 228–460; 228–460; 233–455; 233–455; 233–460; 233–460; 241–455; 241–455; 241–460; 241–460; 242–455; 242–455; 242–460; or 242–460, each in reference to SEQ ID NO:1. In another aspect, D comprises an ANGPTL3 polypeptide comprising at least 95% identity to amino acid residues 242–460 in reference to SEQ ID NO:1 and a K423Q substitution. In another aspect, D comprises an ANGPTL3 polypeptide comprising amino acid residues 242–460 in reference to SEQ ID NO:1 and a K423Q substitution, D1, (SEQ ID NO:19). In another aspect, R^1 is hydrogen

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or methyl, R^{1a} is hydrogen or methyl, or CR^1R^{1a}, taken in combination form a cyclopropan-1,1-diyl group. In another aspect, the variable a is 0. In another aspect, R^3 and R^{3a} are each hydrogen. In another aspect, Y is $C(O)R^4$ and R^4 is C_1-C_6alkyl, C_3-C_6cycloalkyl or C_1-C_2alkoxyC_1-C_2alkyl. In another aspect, R^4 is methyl, ethyl, propyl, isopropyl, 1-methyl-cyclopropyl, or methoxymethyl. In another aspect, Y is $SiR^5R^6R^7$; R^5 and R^6 are each methyl, ethyl, propyl or isopropyl; and R^7 is C_1-C_4 alkyl, ethoxy, propoxy, isopropoxy, butoxy, iso-butoxy, sec-butoxy, 2-ethoxyethoxy, 2-isopropoxy-ethoxy, tetrahydropyranyloxy, or -$(OCHR^3CH_2)_bO$-C_1-C_4alkyl where b is 2, 3, or 4. In another aspect, Z is CH-L-A, CH-A, N-L-A, or N-A; L is an optionally substituted bivalent linker Q-$[Sp\text{-}Q]_h$-Q; Q is independently selected at each occurrence from a bond, O, C(O), N(H), N(C_1-C_4alkyl), C(O)NH, C(O)N(C_1-C_4alkyl), N(H)C(O), N(C_1-C_4alkyl)C(O), N(H)C(O)O, N(C_1-C_4alkyl)C(O)O, OC(O)N(H), OC(O)N(C1-C_4alkyl), N(H)C(O)N(H), N(C_1-C_4alkyl)C(O)N(H), N(H)C(O)N(C_1-C_4alkyl), N(C_1-C4alkyl)C(O)N(C_1-C_4alkyl), C(O)O, OC(O), OC(O)O, S, $S(O)_2$, N(H)$S(O)_2$, N(C_1-C_4alkyl)$S(O)_2$, $S(O)_2$N(H), $S(O)_2$N(C_1-C_4alkyl), C_1-C_2alkyl-C(O)N(H), N(H)C(O)C_1-C_2alkyl, C_1-C_2alkyl-C(O)O, OC(O)C_1-C_2alkyl, 1,2,3-triazole, $OP(O)_2$, $P(O)_2O$, C_1-C_4alkyl-$P(O)_2$-O, or O-$P(O)_2$-C_1-$_4$alkyl; Sp is independently selected at each occurrence from an optionally substituted C_1-C_{20}alkyl, C_2-C_{20}alkenyl, C_2-C_{20}alkynyl, $[W\text{-}O]_g$, C_1-C_8alkyl-$[O\text{-}W]_g$, $[O\text{-}W]_g$-O-C_1-C_8alkyl, C_1-C_8Calkyl-$[O\text{-}W]_g$-O-C_1-C_8alkyl, or oligopeptide; h is an integer of between 1 and 20; g is a weighted average number of between about 2 and about 50; W is C_2-C_4alkyl-1,2-diyl in which a hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom; A is hydrogen, C_1-C_8alkyl, C(O)C_1-C_8alkyl, C(O)OC$_1$-C_8alkyl, C(O)N(H)C_1-C_8alkyl, R^{10}, or R^{11} wherein the alkyl group is optionally substituted with 0 or 1 R^{10}; R^{10} is a reactive functional group suitable for coupling Formula (I) to a carrier; and R^{11} is a carrier. In another aspect, Z is CHR^8 or NR^9; R^8 and R^9 are each independently selected from hydrogen, C_1-C_8 alkyl, C(O)-$(CH_2)_n$-Q-A, C(O)C_1-C_8 alkyl, or $-C(O)(CH_2)_q[O\text{–}W]_g(NHC(O))_m(CH_2)_q[O\text{–}W]_p$-Q-A, wherein the alkyl group is optionally substituted with 0 or 1 Q-A; q is independently at each occurrence 1, 2, or 3; g and p each independently have a weighted average length of between about 2 and about 50; m is 1 or 0; W is C_2-C_4alkyl-1,2-diyl in which a hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom; Q is a bond, O, N(H) or N(C_1-C_4alkyl); A is hydrogen, C_1-C_8alkyl, C(O)C_1-C_8alkyl, C(O)N(H)C_1-C_8alkyl, C(O)OC$_1$-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10}; R^{10} is a reactive functional group suitable for coupling Formula (I) to a carrier; and R^{11} is a carrier. In another aspect, Z is NR^9; R^9 is C(O)-

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$(CH_2)_n$-Q-A or -C(O)$(CH_2)_q$[O-W]$_g$(NHC(O))$_m$$(CH_2)_q$[O-W]$_p$-Q-A; n is an integer of 1 to 8; and A is R^{10} or R^{11}. In another aspect, R^{10} is azidyl, alkynyl, substituted or unsubstituted C_7-C_{12} cycloalkynyl, substituted or unsubstituted C_7-C_{12} heterocycloalkynyl, substituted or unsubstituted C_7-C_{12} cycloalkenyl, norbornyl, substituted or unsubstituted vinyl carboxyl, substituted or unsubstituted vinyl sulfonyl, substituted or unsubstituted C_2-C_8 alkenyl, amino, thiol, substituted or unsubstituted C_1-C_8 carboxyl, substituted or unsubstituted C_1-C_8 carbonyl, -O-NH_2, hydrazidyl, maleimide, alpha-halo carbonyl, furan, substituted or unsubstituted tetrazinyl, lysine, glutamine, cyclodextrin, or adamantanyl. In another aspect, R^{10} comprises a reactive functional group suitable for coupling the drug delivery system or pharmaceutically acceptable salt of Formula (I) to a carrier. In another aspect, R^{11} is biodegradable. In another aspect, R^{11} comprises a polymer or cross-linked polymer. In another aspect, R^{11} comprises a hydrogel comprising one or more cross-linked polymers. In another aspect, R^{11} comprises a polymer, cross-linked polymer, or hydrogel comprising one or more of hyaluronic acid, polyethylene glycol, polypropylene glycol, polyethylene oxide, polypropylene oxide, polyglutamate, polylysine, polysialic acid, polyvinyl alcohol, polyacrylate, polymethacrylate, polyacrylamide, polymethacrylamide, polyvinyl pyrrolidone, polyoxazoline, polyiminocarbonate, polyamino acid, hydrophilic polyester, polyamide, polyurethane, polyurea, dextran, agarose, xylan, mannan, carrageenan, alginate, gelatin, collagen, albumin, cellulose, methylcellulose, ethylcellulose, hydroxypropylmethylcellulose, hydroxyethyl starch, chitosan, nucleic acids, derivatives thereof, co-polymers thereof, or combinations thereof. In another aspect, R^{11} comprises hyaluronic acid, polyethylene glycol, a cross-linked hydrogel of hyaluronic acid, a cross-linked hydrogel of polyethylene glycol, or combinations thereof. In another aspect, R^{11} comprises hyaluronic acid or polyethylene glycol. In another aspect, R^{11} comprises a hydrogel comprising cross-linked hyaluronic acid or cross-linked polyethylene glycol. In another aspect, the hyaluronic acid or polyethylene glycol are functionalized with at least one functional group comprising azidyl, alkynyl, substituted or unsubstituted C_7-C_{12} cycloalkynyl, substituted or unsubstituted C_7-C_{12} heterocycloalkynyl, C_7-C_{12} cycloalkenyl, norbornyl, vinyl carboxyl, vinyl sulfonyl, C_2-C_8 alkenyl, amino, thiol, C_1-C_8 carboxyl, C_1-C_8 carbonyl, -O-NH2, carbohydrazide, maleimide, alpha-halo carbonyl, furan, substituted or unsubstituted tetrazinyl, lysine, glutamine, cyclodextrin, adamantanyl, or combinations thereof. In another aspect, R^{11} comprises a hydrogel comprising cross-linked hyaluronic acid, wherein the hyaluronic acid comprises at least one side chain selected

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from -NH(W1)(O(W1))$_d$-V, wherein W1 is C_2-C_4alkyl-1,2-diyl in which a hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom; d is a number average of 0 to 500; and V is a suitable functional group comprising azidyl, alkynyl, substituted or unsubstituted C_7-C_{12} cycloalkynyl, substituted or unsubstituted C_7-C_{12} heterocycloalkynyl, C_7-C_{12} cycloalkenyl, norbornyl, vinyl carboxyl, vinyl sulfonyl, C_2-C_8 alkenyl, amino, thiol, C_1-C_8 carboxyl, C_1-C_8 carbonyl, -O-NH2, carbohydrazide, maleimide, alpha-halo carbonyl, furan, substituted or unsubstituted tetrazinyl, lysine, glutamine, cyclodextrin, or adamantanyl. In another aspect, V is azide.

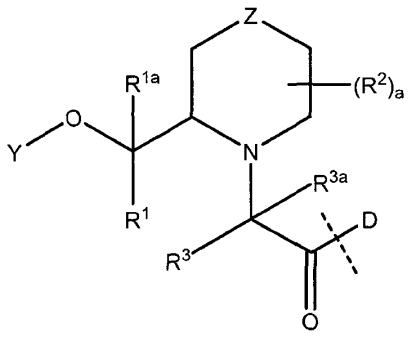
Another embodiment described herein is a process for making a cross-linked carrier formulation, the process comprising: (a) functionalizing a carrier molecule, R^{11}; (b) preparing a reactive cross-linker; and (c) reacting the functionalized carrier molecule with the reactive cross-linker to form a cross-linked carrier by incubation for about 0.5 hours to about 48 hours at a temperature of about 4 °C to about 60 °C. In one aspect, the carrier molecule comprises hyaluronic acid or polyethylene glycol. In another aspect, the carrier molecule is functionalized with azide, sulfhydryl, amine, aminoxy (O-NH_2), or aldehyde moieties to provide reactive functional groups for cross-linking. In another aspect, the preparation of the reactive crosslinker comprises reacting a polyethylene glycol with 1-((tert-butoxycarbonyl)amino)cyclopropane-1-carboxylic acid, 3-((tert-butoxycarbonyl)amino)propanoic acid, 1-(tert-butoxycarbonyl)piperidine-4-carboxylic acid, 2-(1-(((tert-butoxycarbonyl)amino)methyl)cyclopropyl)acetic acid, 2-methyl-3-((tert-butoxycarbonyl)amino)propanoic acid, or 7-((tert-butoxycarbonyl)amino)heptanoic acid and deprotecting the functionalized polyethylene glycol ester. In another aspect, the preparation of the reactive crosslinker further comprises introduction at least two bicyclo[6.1.0]non-4-yn-9-yl)methyl groups after deprotection of the functionalzed polyethylene glycol ester. Another aspect comprises a cross-linked hydrogel obtainable using the methods described herein. In another aspect, the carrier molecule is functionalized with azide.

Another embodiment described herein is a process for preparing a drug adduct, comprising a traceless linker, R, coupled to an ANGPTL3 polypeptide comprising at least one primary amine, D, the process comprising: (a) providing an ANGPTL3 polypeptide comprising at least one primary amine, D; (b) reacting the biologically active agent with a traceless linker, R, that has an activated carbonyl functional group; and (c) purifying the drug adduct from the reagents. One aspect is a drug adduct obtainable using the process described herein.

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Another embodiment described herein is a process for making a drug delivery system, the process comprising: (a) preparing a carrier molecule, R^{11}, wherein R^{11} is a cross-linked hydrogel; optionally, step (a) may further comprise purifying the cross-linked hydrogel carrier molecule R^{11}; (b) separately conjugating the traceless linker, R, to an ANGPTL3 polypeptide comprising at least one primary amine, D, thereby forming the traceless linker-D adduct; step (b) may optionally further comprise purification of the traceless linker-D adduct, (c) conjugating the carrier molecule, R^{11}, with the traceless linker-D adduct; and (d) purifying the drug delivery system from the reagents. One aspect is a drug delivery system made using the method described herein.

Another embodiment described herein is a method for treating macular degeneration comprising administering to a subject in need thereof a drug delivery system or pharmaceutically acceptable salt thereof comprising a conjugate D–R, that is represented by Formula (I), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; R is a linker suitable for release of D:



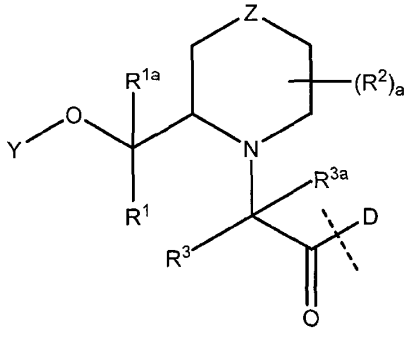
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where the dashed line indicates attachment to the primary amine; R^1 is hydrogen or C_1-C_4alkyl; R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl; R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group; a is 0, 1, 2, 3 or 4; R^3 is hydrogen or C_1-C_4alkyl; R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl; Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$; R^{12} is hydrogen or C_1-C_4alkyl; R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl; R^{13} is C_1-C_4alkyl; or $CHR^{12}OR^{13}$, taken in combination form a 5, 6, or 7-member cyclic ether; R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by hydroxy, amino, C_1-C_4alkoxy or mono- and di- C_1-

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C_4alkylamino; R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl; R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or -$(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups; b is an integer of from 1 to 10; Z is CHR^8 or NR^9; R^8 and R^9 are each independently selected from hydrogen, C_1-C_8 alkyl, $C(O)C_1$-C_8 alkyl, or $-C(O)(CH_2)_q[O-W]_g(NHC(O))_m(CH_2)_q[O-W]_p$-Q-A, wherein the alkyl group is optionally substituted with 0 or 1 Q-A; q is independently at each occurrence 1, 2, or 3; g and p each independently have a weighted average length of between about 2 and about 50; m is 1 or 0; W is C_2-C_4alkyl-1,2-diyl in which the hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom; Q is a bond, O, N(H) or N(C_1-C_4alkyl); A is hydrogen, C_1-C_8alkyl, $C(O)C_1$-C_8alkyl, $C(O)N(H)C_1$-C_8alkyl, $C(O)OC_1$-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10}; R^{10} is a reactive functional group suitable for coupling Formula (I) to a carrier; and R^{11} is a carrier.

Another embodiment described herein is a method for treating musculoskeletal disorders comprising administering to a subject in need thereof a drug delivery system or pharmaceutically acceptable salt thereof comprising a conjugate D–R, that is represented by Formula (I), where D an ANGPTL3 polypeptide comprising at least one primary amine; R is a linker suitable for release of D:



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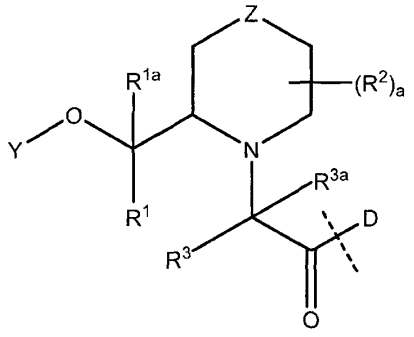
where the dashed line indicates attachment to the primary amine, secondary amine, or ring nitrogen atom of an azaheteroaryl ring; R^1 is hydrogen or C_1-C_4alkyl; R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl; R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group; a is 0, 1, 2, 3 or 4; R^3 is hydrogen or C_1-C_4alkyl;

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R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl; Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$; R^{12} is hydrogen or C_1-C_4alkyl; R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl; R^{13} is C_1-C_4alkyl; or $CHR^{12}OR^{13}$, taken in combination form a 5, 6, or 7-member cyclic ether; R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by hydroxy, amino, C_1-C_4alkoxy or mono- and di- C_1-C_4alkylamino; R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl; R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups; b is an integer of from 1 to 10; Z is CHR^8 or NR^9; R^8 and R^9 are each independently selected from hydrogen, C_1-C_8 alkyl, $C(O)C_1$-C_8 alkyl, or $-C(O)(CH_2)_q[O–W]_g(NHC(O))_m(CH_2)_q[O–W]_p$-Q-A, wherein the alkyl group is optionally substituted with 0 or 1 Q-A; q is independently at each occurrence 1, 2, or 3; g and p each independently have a weighted average length of between about 2 and about 50; m is 1 or 0; W is C_2-C_4alkyl-1,2-diyl in which the hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom; Q is a bond, O, N(H) or N(C_1-C_4alkyl); A is hydrogen, C_1-C_8alkyl, $C(O)C_1$-C_8alkyl, $C(O)N(H)C_1$-C_8alkyl, $C(O)OC_1$-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10}; R^{10} is a reactive functional group suitable for coupling Formula (I) to a carrier; and R^{11} is a carrier.

Another embodiment described herein is a method for treating a disease or disorder comprising administering to a subject in need thereof a drug delivery system or pharmaceutically acceptable salt thereof comprising a conjugate D–R, that is represented by Formula (I), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; R is a linker suitable for release of D:

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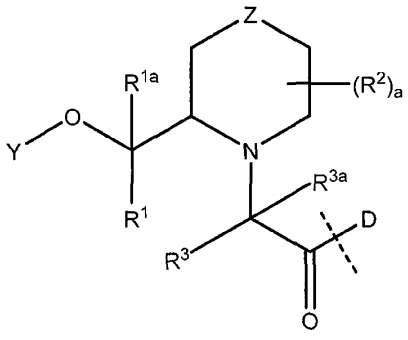
(I),

where the dashed line indicates attachment to the primary amine; R^1 is hydrogen or C_1-C_4alkyl; R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl; R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group; a is 0, 1, 2, 3 or 4; R^3 is hydrogen or C_1-C_4alkyl; R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl; Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$; R^{12} is hydrogen or C_1-C_4alkyl; R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl; R^{13} is C_1-C_4alkyl; or $CHR^{12}OR^{13}$, taken in combination form a 5, 6, or 7-member cyclic ether; R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by hydroxy, amino, C_1-C_4alkoxy or mono- and di- C_1-C_4alkylamino; R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl; R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups; b is an integer of from 1 to 10; Z is CHR^8 or NR^9; R^8 and R^9 are each independently selected from hydrogen, C_1-C_8 alkyl, $C(O)C_1$-C_8 alkyl, or $-C(O)(CH_2)_q[O-W]_g(NHC(O))_m(CH_2)_q[O-W]_p$-Q-A, wherein the alkyl group is optionally substituted with 0 or 1 Q-A; q is independently at each occurrence 1, 2, or 3; g and p each independently have a weighted average length of between about 2 and about 50; m is 1 or 0; W is C_2-C_4alkyl-1,2-diyl in which the hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom; Q is a bond, O, N(H) or N(C_1-C_4alkyl); A is hydrogen, C_1-C_8alkyl, $C(O)C_1$-C_8alkyl, $C(O)N(H)C_1$-C_8alkyl, $C(O)OC_1$-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is

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optionally substituted with 0 or 1 R^{10}; R^{10} is a reactive functional group suitable for coupling Formula (I) to a carrier; and R^{11} is a carrier.

Another embodiment described herein is a means for extending half-life of an ANGPTL3 polypeptide comprising at least one primary amine, D, the means comprising attaching D to R, that is represented by Formula (I), where R is a linker suitable for release of D:



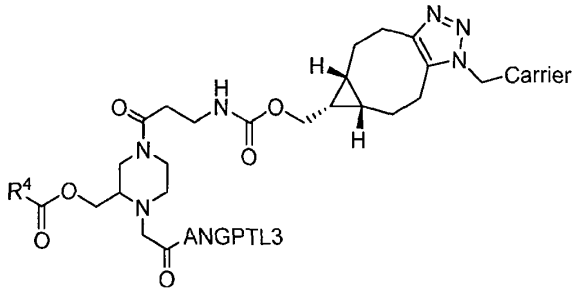
(I),

where the dashed line indicates attachment to the primary amine; R^1 is hydrogen or C_1-C_4alkyl; R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl; R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group; a is 0, 1, 2, 3 or 4; R^3 is hydrogen or C_1-C_4alkyl; R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl; Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$; R^{12} is hydrogen or C_1-C_4alkyl; R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl; R^{13} is C_1-C_4alkyl; or $CHR^{12}OR^{13}$, taken in combination form a 5, 6, or 7-member cyclic ether; R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by hydroxy, amino, C_1-C_4alkoxy or mono- and di- C_1-C_4alkylamino; R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl; R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups; b is an integer of from 1 to 10; Z is CHR^8 or NR^9; R^8 and R^9 are each independently selected from hydrogen, C_1-C_8 alkyl, $C(O)C_1$-C_8 alkyl, or $-C(O)(CH_2)_q[O-W]_g(NHC(O))_m(CH_2)_q[O-W]_p$-Q-A, wherein the alkyl group is optionally substituted with 0 or 1 Q-A; q is independently at each occurrence 1, 2, or 3; g and p each

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independently have a weighted average length of between about 2 and about 50; m is 1 or 0; W is C_2-C_4alkyl-1,2-diyl in which the hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom; Q is a bond, O, N(H) or N(C_1-C_4alkyl); A is hydrogen, C_1-C_8alkyl, C(O)C_1-C_8alkyl, C(O)N(H)C_1-C_8alkyl, C(O)OC_1-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10}; R^{10} is a reactive functional group suitable for coupling Formula (I) to a carrier; and R^{11} is a carrier. In one aspect, D comprises an ANGPTL3 polypeptide having at least 95% identity to any one of SEQ ID NO: 1 or 3–45.

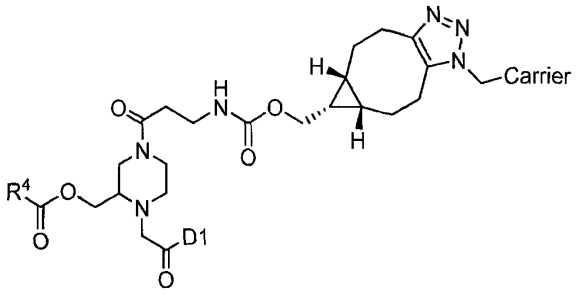
Another embodiment described herein is a drug delivery system comprising Formula (III):



(III),

wherein ANGPTL3 comprises an ANGPTL3 polypeptide having at least 95% identity to any one of SEQ ID NO: 1 or 3–45, or combinations thereof.

Another embodiment described herein is a drug delivery system comprising Formula (IV):



(IV),

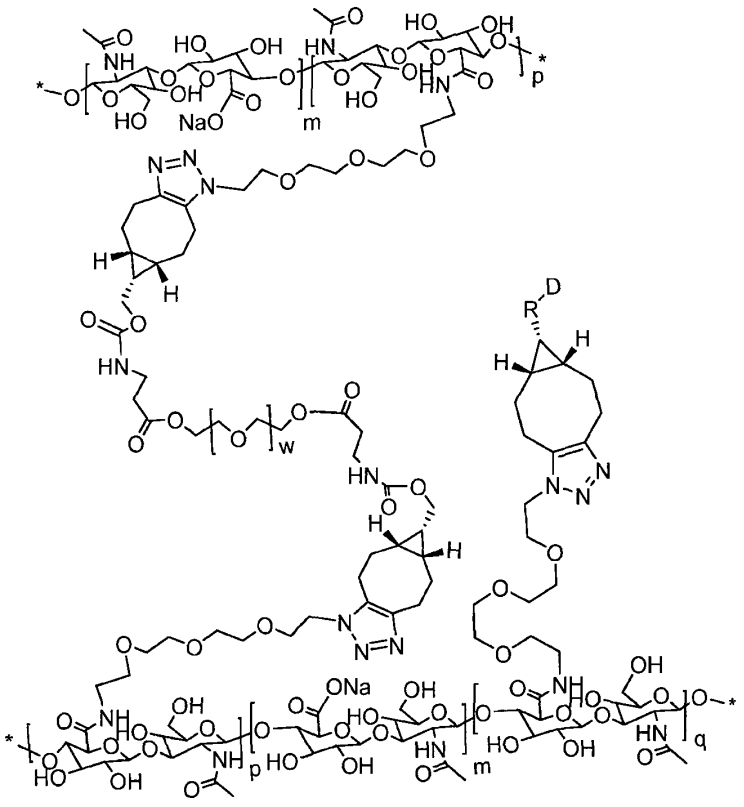
wherein D1 comprises an ANGPTL3 polypeptide comprising amino acid residues 242–460 in reference to SEQ ID NO:1 and a K423Q substitution (SEQ ID NO:19).

Another embodiment described herein is a drug delivery system comprising D–R–R^{11}, wherein R comprises a traceless linker, coupled to a biologically-active agent, D; D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R^{11} comprises a plurality of hyaluronic acid polymers each of which comprises a random series of unfunctionalized D-glucuronic acid monomers, one or more D-glucuronic acid monomers functionalized with a cross-

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linker, or one or more D-glucuronic acid monomers functionalized with a drug adduct, D–R. In one aspect, D comprises an ANGPTL3 polypeptide having at least 95% identity to any one of SEQ ID NO: 1 or 3–45.

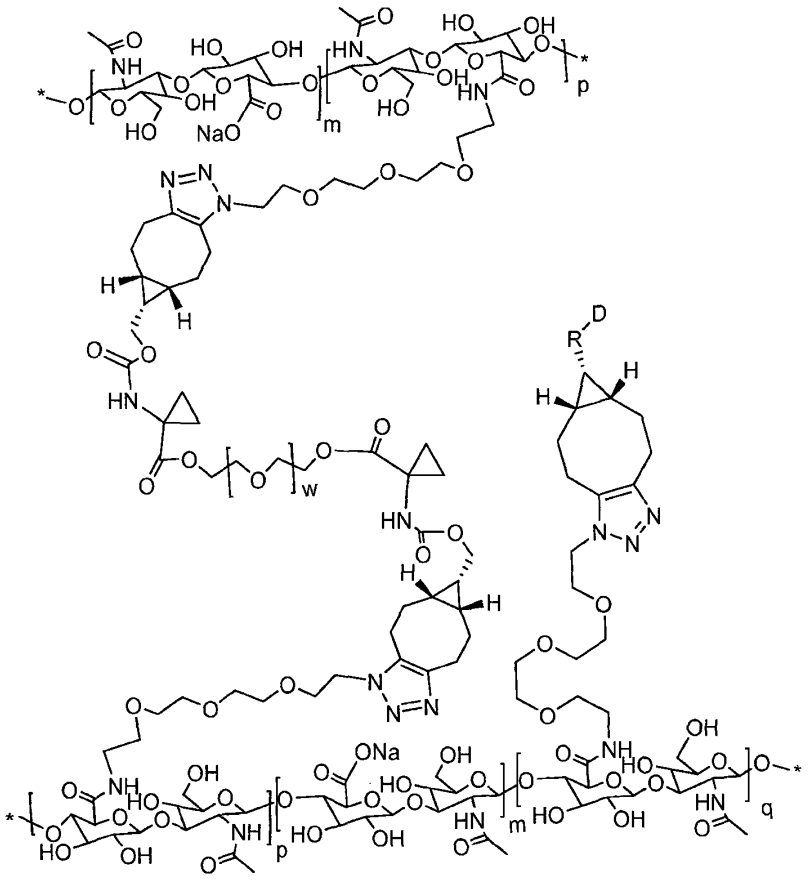
Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXIV), where D is an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release D:



(XXIV).

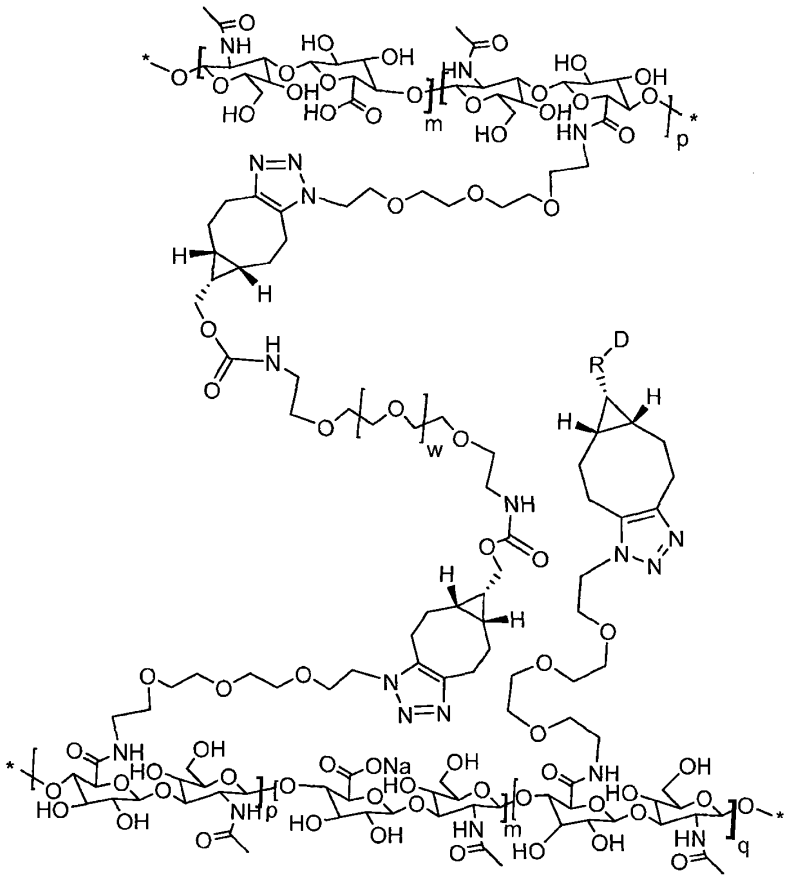
Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXV), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:

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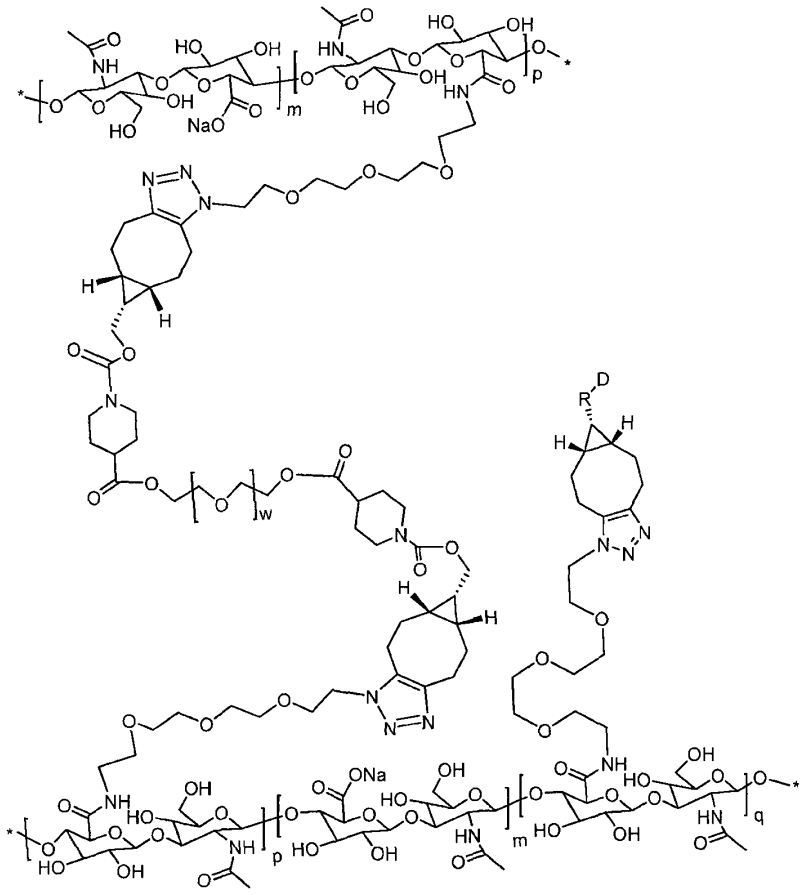
(XXV).

Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXVI), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:



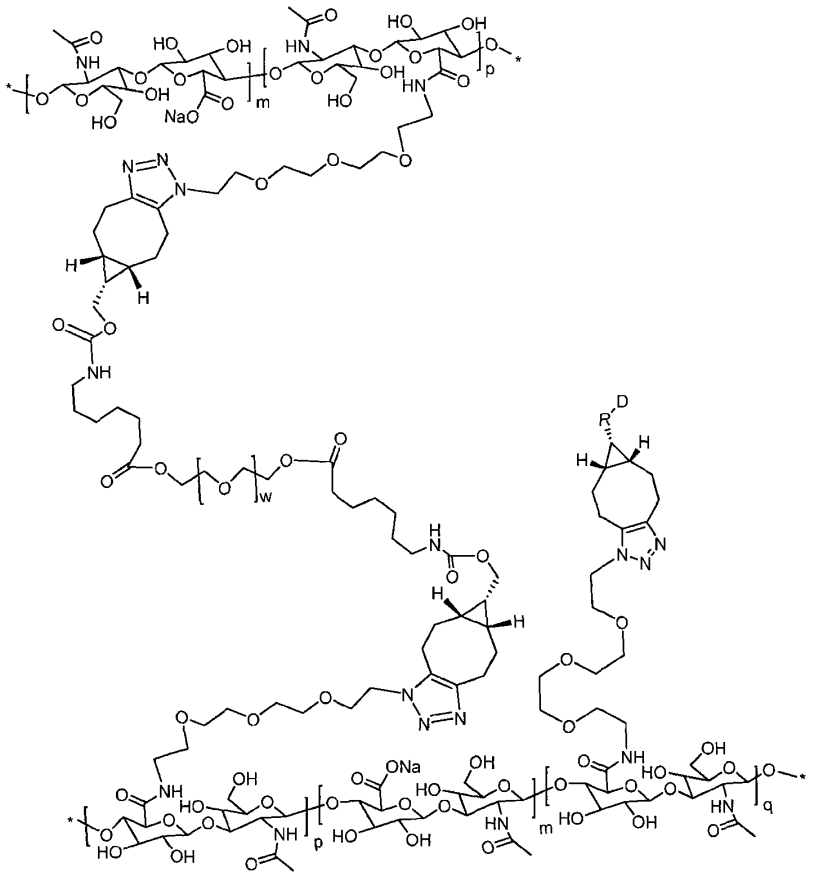
(XXVI).

Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXVII), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:



(XXVII).

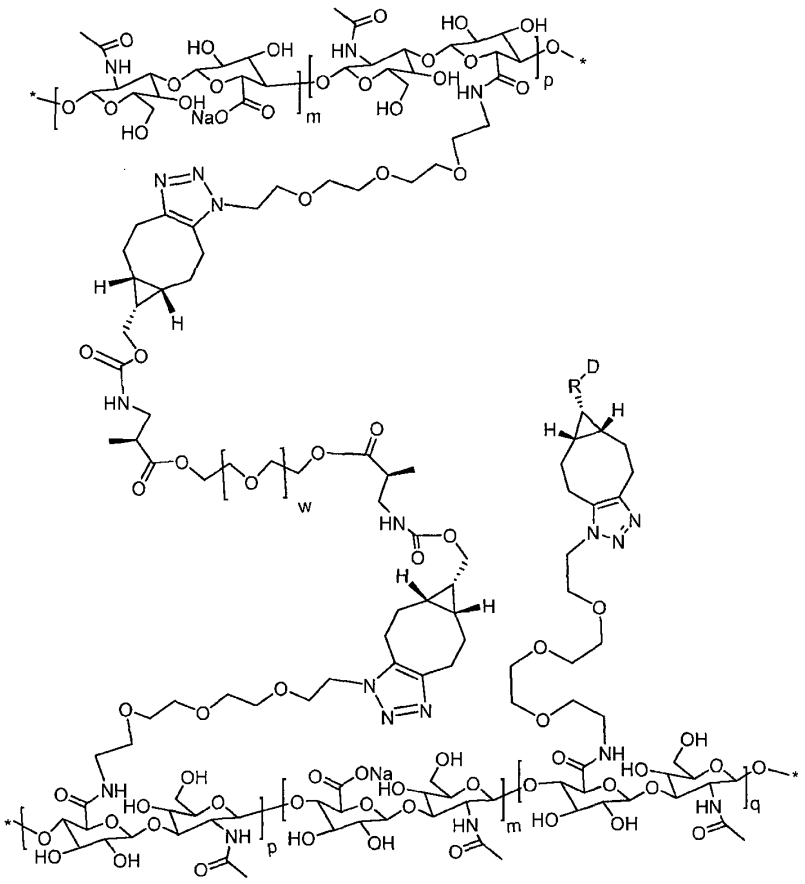
Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXVIII), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:



(XXVIII).

Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXIX), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:

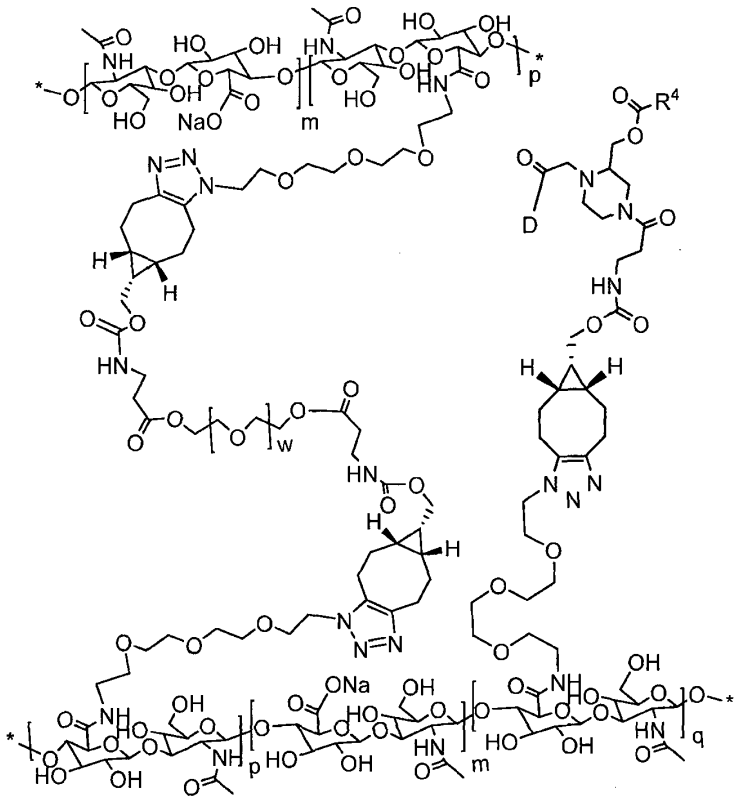
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(XXIX).

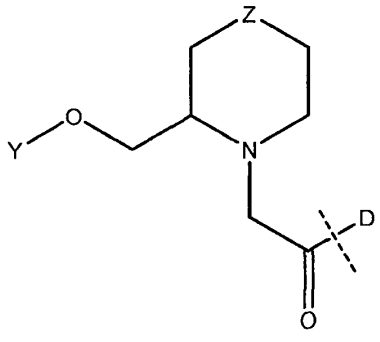
Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof that is represented by Formula (XXX), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R^4 is methyl, ethyl, propyl, isopropyl, 1-methyl-cyclopropyl, or methoxymethyl:

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(XXX).

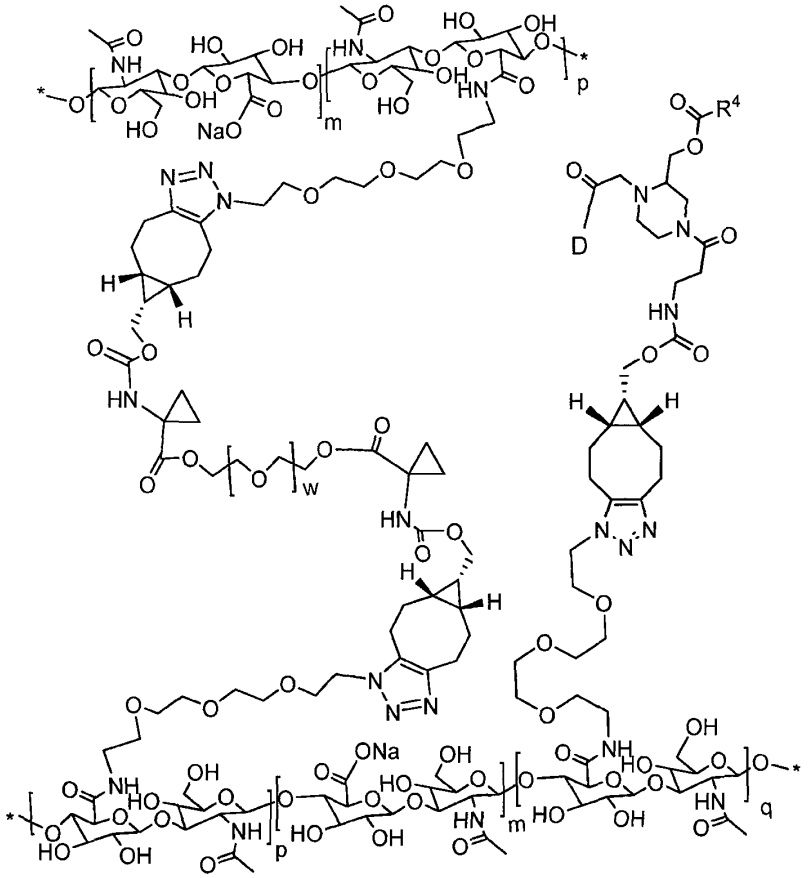
Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXXI), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:



(XXXI),

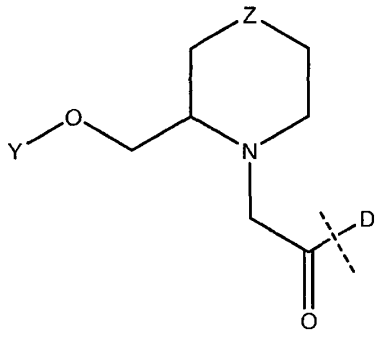
where the dashed line indicates attachment to the primary amine; Y is $C(O)R^4$; R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy; Z is N-L-A; L is $C(O)CH_2CH_2NH$; A is R^{11}; R^{11} is a hydrogel derived from a cross-linked hyaluronic acid, wherein the hyaluronic acid comprises at least one amide-linked side chain of $N(H)(CH_2CH_2O)_3CH_2CH_2N_3$, and wherein the cross-linker used to form the hydrogel comprises PEG(2000)-bis-[3-(((((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methoxy)carbonyl)amino)propanoate].

Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof that is represented by Formula (XXXII), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R^4 is methyl, ethyl, propyl, isopropyl, 1-methyl-cyclopropyl, or methoxymethyl:



(XXXII).

Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula XXXIII, where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:

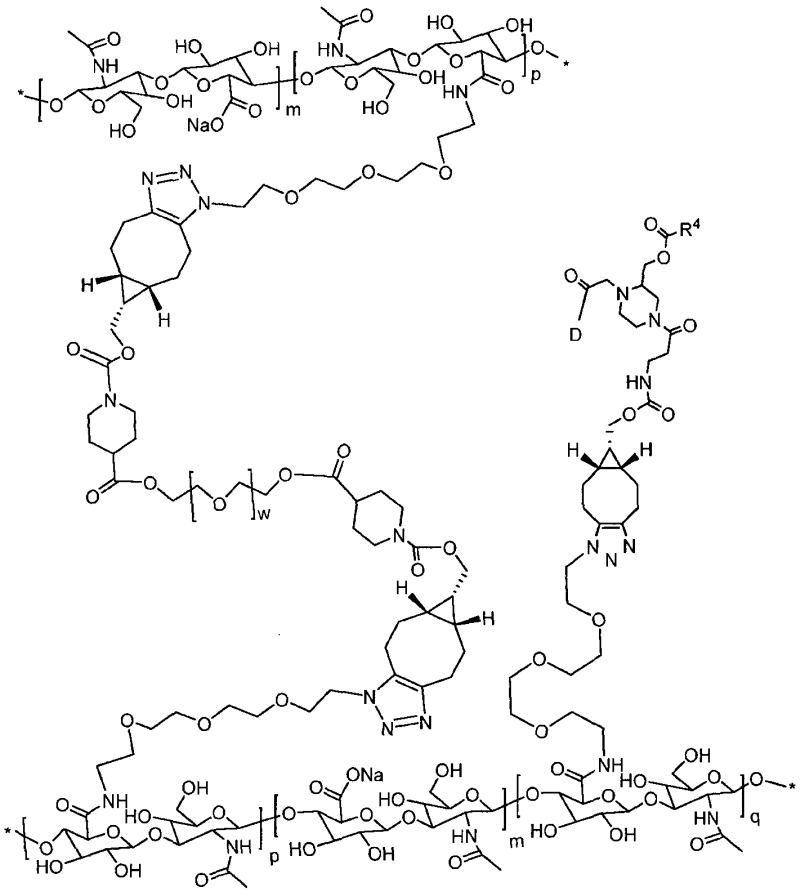


(XXXIII),

where the dashed line indicates attachment to the primary amine; Y is $C(O)R^4$; R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy; Z is N-L-A; L is $C(O)CH_2CH_2NH$; A is R^{11}; R^{11} is a hydrogel derived from a cross-linked hyaluronic acid, wherein the hyaluronic acid comprises at least one amide-linked side chain of $N(H)(CH_2CH_2O)_3CH_2CH_2N_3$, and wherein the cross-linker used to form the hydrogel comprises PEG(2000)-bis-[1-((((1′*R*,8′*S*,9′s)-bicyclo[6.1.0]non-4′-yn-9′-yl)methoxy)carbonyl)amino-cyclopropane-1-carboxylic acid ester].

Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof that is represented by Formula (XXXIV), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R^4 is methyl, ethyl, propyl, isopropyl, 1-methyl-cyclopropyl, or methoxymethyl:

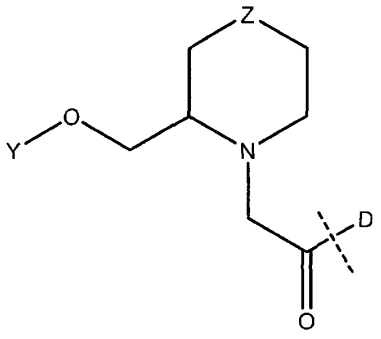
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(XXXIV).

Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula XXXV, where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:

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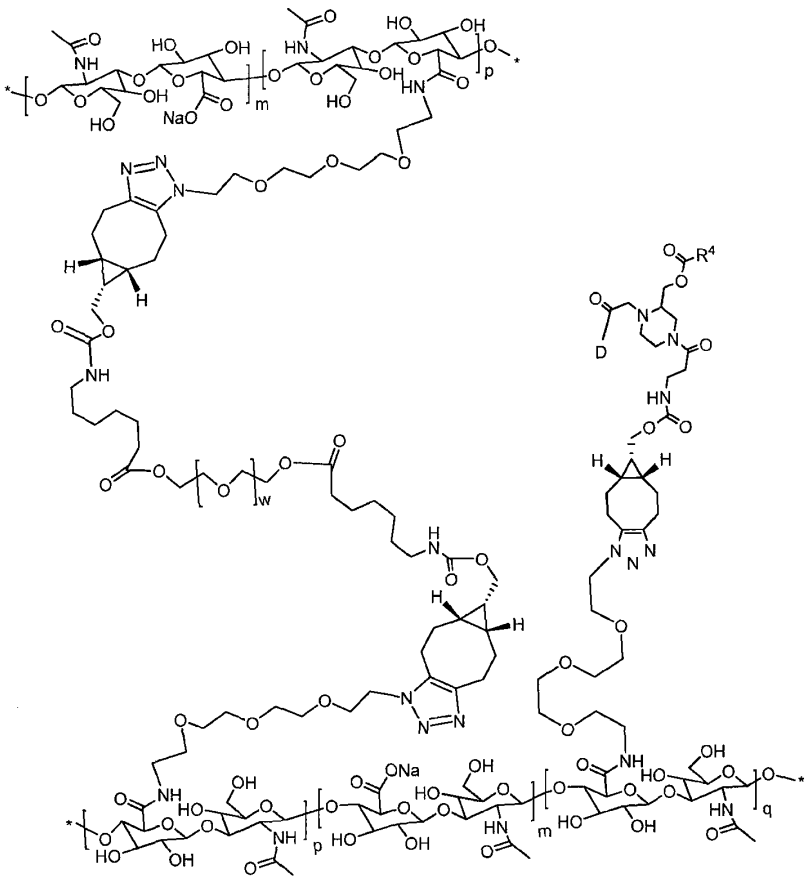


(XXXV),

where the dashed line indicates attachment to the primary amine; Y is $C(O)R^4$; R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy; Z is N-L-A; L is $C(O)CH_2CH_2NH$; A is R^{11}; R^{11} is a hydrogel derived from a cross-linked hyaluronic acid, wherein the hyaluronic acid comprises at least one amide-linked side chain of $N(H)(CH_2CH_2O)_3CH_2CH_2N_3$, and wherein the cross-linker used to form the hydrogel comprises PEG(2000)-bis-[1-((((1′*R*,8′*S*,9′s)-bicyclo[6.1.0]non-4′-yn-9′-yl)methoxy)carbonyl)piperidine-4-carboxylic acid ester].

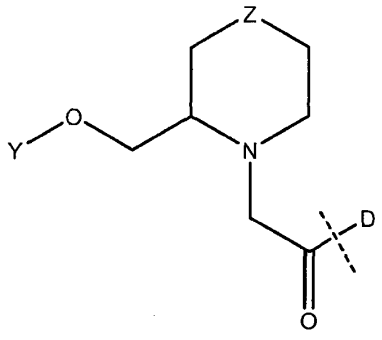
Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof that is represented by Formula (XXXVI), where Drug, D, comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R^4 is methyl, ethyl, propyl, isopropyl, 1-methyl-cyclopropyl, or methoxymethyl:

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(XXXVI).

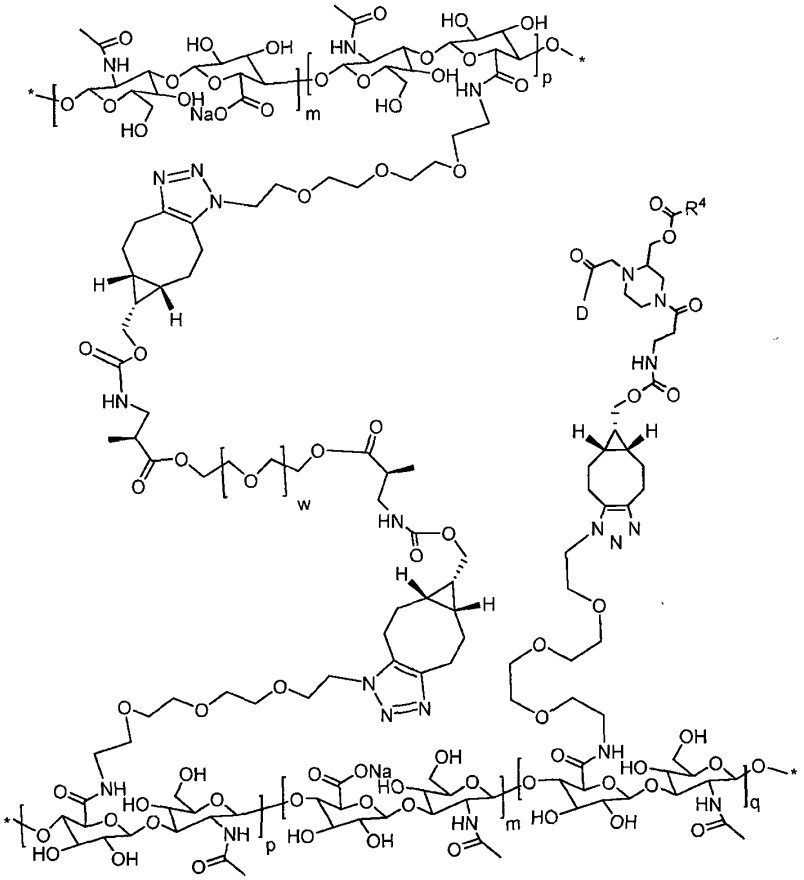
Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula XXXVII, where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:



(XXXVII),

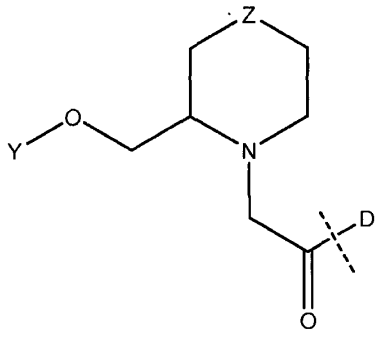
where the dashed line indicates attachment to the primary amine; Y is $C(O)R^4$; R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy; Z is N-L-A; L is $C(O)CH_2CH_2NH$; A is R^{11}; R^{11} is a hydrogel derived from a cross-linked hyaluronic acid, wherein the hyaluronic acid comprises at least one amide-linked side chain of $N(H)(CH_2CH_2O)_3CH_2CH_2N_3$, and wherein the cross-linker used to form the hydrogel comprises PEG(2000)-bis-[7-(((((1′*R*,8′*S*,9′s)-bicyclo[6.1.0]non-4′-yn-9′-yl)methoxy)carbonyl)amino)heptanoate].

Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof that is represented by Formula (XXXVIII), where Drug, D, comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R^4 is methyl, ethyl, propyl, isopropyl, 1-methyl-cyclopropyl, or methoxymethyl:



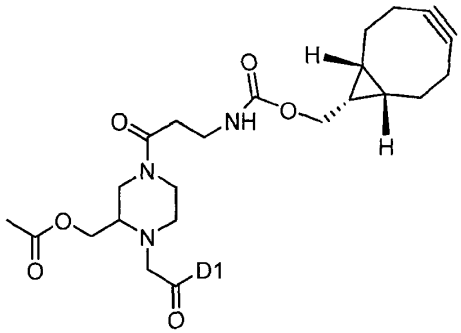
(XXXVIII).

Another embodiment described herein is a drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXXIX), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:



(XXXIX),

where the dashed line indicates attachment to the primary amine, secondary amine, or ring nitrogen atom of an azaheteroaryl ring; Y is $C(O)R^4$; R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy; Z is N-L-A; L is $C(O)CH_2CH_2NH$; A is R^{11}; R^{11} is a hydrogel derived from a cross-linked hyaluronic acid, wherein the hyaluronic acid comprises at least one amide-linked side chain of $N(H)(CH_2CH_2O)_3$ $CH_2CH_2N_3$, and wherein the cross-linker used to form the hydrogel comprises PEG(2000)-bis-[2-methyl-3-(((((1′*R*,8′*S*,9′s)-bicyclo[6.1.0]non-4′-yn-9′-yl)methoxy)carbonyl)amino)propanoate]. In one aspect, D is D1 (SEQ ID NO:19) and R^4 is -CH3, $-CH_2CH_3$, $-CH(CH_3)CH_3$, or methylcyclopropyl. In another aspect, D is D1 (SEQ ID NO:19) and R^4 is $-CH_3$. In another aspect, the D–R adduct comprises:



BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a cartoon of a drug delivery system comprising a drug conjugated to a carrier by a traceless linker, and the drug after release. Reaction of the trigger generates a nucleophilic functional group that cleaves the amide bond linking the drug to the drug delivery system. In this non-limiting example, the drug is linked via a primary amine group.

FIG. 2 shows a partial chemical representation of a hydrogel-drug conjugate, where the hydrogel is comprised of hyaluronic acid cross-linked with a PEG and drug is conjugated to the hydrogel by a traceless linker with trigger Y. In FIG. 2 and throughout the specification, polymeric molecules, such as hyaluronic acid, are represented as $[P]_n$, where P represents the monomeric repeating unit and n represents an average number of repeating unit of the monomer. The number of repeating units is a random distribution for any given polymer population. In addition, the relative connectivity of the independent molecules for hydrogels or drug delivery systems described herein is random within a population. Structural depictions of hydrogels or drug delivery systems represent a single potential structural unit in two dimensions, whereas these complexes are three-dimensional with many structural subunits.

FIG. 3A shows an exemplary reaction of a traceless linker with an azide-functionalized hyaluronic acid polymer.

FIG. 3B shows an exemplary reaction of a traceless linker with an azide-functionalized hydrogel.

FIG. 4A (illustrating Example 8.1) shows an example of the in vitro release of D1 (SEQ ID NO:19) from a hydrogel conjugate.

FIG. 4B shows in vitro release of D1 (SEQ ID NO:19) from hydrogel conjugate C2-2.

FIG. 5 (illustrating Example 9) shows the in vivo release of D1 (SEQ ID NO:19) from hydrogel conjugate C2-2 administered in rats via intra-articular injection.

DETAILED DESCRIPTION

Described herein are drug delivery systems for delivering biologically active agents comprising primary or secondary amines, or a ring nitrogen atom of an azaheteroaryl ring, pharmaceutically acceptable salts thereof, drug delivery reagents related thereto, pharmaceutical compositions comprising the drug delivery systems, and the use of the drug delivery systems as sustained release therapeutics.

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As used herein, the term "alkyl" refers to a straight chain, branched or cyclic carbon chain. Unless otherwise specified, one or more hydrogen atoms of an alkyl carbon may be replaced by a substituent. Representative examples of alkyl include, but are not limited to, methyl, ethyl, n-propyl, iso-propyl, n-butyl, sec-butyl, iso-butyl, tert-butyl, n-pentyl, isopentyl, neopentyl, n-hexyl, 3-methylhexyl, 2,2-dimethylpentyl, 2,3-dimethylpentyl, n-heptyl, n-octyl, n-nonyl, n-decyl, and the like. When two moieties of a molecule are linked by the alkyl group, it is referred to also as alkylene. "Lower alkyl" as used herein, is a subset of alkyl, in some embodiments preferred, and refers to a straight or branched chain hydrocarbon group containing from 1 to 4 carbon atoms. Representative examples of lower alkyl include, but are not limited to, methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, and the like. The term "alkyl" or "lower alkyl" is intended to include both substituted and unsubstituted alkyl or lower alkyl unless otherwise indicated and these groups may be substituted with additional organic and/or inorganic groups, including but not limited to groups selected from halo (e.g., to form haloalkyl), alkyl, haloalkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, aryl, arylalkyl, heterocyclic, heterocycloalkyl, hydroxyl, alkoxy (thereby creating a polyalkoxy such as polyethylene glycol), alkenyloxy, alkynyloxy, haloalkoxy, cycloalkoxy, cycloalkylalkyloxy, aryloxy, arylalkyloxy, heterocyclooxy, heterocyclolalkyloxy, mercapto, amino, alkylamino, alkenylamino, alkynylamino, haloalkylamino, cycloalkylamino, cycloalkylalkylamino, arylamino, arylalkylamino, heterocycloamino, heterocycloalkylamino, disubstituted-amino, acylamino, acyloxy, ester, amide, sulfonamide, urea, alkoxyacylamino, aminoacyloxy, nitro, cyanoalkyl-$S(O)_m$, haloalkyl-$S(O)_m$, alkenyl-S(O)n, alkynyl-$S(O)_m$, cycloalkyl-$S(O)_m$, cycloalkylalkyl-$S(O)_m$, aryl-$S(O)_m$, arylalkyl-$S(O)_m$, heterocyclo-$S(O)_m$, or heterocycloalkyl-$S(O)_m$, where m = 0, 1, 2, or 3.

As used herein, the term "alkenyl" alone or as part of another group, refers to a straight or branched chain hydrocarbon containing from 1 to 10, 20, or 30 or more carbon atoms (or in lower alkenyl 1 to 4 carbon atoms), which include 1 to 4, 5, or 6 or more double bonds in the normal chain. Representative examples of alkenyl include, but are not limited to, vinyl, 2-propenyl, 3-butenyl, 2-butenyl, 4-pentenyl, 3-pentenyl, 2-hexenyl, 3-hexenyl, 2,4-heptadiene, and the like. The term "alkenyl" or "lower alkenyl" is intended to include both substituted and unsubstituted alkenyl or lower alkenyl unless otherwise indicated and these groups may be substituted with groups as described in connection with alkyl and lower alkyl above.

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As used herein, the term "alkynyl" As used herein, alone or as part of another group, refers to a straight or branched chain hydrocarbon containing from 1 to 10, 20, 30, or 40 or more carbon atoms (or in lower alkynyl 1 to 4 carbon atoms) which include 1, 2, or 3 or more triple bonds in the normal chain. Representative examples of alkynyl include, but are not limited to, 2-propynyl, 3-butynyl, 2- butynyl, 4-pentynyl, 3-pentynyl, and the like. The term "alkynyl" or "lower alkynyl" is intended to include both substituted and unsubstituted alkynyl or lower alknynyl unless otherwise indicated and these groups may be substituted with the same groups as set forth in connection with alkyl and lower alkyl above.

As used herein, the term "cycloalkynyl" refers to a cyclic hydrocarbon ring system having between 6 and 16 carbon atoms and 1, 2, or 3 rings that are fused or bridged including at least 1 or more triple bonds in the ring structure.

As used herein, the term "heterocycloalkynyl" refers to a cyclic hydrocarbon ring system having between 6 and 16 carbon atoms, 1, 2, 3, or 4 heteroatoms independently selected from oxygen, nitrogen and sulfur, and 1, 2 or 3 rings which are fused or bridged including at least 1 or more triple bonds in the ring structure.

As used herein, the term "cycloalkyl" alone or as part of another group, refers to a saturated or partially unsaturated cyclic hydrocarbon group containing from 3, 4, 5, 6, 7, or 8 carbons (which carbons may be replaced in a heterocyclic group as discussed below). Representative examples of cycloalkyl include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl. These rings may be optionally substituted with additional substituents as described herein such as halo or lower alkyl. The term "cycloalkyl" is generic and intended to include heterocyclic groups as discussed below unless specified otherwise.

As used herein, the term "heterocyclic" or "heterocyclyl" alone or as part of another group, refers to an aliphatic (e.g., fully or partially saturated heterocyclic) or aromatic (e.g., heteroaryl) monocyclic- or bicyclic-ring system. Monocyclic ring systems are exemplified by any 3- to 8-membered ring containing 1, 2, 3, or 4 heteroatoms independently selected from oxygen, nitrogen and sulfur. The 5-membered ring has from 0–2 double bonds and the 6-membered ring has from 0–3 double bonds. Representative examples of monocyclic ring systems include, but are not limited to, azetidine, azepine, aziridine, diazepine, 1,3-dioxolane, dioxane, dithiane, furan, imidazole, imidazoline, imidazolidine, isothiazole, isothiazoline, isothiazolidine, isoxazole, isoxazoline, isoxazolidine, morpholine, oxadiazole, oxadiazoline, oxadiazolidine, oxazole,

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oxazoline, oxazolidine, piperazine, piperidine, pyran, pyrazine, pyrazole, pyrazoline, pyrazolidine, pyridine, pyrimidine, pyridazine, pyrrole, pyrroline, pyrrolidine, tetrahydrofuran, tetrahydrothiophene, tetrazine, tetrazole, thiadiazole, thiadiazoline, thiadiazolidine, thiazole, thiazoline, thiazolidine, thiophene, thiomorpholine, thiomorpholine sulfone, thiopyran, triazine, triazole, trithiane, and the like. Bicyclic ring systems are exemplified by any of the above monocyclic ring systems fused to an aryl group as defined herein, a cycloalkyl group as defined herein, or another monocyclic ring system as defined herein. Representative examples of bicyclic ring systems include but are not limited to, for example, benzimidazole, benzothiazole, benzothiadiazole, benzothiophene, benzoxadiazole, benzoxazole, benzofuran, benzopyran, benzothiopyran, benzodioxine, 1,3-benzodioxole, cinnoline, indazole, indole, indoline, indolizine, naphthyridine, isobenzofuran, isobenzothiophene, isoindole, isoindoline, isoquinoline, phthalazine, purine, pyranopyridine, quinoline, quinolizine, quinoxaline, quinazoline, tetrahydroisoquinoline, tetrahydroquinoline, thiopyranopyridine, and the like. These rings include quaternized derivatives thereof and may be optionally substituted with additional organic and/or inorganic groups, including but not limited to groups selected from halo, alkyl, haloalkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, aryl, arylalkyl, heterocyclic, heterocycloalkyl, hydroxyl, alkoxy, alkenyloxy, alkynyloxy, haloalkoxy, cycloalkoxy, cycloalkylalkyloxy, aryloxy, arylalkyloxy, heterocyclooxy, heterocyclolalkyloxy, mercapto, amino, alkylamino, alkenylamino, alkynylamino, haloalkylamino, cycloalkylamino, cycloalkylalkylamino, arylamino, arylalkylamino, heterocycloamino, heterocycloalkylamino, disubstituted-amino, acylamino, acyloxy, ester, amide, sulfonamide, urea, alkoxyacylamino, aminoacyloxy, nitro, cyano, alkyl-$S(O)_m$ haloalkyl-$S(O)_m$, alkenyl-$S(O)_m$, alkynyl-$S(O)_m$, cycloalkyl-$S(O)_m$, cycloalkylalkyl-$S(O)_m$, aryl-$S(O)_m$, arylalkyl-$S(O)_m$, heterocyclo-$S(O)_m$, heterocycloalkyl-$S(O)_m$, where m = 0, 1, 2, or 3.

As used herein, the term "heteroaryl" is as described in connection with heterocyclic above.

As used herein, the term "cycloalkylalkyl," "cycloalkylalkenyl," and "cycloalkylalkynyl" As used herein, alone or as part of another group, refers to a cycloalkyl group, as defined herein, appended to the parent molecular moiety through an alkyl, alkenyl, or alkynyl group, respectively, as defined herein.

As used herein, the term "alkoxy" alone or as part of another group, refers to an alkyl or lower alkyl group, as defined herein (and thus including substituted versions such as polyalkoxy), appended to the parent molecular moiety through an oxy group, -O-. Representative examples of

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alkoxy include, but are not limited to, methoxy, ethoxy, propoxy, 2-propoxy, butoxy, tert-butoxy, pentyloxy, hexyloxy and the like. In some aspects, alkoxy groups, when part of a more complex molecule, comprise an alkoxy substituent attached to an alkyl or lower alkyl via an ether linkage.

As used herein, the term "halo" or "halogen" refers to any suitable halogen, including –F, –Cl, –Br, or –I.

As used herein, the term "acyl" alone or as part of another group refers to a –C(O)R′ radical, wherein R′ is any suitable substituent such as aryl, alkyl, alkenyl, alkynyl, cycloalkyl or other suitable substituent as described herein.

As used herein, the term "alkylthio" alone or as part of another group, refers to an alkyl group, as defined herein, appended to the parent molecular moiety through a thiol moiety, as defined herein. Representative examples of alkylthio include, but are not limited, methylthio, ethylthio, tert-butylthio, hexylthio, and the like.

As used herein, the term "amide", "amido", or "amidyl" As used herein, alone or as part of another group refers to a –C(O)NR_aR_b radical, where R_a and R_b are independently any suitable substituent such as alkyl, hydrogen, cycloalkyl, alkenyl, alkynyl or aryl.

As used herein, the term "ester" alone or as part of another group refers to a –C(O)OR′ radical, where R′ is any suitable substituent such as alkyl, cycloalkyl, alkenyl, alkynyl or aryl.

As used herein, the term "ether" alone or as part of another group refers to a –COR′ radical where R′ is any suitable substituent such as alkyl, cycloalkyl, alkenyl, alkynyl, or aryl.

As used herein, the term "sulfone" alone or as part of another group refers to a –S(O)(O)R′ radical, where R′ is any suitable substituent such as alkyl, cycloalkyl, alkenyl, alkynyl or aryl.

As used herein, the term "sulfonamide" alone or as part of another group refers to a –$S(O)_2NR_aR_b$ radical, where R_a and R_b are independently any suitable substituent such as H, alkyl, cycloalkyl, alkenyl, alkynyl or aryl.

As used herein, the term "carboxyl" refers to refers to the radical –C(O)OH.

As used herein, the term "hydroxyl" refers to the radical –OH.

As used herein, the term "amino" refers to the radical $–NH_2$.

As used herein, vinyl refers to the radical $–CH_2CH_2$.

As used herein, the term "sulfonate" refers to the radical –S(O)(O)OR′, where R′ is any suitable substituent such as alkyl, cycloalkyl, alkenyl, alkynyl or aryl.

As used herein, the term "sulfonyl" refers to the radical –S(O)(O)R′, where R′ is any suitable substituent such as alkyl, cycloalkyl, alkenyl, alkynyl or aryl.

As used herein, thiol refers to the radical –SH.

As used herein, oxyamine or aminoxy refers to the radical $–ONH_2$.

As used herein, "hydrazide" or "hydrazidyl" refers to the radical $–C(O)-NH-NH_2$.

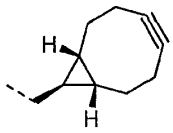
As used herein, "maleimide or maleimidyl" refers to a cyclic compound with the molecular formula $C_2H_2(C(O))_2NH$ or the radical $–N(C(O))_2C_2H_2$ having at least one C-C double bond.

As used herein, furan refers to a five-membered aromatic ring with four carbon atoms and one oxygen.

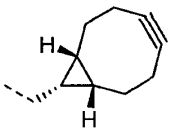
As used herein, "tetrazine" or "tetrazinyl" refers to a six-membered aromatic ring containing four nitrogen atoms with the molecular formula $C_2H_2N_4$ or the radical $–C_2HN_4$.

As used herein, the term "azide," "azidyl," or "azido" refers to an $–N_3$ group.

As used herein, the term "BCN" refers to a bicyclo[6.1.0]non-4-yn-9-yl)methyl radical, in which the exocyclic methylene can have an exo or endo orientation relative to the bicycle as shown:



exo BCN

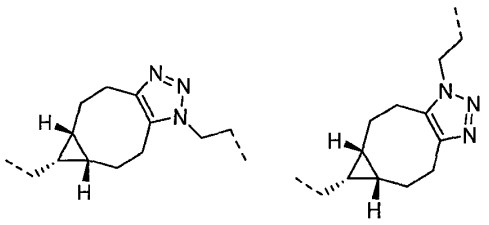


endo BCN

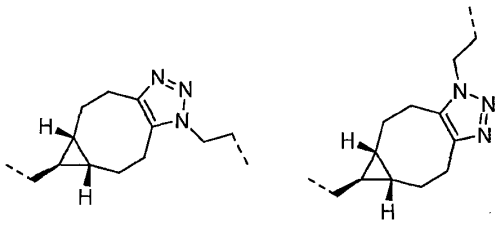
As used herein, throughout the specification, as a matter of convenience most structures do not depict stereochemistry and thus represent all possible stereoisomers.

Specifically with regard to the structure of the triazole products of the reaction between a BCN and azido compound, As used herein, throughout the specification, the N-linked substitutent on the triazole ring is shown in a single regiochemistry position as a matter of convenience. One of ordinary skill in the art will recognize that the reaction of a BCN alkyne with an azido compound will result in a stereoisomeric mixture of products with the N-linked substituent on the 1- and 3-position of the triazole as shown:

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Stereoisomer reaction products of endo BCN with alkyl azide



Stereoisomer reaction products of exo BCN with alkyl azide

As used herein, "reactive functional group" refers to a functional group, which is suitable for orthogonal coupling reactions. Suitable reactive functional groups are those that readily undergo orthogonal reactions. Exemplary and non-limiting orthogonal chemical reactions include functional groups shown in Table 2.

As used herein, "trigger" refers to a functional group present in the drug traceless linker adduct D–R, which is capable of undergoing a chemical reaction resulting in a new functional group. The presence of the new functional group substantially decreases the stability of the bond between the drug and the traceless linker relative and results in an increased probability of drug release.

The term "number average molecular weight" or "M_n" refers to the statistical average molecular weight of all molecules in the sample expressed in units of g/mol. The number average molecular weight may be determined by techniques known in the art, such as gel permeation chromatography (wherein M_n can be calculated based on known standards based on an online detection system such as a refractive index, ultraviolet, or other detector), viscometry, mass spectrometry, or colligative methods (e.g., vapor pressure osmometry, end-group determination, or proton NMR). The number average molecular weight is defined by the equation below,

$$M_n = \frac{\sum N_i M_i}{\sum N_i}$$

wherein M_i is the molecular weight of a molecule and N_i is the number of molecules of that molecular weight.

The term "weight average molecular weight" or "M_w" refers to the statistical average molecular weight of all molecules, taking into account the weight of each molecule in determining its contribution to the molecular weight average, expressed in units of g/mol. The higher the molecular weight of a given molecule, the more that molecule will contribute to the M_w value. The weight average molecular weight may be calculated by techniques known in the art that are

sensitive to molecular size, such as static light scattering, small angle neutron scattering, X-ray scattering, and sedimentation velocity. The weight average molecular weight is defined by the equation below,

$$M_w = \frac{\sum N_i M_i^2}{\sum N_i M_i}$$

wherein 'M_i' is the molecular weight of a molecule and 'N_i' is the number of molecules of that molecular weight.

The term "viscosity average molecular weight" or "M_v" refers to the statistical average molecular weight of all molecules, taking into account the weight of each molecule in determining its contribution to the molecular weight average, expressed in units of g/mol. Large molecules or polymers have higher viscosities than smaller molecules. The viscosity average molecular weight is defined by the equation below,

$$M_v = \left(\frac{\sum N_i M_i^{(1+a)}}{\sum N_i M_i}\right)^{1/a}$$

wherein 'M_i' is the molecular weight of a molecule and 'N_i' is the number of molecules of that molecular weight, and a is constant determined by the molecule, solvent, and temperature. Flexible polymeric molecules have a values of $0.5 \leq a \leq 0.8$. Semi-flexible polymeric molecules have a ≥ 0.8. The viscosity average molecular weight can be determined from intrinsic viscosity experiments or size exclusion chromatography.

As used herein, a "drug" comprises one or more biologically active moieties comprising at least one primary or secondary amine or a ring nitrogen atom of an azaheteroaryl ring. The biologically active moieties may be small molecules, macromolecules such as proteins, peptides, or nucleic acids, or combinations thereof. With specific regard to a traceless linker, R, the drug or biologically active moiety comprises "D" in the D–R representation. The terms or phrases "drug", "biologically active molecule," "biologically active moiety," "biologically active agent," "active agent," or "D," refer to any substance that can affect any physical or biochemical properties of a biological organism, including but not limited to viruses, bacteria, fungi, plants, animals, and humans. In particular, drugs or biologically active molecules include any substance intended for diagnosis, cure, mitigation, treatment, or prevention of disease in humans or other animals, or to otherwise enhance physical or mental well-being of humans or animals.

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In one embodiment, the biologically active moiety, D, comprises angiopoietin-like 3 (ANGPTL3) polypeptides. "ANGPTL3" refers to a member of the angoipoietin protein family. An amino acid sequence of human ANGPTL3 (GenBank Accession No. NP_055310.1) is set forth in SEQ ID NO:1; and the corresponding polynucleotide sequence of which is set forth as SEQ ID NO: 2 (NCBI reference sequence number NM014495.2, wherein the ANGPTL3 coding sequence comprises nucleotides 52–1434 of SEQ ID NO:2). *See* Table 1. For the purposes of the present disclosure, the numbering of an amino acid is typically determined with reference to the full-length wildtype human ANGPTL3 polypeptide sequence (SEQ ID NO:1). Thus, in embodiments in which a polypeptide of the invention contains only a C-terminal portion of full-length ANGPTL3, but not the N-terminal portion, although the peptide is less than 460 amino acids in length, the numbering of the positions is based on SEQ ID NO:1. For example, reference to amino acid position 423 of an ANGPTL3 polypeptide refers to position 423 of SEQ ID NO:1, even though the ANGPTL3 polypeptide of the invention itself may only be 200 amino acids in length. In determining an amino acid in a sequence of interest that "corresponds to" a position in a reference sequence, such as SEQ ID NO:1, this is performed by optimally aligning the sequences, e.g., using the default CLUSTAL alignment parameters or default BLAST 2 alignment parameters and comparing the sequences. For example, position 423 in a sequence of interest that is "determined with reference to SEQ ID NO:1", or an amino acid that "corresponds to" position 423 of SEQ ID NO:1, means the amino acid that aligns with position 423 of SEQ ID NO:1 when the sequence of interest is optimally aligned with SEQ ID NO:1.

In one embodiment, the biologically active moiety, D, comprises the ANGPTL3 polypeptide D1. As used herein, D1 refers to the ANGPTL3 polypeptide having the sequence of SEQ ID NO:19.

The terms "peptidomimetic" and "mimetic" refer to a synthetic chemical compound that has substantially the same functional characteristics of a naturally or non-naturally occurring polypeptide (e.g., ANGPTL3), but different (though typically similar) structural characteristics. Peptide analogs are commonly used in the field as non-peptide active compounds (e.g., drugs) with properties analogous to those of a template peptide. Such non-peptide compounds are termed "peptide mimetics" or "peptidomimetics." Fauchere, *J. Adv. Drug Res.* 15:29 (1986); Veber and Freidinger TINS p. 392 (1985); Evans et al. *J. Med. Chem.* 30:1229 (1987). Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent or

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enhanced therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biological or pharmacological activity), such as found in a polypeptide of interest, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of, e.g., $-CH_2NH-$, $-CH_2S-$, $-CH_2-CH_2-$, $-CH=CH-$ (*cis* and *trans*), $-COCH_2-$, $-CH(OH)CH_2-$, and $-CH_2SO-$. A mimetic can be either entirely composed of synthetic, non-natural analogues of amino acids, or, is a chimeric molecule of partly natural peptide amino acids and partly non-natural analogs of amino acids. A mimetic can also incorporate any amount of natural amino acid conservative substitutions as long as such substitutions also do not substantially alter the mimetic's structure and/or activity. For example, a mimetic composition is within the scope of the polypeptides described herein if it is capable of chondrogenic activity of an ANGPTL3 polypeptide.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. Polypeptides, peptides, and proteins described herein comprise protease resistant ANGPTL3 peptidomimetics having chondrogenic activity.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ-carboxyglutamate, and O-phosphoserine Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an .alpha. carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Naturally encoded amino acids are the 20 common amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) as well as pyrrolysine, pyrroline-carboxy-lysine, and selenocysteine.

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"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG, and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every polypeptide sequence herein which is encoded by a polynucleotide encompasses every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in each described sequence.

One of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids with reference to an original encoded amino acid sequence results in a "conservatively modified variant" where the alteration produces substitution of an amino acid with a chemically similar amino acid and/or a polypeptide sequence that produces a structurally similar protein having similar functional activity to the original protein. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles as described herein.

The term "conservative amino acid substitutions" refers to the substitution (conceptually or otherwise) of an amino acid from one such group with a different amino acid from the same group. One example of substitutions is based on analyzing the normalized frequencies of amino acid changes between corresponding proteins of homologous organisms. According to such analyses, groups of amino acids may be defined where amino acids within a group exchange preferentially with each other and, therefore, resemble each other most in their impact on the overall protein structure. *See* Schulz and Schirmer, *Principles of Protein Structure*, Springer-

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Verlag. One example of a set of amino acid groups defined in this manner include: (i) a charged group, consisting of Glu and Asp, Lys, Arg and His; (ii) a positively-charged group, consisting of Lys, Arg and His; (iii) a negatively-charged group, consisting of Glu and Asp; (iv) an aromatic group, consisting of Phe, Tyr and Trp; (v) a nitrogen ring group, consisting of His and Trp; (vi) a large aliphatic nonpolar group, consisting of Val, Leu and Ile; (vii) a slightly-polar group, consisting of Met and Cys; (viii) a small-residue group, consisting of Ser, Thr, Asp, Asn, Gly, Ala, Glu, Gln and Pro; (ix) an aliphatic group consisting of Val, Leu, Ile, Met and Cys; and (x) a small hydroxyl group consisting of Ser and Thr. Other examples of conservative substitutions based on shared physical properties are the substitutions within the following groups: (1) Alanine (A), Glycine (G); (2) Aspartic acid (D), Glutamic acid (E); (3) Asparagine (N), Glutamine (Q); (4) Arginine (R), Lysine (K); (5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); (6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); (7) Serine (S), Threonine (T); and (8) Cysteine (C), Methionine (M). *See* Creighton, *Proteins* (1984).

"Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the amino acid sequence or polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (e.g., an ANGPTL3 polypeptide), 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 nucleic acid base or amino acid 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.

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same sequences. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (i.e., 95% identity, optionally 96%, 97%, 98%, or 99% identity over a specified region, or, when not specified, over the entire sequence), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Polypeptides that are substantially identical to the polypeptides, respectively, exemplified herein (e.g., any of SEQ ID NOs: 1, 3–45), as well as uses

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thereof including but not limited to use for treating or preventing arthritis or joint injury. Optionally, for nucleic acids, the identity exists over a region that is at least about 150 nucleotides in length, or more preferably over a region that is 300 to 450 or 600 or more nucleotides in length, or the entire length of the reference sequence. For amino acid sequence, optionally, identity exists over a region that is at least about 50 amino acids in length, or more preferably over a region that is 100 to 150 or 200 or more amino acids in length, or the entire length of the reference sequence.

For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 50 to 600, usually about 75 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith and Waterman (1970) *Adv. Appl. Math.* 2:482c, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Nat'l. Acad. Sci. USA* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA), or by manual alignment and visual inspection. *See e.g.*, Ausubel et al., Current Protocols in Molecular Biology (1995).

Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1977) *Nuc. Acids Res.* 25:3389-3402, and Altschul et al. (1990) *J. Mol. Biol.* 215:403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which

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either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix. *See* Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences. *See* Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873-5787 (1993). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

The term "isolated," when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is purified to be essentially free of other cellular components with which it is associated in the natural state. It is often in a homogeneous or nearly homogeneous state. It can be in either a dry or aqueous solution. Purity and homogeneity may be determined using analytical chemistry techniques known and used typically in the art, e.g., polyacrylamide gel electrophoresis, high performance liquid chromatography, etc. A protein that is the predominant species present in a

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preparation is substantially purified. The term "purified" in some aspects denotes that a protein gives rise to essentially one band in an electrophoretic gel. Typically, it means that a protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

As used herein, the ANGPTL3 polypeptide may be natural or recombinant and from any species; the ANGPTL3 peptide may also be synthetic. In one aspect, the ANGPTL3 polypeptide comprises the sequence of *Homo sapiens* ANGPTL3, mutant thereof, deletion thereof, or truncated version thereof. Particularly useful are ANGPTL3 polypeptides comprising mutants or deletions of amino acid K423 using the numbering for the full length wild-type human ANGPTL3 sequence (SEQ ID NO:1). In another aspect, the ANGPTL3 polypeptide is described in U.S. Patent Nos. 9,301,971; 9,649,359; 9,745,358; 9,868,771, or European Patent No. EP2 964 250, each of which are herein incorporated by reference for the specific teachings related to ANGPTL3 polypeptides and the sequences thereof. In another aspect, the ANGPTL3 polypeptide is a protease resistant polypeptide. In one aspect, the ANGPTL3 polypeptide is a protease-resistant polypeptide that has an amino acid substitution, relative to a native wildtype peptide sequence, at an R or a K residue. In another aspect, the ANGPTL3 polypeptide is D1 (SEQ ID NO:19) which comprises a truncated version of human ANGPTL3 (e.g., amino acids 242–460) having a K423Q mutation using the numbering for the full length wild-type human ANGPTL3 sequence (SEQ ID NO:1). In another aspect, the ANGPTL3 polypeptide comprises any of SEQ ID NO:1 or 3–45 as shown in Table 1. In another aspect, the ANGPTL3 polypeptide comprises the polypeptide encoded by the DNA sequence of SEQ ID NO:2 or mutations or deletions thereof. In one aspect, the ANGPTL3 polypeptide comprises a sequence having 80–99% identity to SEQ ID NO:1 or 3–45, including all integers within the specified range. In another aspect, the ANGPTL3 polypeptide comprises an amino acid sequence that has at least 95% identity, at least 96% identity, at least 97% identity, at least 98% identity, or at least 99% identity to any Sequence shown in Table 1, wherein the polypeptide comprises an amino acid that is a polar amino acid other than K or R at position 423, as determined with reference to SEQ ID NO:1. In one aspect, the ANGPTL3 polypeptides have chondrogenic activity. In another aspect, the ANGPTL3 polypeptide comprises the amino acid sequence that has at least 95% identity, at least 96% identity, at least 97% identity, at least 98% identity, or at least 99% identity to any one of SEQ ID NOs:1 or 3–45. In another aspect, the ANGPTL3 polypeptide comprises a polypeptide encoded by a DNA sequence having 90–100%

identity to the coding portion of SEQ ID NO:2 (e.g., nucleotides 52–1434), including DNA sequences encoding mutations, truncations, or deletions.

Table 1. ANGPTL3 Sequences	
SEQ ID NO:	Description
1	*Homo sapiens* Angiopoietin-like 3 (ANGPTL3)
2	ANGPTL3 DNA Sequence
3	ANGPTL3 K423Q
4	ANGPTL3 K423S
5	ANGPTL3 207-460 K423Q
6	ANGPTL3 207-460 K423S
7	ANGPTL3 225-460 K423Q
8	ANGPTL3 225-460 K423S
9	ANGPTL3 225-460 S424T
10	ANGPTL3 226-460 K423Q
11	ANGPTL3 226-460 K423S
12	ANGPTL3 228-460 K423Q
13	ANGPTL3 228-460 K423S
14	ANGPTL3 228-460 S424T
15	ANGPTL3 233-460 K423Q
16	ANGPTL3 233-460 K423S
17	ANGPTL3 241-460 K423Q
18	ANGPTL3 241-460 K423S
19	ANGPTL3 242-460 K423Q
20	ANGPTL3 242-460 K423S
21	ANGPTL3 225-455 K423Q
22	ANGPTL3 225-455 K423S
23	ANGPTL3 226-455 K423Q
24	ANGPTL3 226-455 K423S
25	ANGPTL3 228-455 K423Q
26	ANGPTL3 228-455 K423S
27	ANGPTL3 233-455 K423Q
28	ANGPTL3 233-455 K423S
29	ANGPTL3 241-455 K423Q
30	ANGPTL3 241-455 K423S
31	ANGPTL3 242-455 K423Q
32	ANGPTL3 242-455 K423S
33	ANGPTL3 201-460 K423del
34	ANGPTL3 225-460 K423del
35	ANGPTL3 226-460 K423del
36	ANGPTL3 228-460 K423del
37	ANGPTL3 233-460 K423del

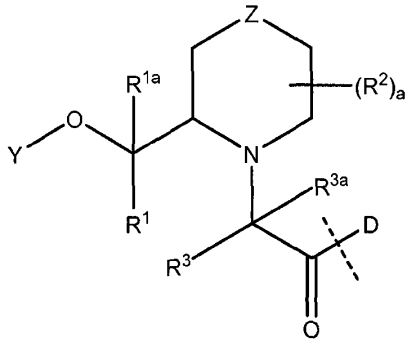
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38	ANGPTL3 241-460 K423del
39	ANGPTL3 242-460 K423del
40	ANGPTL3 225-455 K423del
41	ANGPTL3 226-455 K423del
42	ANGPTL3 228-455 K423del
43	ANGPTL3 233-455 K423del
44	ANGPTL3 241-455 K423del
45	ANGPTL3 242-455 K423del

As used herein, a "biologically active moiety comprising at least one primary amine, secondary amine, or ring nitrogen atom of an azaheteroaryl ring" refers to both the free biologically active moiety prior to attachment to a traceless linker or to the free biologically active moiety "D–H," which results after cleavage from the traceless linker adduct "D–R." In some aspects, the drug adduct, D–R, may have biological activity. In some aspects, biologically active moiety comprises an ANGPTL3 polypeptide as described herein.

As used herein, "free form" of a drug or biologically active moiety refers to the drug in its unmodified, pharmacologically active form, such as prior to being attached to a traceless linker or after being released from a traceless linker in a drug delivery system.

As used herein, a "traceless linker," R, is a linker that is represented by Formula (I) suitable for release of biologically active moiety D comprising at least one primary or secondary amine or a ring nitrogen atom of an azaheteroaryl ring:



(I),

where the dashed line indicates attachment to the primary amine, secondary amine, or ring nitrogen atom of an azaheteroaryl ring;

R^1 is hydrogen or C_1-C_4alkyl;

R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

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R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group;

a is 0, 1, 2, 3 or 4;

R^3 is hydrogen or C_1-C_4alkyl;

R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$;

R^{12} is hydrogen or C_1-C_4alkyl;

R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^{13} is C_1-C_4alkyl; or

$CHR^{12}OR^{13}$, taken in combination form a 5, 6, or 7-member cyclic ether;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy;

R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl;

R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups;

b is an integer of from 1 to 10;

Z is CH-L-A, CH-A, N-L-A, or N-A;

L is an optionally substituted bivalent linker;

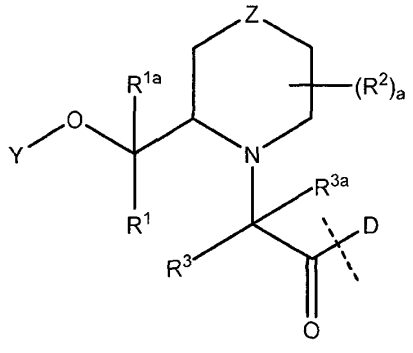
A is hydrogen, C_1-C_8alkyl, $C(O)C_1$-C_8alkyl, $C(O)N(H)C_1$-C_8alkyl, $C(O)OC_1$-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10};

R^{10} is a reactive functional group suitable for coupling Formula I to a carrier; and

R^{11} is a carrier.

In another embodiment, R, is a linker that is represented by Formula (I) suitable for release of biologically active moiety D comprising at least one primary or secondary amine or a ring nitrogen atom of an azaheteroaryl ring:

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(I),

where the dashed line indicates attachment to the primary amine, secondary amine, or ring nitrogen atom of an azaheteroaryl ring;

R^1 is hydrogen or C_1-C_4alkyl;

R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group;

a is 0, 1, 2, 3 or 4;

R^3 is hydrogen or C_1-C_4alkyl;

R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$;

R^{12} is hydrogen or C_1-C_4alkyl;

R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^{13} is C_1-C_4alkyl; or

$CHR^{12}OR^{13}$, taken in combination form a 5, 6, or 7-member cyclic ether;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy;

R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl;

R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups;

b is an integer of from 1 to 10;

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Z is CH-L-A, CH-A, N-L-A, or N-A;

L is an optionally substituted bivalent linker Q-[Sp-Q]$_h$-Q;

Q is independently selected at each occurrence from a bond, O, C(O), N(H), N(C_1-C_4alkyl), C(O)NH, C(O)N(C_1-C_4alkyl), N(H)C(O), N(C_1-C_4alkyl)C(O), N(H)C(O)O, N(C_1-C_4alkyl)C(O)O, OC(O)N(H), OC(O)N(C1-C_4alkyl), N(H)C(O)N(H), N(C_1-C_4alkyl)C(O)N(H), N(H)C(O)N(C_1-C_4alkyl), N(C_1-C4alkyl)C(O)N(C_1-C_4alkyl), C(O)O, OC(O), OC(O)O, S, S(O)$_2$, N(H)S(O)$_2$, N(C_1-C_4alkyl)S(O)$_2$, S(O)$_2$N(H), S(O)$_2$N(C_1-C_4alkyl), C_1-C_2alkyl-C(O)N(H), N(H)C(O)C_1-C_2alkyl, C_1-C_2alkyl-C(O)O, OC(O)C_1-C_2alkyl, 1,2,3-triazole, OP(O)$_2$, P(O)$_2$O, C_1-C_4alkyl-P(O)$_2$-O, or O-P(O)$_2$-C_1-$_4$alkyl;

Sp is independently selected at each occurrence from an optionally substituted C_1-C_{20}alkyl, C_2-C_{20}alkenyl, C_2-C_{20}alkynyl, [W-O]$_g$, C_1-C_8alkyl-[O-W]$_g$, [O-W]$_g$-O-C_1-C_8alkyl, C_1-C_8Calkyl-[O-W]$_g$-O-C_1-C_8alkyl, oligopeptide;

h is an integer of between 1 and 20;

g is a weighted average number of between about 2 and about 50;

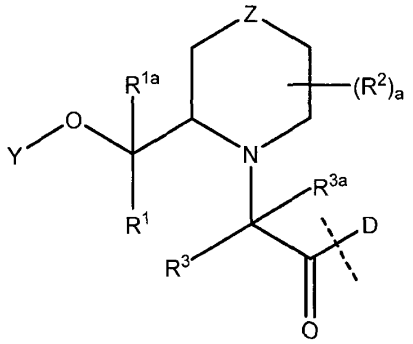
W is C_2-C_4alkyl-1,2-diyl in which the hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom;

A is hydrogen, C_1-C_8alkyl, C(O)C_1-C_8alkyl, C(O)OC$_1$-C_8alkyl, C(O)N(H)C_1-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10};

R^{10} is a reactive functional group suitable for coupling Formula I to a carrier; and

R^{11} is a carrier.

In another embodiment, R, is a linker that is represented by Formula (I) suitable for release of biologically active moiety D comprising at least one primary or secondary amine or a ring nitrogen atom of an azaheteroaryl ring:



(I),

where the dashed line indicates attachment to the primary amine, secondary amine, or ring nitrogen atom of an azaheteroaryl ring;

R^1 is hydrogen or C_1-C_4alkyl;

R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group;

a is 0, 1, 2, 3 or 4;

R^3 is hydrogen or C_1-C_4alkyl;

R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$;

R^{12} is hydrogen or C_1-C_4alkyl;

R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^{13} is C_1-C_4alkyl; or

$CHR^{12}OR^{13}$, taken in combination form a 5, 6, or 7-member cyclic ether;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy;

R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl;

R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups;

b is an integer of from 1 to 10;

Z is CHR^8 or NR^9;

R^8 and R^9 are each independently selected from hydrogen, C_1-C_8 alkyl, $C(O)C_1$-C_8 alkyl, or $-C(O)(CH_2)_q[O–W]_g(NHC(O))_m(CH_2)_q[O–W]_p$-Q-A, wherein the alkyl group is optionally substituted with 0 or 1 Q-A;

q is independently at each occurrence 1, 2, or 3;

g and p each independently have a weighted average length of between about 2 and about 50;

m is 1 or 0;

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W is C_2-C_4alkyl-1,2-diyl in which the hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom;

Q is a bond, O, N(H) or N(C_1-C_4alkyl);

A is hydrogen, C_1-C_8alkyl, C(O)C_1-C_8alkyl, C(O)N(H)C_1-C_8alkyl, C(O)OC_1-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10};

R^{10} is a reactive functional group suitable for coupling Formula I to a carrier; and

R^{11} is a carrier.

In one aspect, the traceless linker is a linker suitable for sustained release of a biologically active moiety. In one aspect, "R" is a traceless linker in the representation D–R, wherein "D" refers to a drug comprising a biologically active moiety comprising at least one primary amine, secondary amine, or ring nitrogen atom of an azaheteroaryl ring.

As used herein, a "drug adduct" is a drug, D linked to a traceless linker, R. With specific regard to a traceless linker, R, the drug adduct comprises "D–R."

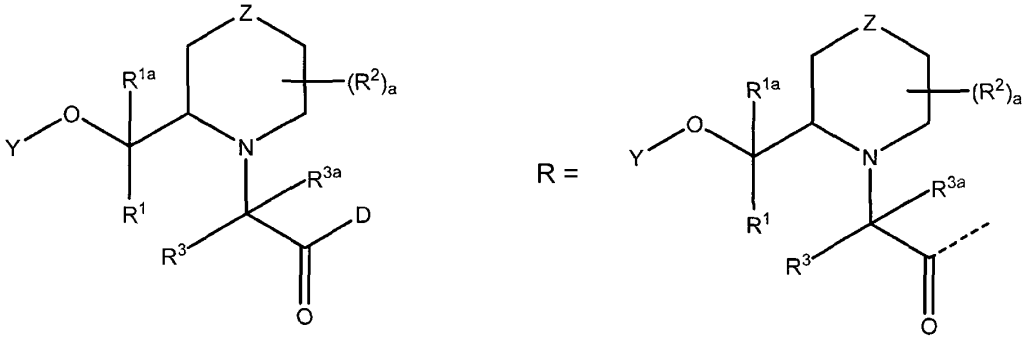
As used herein, a "carrier" is a soluble polymer, biopolymer, or a cross-linked polymer or biopolymer. Carriers comprise proteins, nucleic acids, carbohydrates, or polyethylene glycols. In one aspects, the carrier or mutliple carriers are cross-linked intermolecularly, intramolecularly, or a combination thereof. In one aspect, the cross-linked carrier comprises a hydrogel. With specific regard to a traceless linker, R, the carrier comprises R^{11}. In one aspect, Z is linked to a carrier, typically a polymer or a hydrogel. The carrier is attached to the traceless linker, R, either directly or via a non-cleavable spacer. As non-limiting examples, carriers can comprise polyethylene glycols, hyaluronic acid polymers, or cross-linked hyaluronic acid or polyethylene glycol that are capable of forming hydrogels.

As used herein, a "polymer" refers to a molecule comprised of repeating structural units (monomers) connected by chemical bonds in a linear, circular, branched, or dendrimeric way or a combination thereof, that can be of synthetic or biological origin or a combination of both. Typically, a polymer has an average molecular weight of at least 1 kDa. A copolymer is a polymer comprised of at least two chemically distinct monomers. Typically, a polymer is comprised of molecules having a distribution of molecular weights. One way to describe the molecular weight distribution of a polymer is the average molecular weight. Typically, a polymer is comprised of molecules having a distribution of degree of polymerization. One way to describe the degree of polymerization distribution of a polymer is the average degree of polymerization.

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As described herein and depicted in the structures herein, polymeric molecules, such as polyethylene glycol or hyaluronic acid, are represented as $[P]_n$, where P represents the monomeric repeating unit and n represents the average degree of polymerization of the monomer in the polymer. One of ordinary skill in the art will understand that two polymeric molecules depicted with identical repeating unit P but different n are considered equivalent if the difference is about 10% or less of n. For the hyaluronic acid copolymers described and depicted in the structures herein, the distribution of monomers in the polymer is undefined and assumed to be random. In addition, the relative connectivity of the independent molecules for hydrogels or drug delivery systems described herein is random within a population. Structural depictions of hydrogels or drug delivery systems represent a single potential structural unit in two dimensions, whereas these complexes are three-dimensional with many structural subunits.

As described herein, the exact position of amide bond formation is not precisely known for a traceless linker-drug adduct, R–D. This can occur when D contains more than one primary or secondary amine, or ring nitrogen atom of an azaheteroaryl ring capable of forming an amide bond with a linker R (e.g., a protein or polypeptide). As a matter of convenience, adducts where the position of amide bond formation is not precisely known are depicted herein as follows:



As used herein, a "hydrogel" refers to a three-dimensional, hydrophilic or amphiphilic polymeric network capable of taking up large quantities of water. The networks are composed of homopolymers or copolymers, and are insoluble due to the presence of covalent chemical or physical (ionic, hydrophobic interactions, entanglements) crosslinks. The crosslinks provide the network structure and physical integrity. Hydrogels exhibit a thermodynamic compatibility with water that allows them to swell in aqueous media. The chains of the network are connected in such a fashion that pores exist and water soluble solutes with dimensions smaller than the pores are able to diffuse in and out of the network.

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As used herein, a "linker" or "non-biologically active linker" refers to a linker that does not produce any pharmacological effects. In some embodiments, the linker comprises a bi- or multi-valent organic linker that is compatible with biological systems. "Bivalent" refers to having a reactive group suitable for attachment to a traceless linker or drug, D, at each terminus of the polymer. "Multivalent" refers having a reactive group suitable for attachment to a traceless linker or drug, D, at each terminus of the polymer with additional reactive moieties interspersed along the linker molecule.

As used herein, a "drug delivery system" comprises a carrier linked to a drug adduct, D–R–R^{11}, wherein the carrier comprises R^{11}. The drug delivery system as described herein is the molecular conjugate comprising one or more drugs, D; one or more traceless linkers, R; and one or more carriers, R^{11}. In some embodiments, there can be multiple, distinct drug species conjugated to a drug delivery system. For example, two different drugs, such as D1 and another ANGPTL3 polypeptide as described herein may be conjugated in a single drug delivery system.

As used herein, the phrase "water-insoluble" refers to a swellable three-dimensionally crosslinked molecular network forming the hydrogel. The hydrogel if suspended in a large surplus of water or aqueous buffer of physiological osmolality may take up a substantial amount of water, e.g., up to 10-fold on a weight per weight basis, and is therefore swellable but after removing excess water still retains the physical stability of a gel and a shape. Such shape may be of any geometry and it is understood that such an individual hydrogel object is to be considered as a single molecule consisting of components wherein each component is connected to each other component through chemical bonds.

As used herein, "sustained release" or "sustained release rate" refers to the situation where the intervals between subsequent doses of the respective drug delivery system required to achieve a desired therapeutic effect are expanded. Drugs with a daily dosage may for example be turned into a sustained release form with a week-long or even longer interval between two administrations.

As used herein, a "functional group" refers to a group of atoms within molecules that exhibit a specific chemical activity. Examples are amides, amines, alcohols, carbonyls, carboxylic acids, thiols.

As used herein, a "protective group" refers to a moiety that temporarily protects a functional group of a molecule during synthesis to obtain chemoselectivity in subsequent chemical

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reactions. Protective groups for alcohols are, for example, benzyl and trityl, protective groups for amines are, for example, tert-butyloxycarbonyl, 9-fluorenylmethyloxycarbonyl and benzyl and for thiols examples of protective groups are 2,4,6-trimethoxybenzyl, phenylthiomethyl, acetamidomethyl, p-methoxybenzyloxycarbonyl, tert-butylthio, triphenylmethyl, 3-nitro-2-pyridylthio, 4-methyltrityl.

As used herein, a "protected functional group" means a functional group protected by one or more protective groups.

As used herein, "PBS" refers to phosphate buffered saline.

As used herein, the term "pharmaceutically acceptable salts" refers to salts that retain the biological effectiveness and properties of the biologically active agent and, that typically are not biologically or otherwise undesirable. In many cases, the biologically active agent is capable of forming acid and/or base salts by virtue of the presence of amino and/or carboxyl groups or groups similar thereto.

Pharmaceutically acceptable acid addition salts can be formed with inorganic acids and organic acids, e.g., acetate, aspartate, benzoate, besylate, bromide/hydrobromide, bicarbonate/carbonate, bisulfate/sulfate, camphorsulfonate, chloride/hydrochloride, chlortheophyllinate, citrate, ethandisulfonate, fumarate, gluceptate, gluconate, glucuronate, hippurate, hydroiodide/iodide, isethionate, lactate, lactobionate, laurylsulfate, malate, maleate, malonate, mandelate, mesylate, methylsulphate, naphthoate, napsylate, nicotinate, nitrate, octadecanoate, oleate, oxalate, palmitate, pamoate, phosphate/hydrogen phosphate/dihydrogen phosphate, polygalacturonate, propionate, stearate, succinate, subsalicylate, tartrate, tosylate and trifluoroacetate salts.

Inorganic acids from which salts can be derived include, for example, hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like.

Organic acids from which salts can be derived include, for example, acetic acid, propionic acid, glycolic acid, oxalic acid, maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, toluenesulfonic acid, sulfosalicylic acid, and the like.

Pharmaceutically acceptable base addition salts can be formed with inorganic and organic bases.

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Inorganic bases from which salts can be derived include, for example, ammonium salts and metals from columns I to XII of the periodic table. In certain embodiments, the salts are derived from sodium, potassium, ammonium, calcium, magnesium, iron, silver, zinc, and copper; particularly suitable salts include ammonium, potassium, sodium, calcium and magnesium salts.

Organic bases from which salts can be derived include, for example, primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines, basic ion exchange resins, and the like. Certain organic amines include isopropylamine, benzathine, cholinate, diethanolamine, diethylamine, lysine, meglumine, piperazine, or tromethamine.

The pharmaceutically acceptable salts can be synthesized from a parent compound, a basic or acidic moiety, by conventional chemical methods.

Generally, such salts can be prepared by reacting free acid forms of these compounds with a stoichiometric amount of the appropriate base (such as sodium, potassium, calcium, or magnesium hydroxides, carbonates, bicarbonates, or the like), or by reacting free base forms of these compounds with a stoichiometric amount of the appropriate acid. Such reactions are typically carried out in water or in an organic solvent, or in a mixture of the two. Generally, use of non-aqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile is desirable, where practicable. Lists of additional suitable salts can be found, e.g., in "Remington's Pharmaceutical Sciences", 18th ed., Mack Publishing Company, Easton, Pa., (1990); and in "Handbook of Pharmaceutical Salts: Properties, Selection, and Use" by Stahl and Wermuth (Wiley-VCH, Weinheim, Germany, 2002).

As used herein, the term "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (e.g., antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, and the like and combinations thereof, as would be known to those skilled in the art (*see, e.g.*, Remington's Pharmaceutical Sciences, 18th ed. Mack Printing Company, 1990, pp. 1289-1329). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the therapeutic or pharmaceutical compositions is contemplated.

The term "a therapeutically effective amount" of a biologically active agent refers to an amount of the biologically active agent that will elicit the biological or medical response of a

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subject, for example, amelioration of a symptom, alleviation of a condition, slow or delay disease progression, or prevention of a disease, etc.

In another non-limiting embodiment, the term "a therapeutically effective amount" refers to the amount of the biologically active agent that, when administered to a cell, or a tissue, or a non-cellular biological material, or a medium, is effective to at least partially treat the disease or disorder. As will be appreciated by those of ordinary skill in the art, the absolute amount of a particular agent that is effective may vary depending on such factors as the desired biological endpoint, the agent to be delivered, the target tissue, etc. Those of ordinary skill in the art understand that "a therapeutically effective amount" may be administered in a single dose or may be achieved by administration of multiple doses. For example, in the case of an agent to treat heart failure, an effective amount may be an amount sufficient to result in clinical improvement of the patient, e.g., increased exercise tolerance/capacity, increased blood pressure, decrease fluid retention, and/or improved results on a quantitative test of cardiac functioning, e.g., ejection fraction, exercise capacity (time to exhaustion), etc.

As used herein, the term "subject" refers to an animal. Typically, the animal is a mammal. A subject also refers to for example, primates (e.g., humans and non-human primates), cows, sheep, goats, horses, dogs, cats, rabbits, rats, mice, fish, birds, and the like. In certain embodiments, the subject is a primate. In yet other embodiments, the subject is a human.

As used herein, the term "inhibit," "inhibition," or "inhibiting" refers to the reduction or suppression of a given condition, symptom, or disorder, or disease, or a significant decrease in the baseline activity of a biological activity or process.

As used herein, the term "treat," "treating," or "treatment" of any disease or disorder refers in one embodiment, to ameliorating the disease or disorder (i.e., slowing or arresting or reducing the development of the disease or at least one of the clinical symptoms thereof). In another embodiment "treat," "treating," or "treatment" refers to alleviating or ameliorating at least one physical parameter including those that may not be discernible by the patient. In yet another embodiment, "treat," "treating," or "treatment" refers to modulating the disease or disorder, either physically, (e.g., stabilization of a discernible symptom), physiologically, (e.g., stabilization of a physical parameter), or both. In yet another embodiment, "treat," "treating," or "treatment" refers to preventing or delaying the onset or development or progression of the disease or disorder. As used herein, the terms "prevent," "preventing," and "prevention" refer to the prevention of the

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recurrence, onset, or development of one or more symptoms of a disorder in a subject resulting from the administration of a therapy (e.g., a therapeutic agent), or the administration of a combination of therapies (e.g., a combination of therapeutic agents).

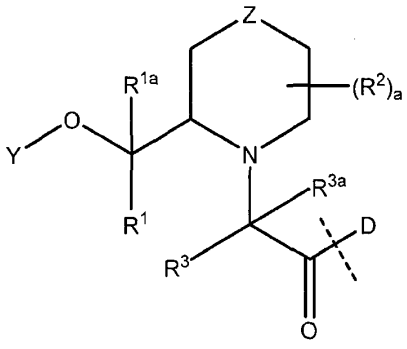
As used herein, a subject is "in need of" or "in need thereof" a treatment if such subject would benefit biologically, medically, or in quality of life from such treatment.

As used herein, the term "a," "an," "the" and similar terms used herein (especially in the context of the claims) are to be construed to cover both the singular and plural unless otherwise indicated herein or clearly contradicted by the context.

As used herein, the term "about" refers to any values, including both integers and fractional components that are within a variation of up to ± 10% of the value modified by the term "about."

The term "or" can be conjunctive or disjunctive such that "or" encompasses "and/or."

One embodiment described herein is a drug adduct comprising a drug and a traceless linker, D–R, wherein R, is a linker that is represented by Formula (I) suitable for release of D comprising an ANGPTL3 polypeptide comprising at least one primary or secondary amine or a ring nitrogen atom of an azaheteroaryl ring:



(I),

where the dashed line indicates attachment to the primary amine, secondary amine, or ring nitrogen atom of an azaheteroaryl ring;

R^1 is hydrogen or C_1-C_4alkyl;

R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group;

a is 0, 1, 2, 3 or 4;

R^3 is hydrogen or C_1-C_4alkyl;

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R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$;

R^{12} is hydrogen or C_1-C_4alkyl;

R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^{13} is C_1-C_4alkyl; or

$CHR^{12}OR^{13}$, taken in combination form a 5, 6, or 7-member cyclic ether;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy;

R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl;

R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups;

b is an integer of from 1 to 10;

Z is CH-L-A, CH-A, N-L-A, or N-A;

L is an optionally substituted bivalent linker;

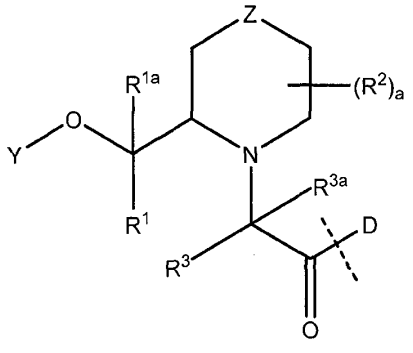
A is hydrogen, C_1-C_8alkyl, $C(O)C_1$-C_8alkyl, $C(O)N(H)C_1$-C_8alkyl, $C(O)OC_1$-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10};

R^{10} is a reactive functional group suitable for coupling Formula I to a carrier; and

R^{11} is a carrier.

Another embodiment described herein is a drug adduct comprising a drug and a traceless linker, D–R, wherein R, is a linker that is represented by Formula (I) suitable for release of D comprising an ANGPTL3 polypeptide comprising at least one primary or secondary amine or a ring nitrogen atom of an azaheteroaryl ring:

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(I),

where the dashed line indicates attachment to the primary amine, secondary amine, or ring nitrogen atom of an azaheteroaryl ring;

R^1 is hydrogen or C_1-C_4alkyl;

R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group;

a is 0, 1, 2, 3 or 4;

R^3 is hydrogen or C_1-C_4alkyl;

R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$;

R^{12} is hydrogen or C_1-C_4alkyl;

R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^{13} is C_1-C_4alkyl; or

$CHR^{12}OR^{13}$, taken in combination form a 5, 6, or 7-member cyclic ether;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy;

R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl;

R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups;

b is an integer of from 1 to 10;

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Z is CH-L-A, CH-A, N-L-A, or N-A;

L is an optionally substituted bivalent linker Q-[Sp-Q]$_h$-Q;

Q is independently selected at each occurrence from a bond, O, C(O), N(H), N(C_1-C_4alkyl), C(O)NH, C(O)N(C_1-C_4alkyl), N(H)C(O), N(C_1-C_4alkyl)C(O), N(H)C(O)O, N(C_1-C_4alkyl)C(O)O, OC(O)N(H), OC(O)N(C1-C_4alkyl), N(H)C(O)N(H), N(C_1-C_4alkyl)C(O)N(H), N(H)C(O)N(C_1-C_4alkyl), N(C_1-C4alkyl)C(O)N(C_1-C_4alkyl), C(O)O, OC(O), OC(O)O, S, S(O)$_2$, N(H)S(O)$_2$, N(C_1-C_4alkyl)S(O)$_2$, S(O)$_2$N(H), S(O)$_2$N(C_1-C_4alkyl), C_1-C_2alkyl-C(O)N(H), N(H)C(O)C_1-C_2alkyl, C_1-C_2alkyl-C(O)O, OC(O)C_1-C_2alkyl, 1,2,3-triazole, OP(O)$_2$, P(O)$_2$O, C_1-C_4alkyl-P(O)$_2$-O, or O-P(O)$_2$-C_1-$_4$alkyl;

Sp is independently selected at each occurrence from an optionally substituted C_1-C_{20}alkyl, C_2-C_{20}alkenyl, C_2-C_{20}alkynyl, [W-O]$_g$, C_1-C_8alkyl-[O-W]$_g$, [O-W]$_g$-O-C_1-C_8alkyl, C_1-C_8Calkyl-[O-W]$_g$-O-C_1-C_8alkyl, oligopeptide;

h is an integer of between 1 and 20;

g is a weighted average number of between about 2 and about 50;

W is C_2-C_4alkyl-1,2-diyl in which the hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom;

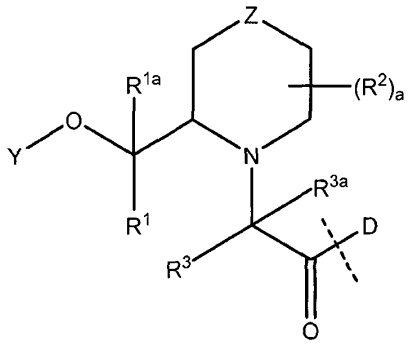
A is hydrogen, C_1-C_8alkyl, C(O)C_1-C_8alkyl, C(O)OC_1-C_8alkyl, C(O)N(H)C_1-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10};

R^{10} is a reactive functional group suitable for coupling Formula I to a carrier; and

R^{11} is a carrier.

Another embodiment described herein is a drug adduct comprising a drug and a traceless linker, D–R, wherein R, is a linker that is represented by Formula (I) suitable for release of D comprising an ANGPTL3 polypeptide comprising at least one primary or secondary amine or a ring nitrogen atom of an azaheteroaryl ring:

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(I),

where the dashed line indicates attachment to the primary amine, secondary amine, or ring nitrogen atom of an azaheteroaryl ring;

R^1 is hydrogen or C_1-C_4alkyl;

R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group;

a is 0, 1, 2, 3 or 4;

R^3 is hydrogen or C_1-C_4alkyl;

R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$;

R^{12} is hydrogen or C_1-C_4alkyl;

R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^{13} is C_1-C_4alkyl; or

$CHR^{12}OR^{13}$, taken in combination form a 5, 6, or 7-member cyclic ether;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy;

R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl;

R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups;

b is an integer of from 1 to 10;

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Z is CHR^8 or NR^9;

R^8 and R^9 are each independently selected from hydrogen, C_1-C_8 alkyl, $C(O)C_1$-C_8 alkyl, or $-C(O)(CH_2)_q[O-W]_g(NHC(O))_m(CH_2)_q[O-W]_p$-Q-A, wherein the alkyl group is optionally substituted with 0 or 1 Q-A;

q is independently at each occurrence 1, 2, or 3;

g and p each independently have a weighted average length of between about 2 and about 50;

m is 1 or 0;

W is C_2-C_4alkyl-1,2-diyl in which the hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom;

Q is a bond, O, N(H) or $N(C_1$-C_4alkyl);

A is hydrogen, C_1-C_8alkyl, $C(O)C_1$-C_8alkyl, $C(O)N(H)C_1$-C_8alkyl, $C(O)OC_1$-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10};

R^{10} is a reactive functional group suitable for coupling Formula I to a carrier; and

R^{11} is a carrier.

In some aspects, R^1–R^8 of Formula (I) have the following meanings. In one aspect, R^1 is hydrogen or methyl. In another aspect, R^{1a} is hydrogen, or methyl. In another aspect, R^1 and R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl. In another aspect, R^2 is methyl. In another aspect, two R^2 groups taken in combination form a fused or spiro C_3-C_6 cycloalkyl group. In another aspect, R^3 is hydrogen or methyl. In another aspect, R^{3a} is hydrogen or methyl. In another aspect, R^3 and R^{3a} taken in combination form a C_3-C_6cycloalk-1,1-diyl. In another aspect, Y is $C(O)R^4$ and R^4 is C_1-C_6alkyl, C_1-C_2alkoxy, C_1-C_2alkyl or C_1-C_6alkoxy. In another aspect, R^4 is methyl, ethyl, propyl, isopropyl, 1-methyl-cyclopropyl, or methoxymethyl. In another aspect, Y is $SiR^5R^6OR^7$; and R^5 and R^6 are each methyl, ethyl, propyl or isopropyl; and R^7 is ethyl, propyl, isopropyl, butyl, isobutyl, secbutyl, ethoxyethyl, ethoxyisopropyl, tetrahydropyranyl, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl where b is 2, 3, or 4.

In some aspects, R^8 and R^9 of Formula (I) have the following meanings. In one aspect, R^9 is, $C(O)C_2$ carbamoyl. In another aspect, R^9 is $C(O)C_2$ amidyl. In another aspect, R^9 is $C(O)C_5$ alkyl. In another aspect, R^9 is $C(O)C_2$ alkyl. In another aspect, R^9 is $C(O)C_1$ alkyl. In another aspect, R^9 is $C(O)CH_2CH_2NHC(O)CH_2CH_2CH_2CH_2$. In another aspect, R^9 is $C(O)(CH_2CH_2O)_bCH_2CH_2$ where b is 1, 2, 3, or 4. In another aspect, R^9 is amidyl. In another

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aspect, R^9 is carbamoyl. In another aspect, R^9 is C_1 alkyl amidyl. In another aspect, R^9 is C_2 alkyl amidyl.

In some aspects, Z is CH-L-A, CH-A, N-L-A, or N-A;

L is an optionally substituted bivalent linker;

A is hydrogen, C_1-C_8alkyl, C(O)C_1-C_8alkyl, C(O)N(H)C_1-C_8alkyl, C(O)OC_1-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10};

R^{10} is a reactive functional group suitable for coupling Formula I to a carrier; and

R^{11} is a carrier.

In some aspects, Z is CH-L-A, CH-A, N-L-A, or N-A;

L is an optionally substituted bivalent linker Q-[Sp-Q]$_h$-Q;

Q is independently selected at each occurrence from a bond, O, C(O), N(H), N(C_1-C_4alkyl), C(O)NH, C(O)N(C_1-C_4alkyl), N(H)C(O), N(C_1-C_4alkyl)C(O), N(H)C(O)O, N(C_1-C_4alkyl)C(O)O, OC(O)N(H), OC(O)N(C1-C_4alkyl), N(H)C(O)N(H), N(C_1-C_4alkyl)C(O)N(H), N(H)C(O)N(C_1-C_4alkyl), N(C_1-C4alkyl)C(O)N(C_1-C_4alkyl), C(O)O, OC(O), OC(O)O, S, S(O)$_2$, N(H)S(O)$_2$, N(C_1-C_4alkyl)S(O)$_2$, S(O)$_2$N(H), S(O)$_2$N(C_1-C_4alkyl), C_1-C_2alkyl-C(O)N(H), N(H)C(O)C_1-C_2alkyl, C_1-C_2alkyl-C(O)O, OC(O)C_1-C_2alkyl, 1,2,3-triazole, OP(O)$_2$, P(O)$_2$O, C_1-C_4alkyl-P(O)$_2$-O, or O-P(O)$_2$-C_1-$_4$alkyl;

Sp is independently selected at each occurrence from an optionally substituted C_1-C_{20}alkyl, C_2-C_{20}alkenyl, C_2-C_{20}alkynyl, [W-O]$_g$, C_1-C_8alkyl-[O-W]$_g$, [O-W]$_g$-O-C_1-C_8alkyl, C_1-C_8Calkyl-[O-W]$_g$-O-C_1-C_8alkyl, oligopeptide;

h is an integer of between 1 and 20;

g is a weighted average number of between about 2 and about 50;

W is C_2-C_4alkyl-1,2-diyl in which the hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom;

A is hydrogen, C_1-C_8alkyl, C(O)C_1-C_8alkyl, C(O)OC_1-C_8alkyl, C(O)N(H)C_1-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10};

R^{10} is a reactive functional group suitable for coupling Formula I to a carrier; and

R^{11} is a carrier.

In certain aspects, g and h are 1 to about 25 or from 1 to about 10.

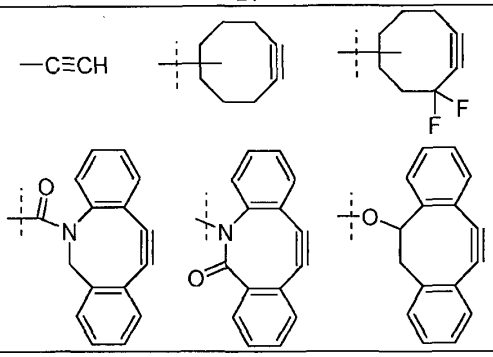
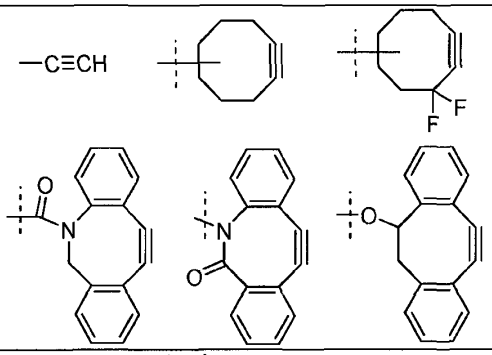
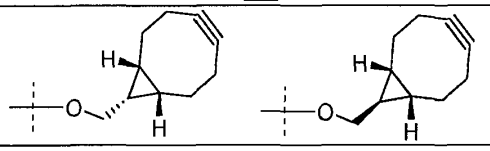
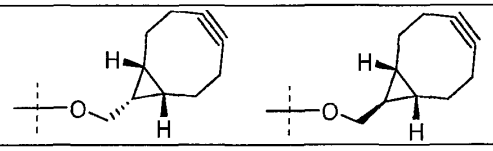
In some aspects, Z of Formula (I) is NR^9. In one aspect, R^9 is further substituted with R^{10}. In another aspect, R^9 is further substituted with R^{11}.

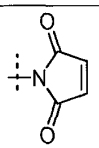
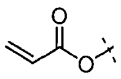
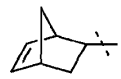
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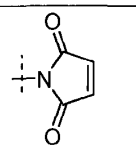
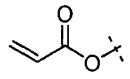
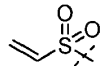
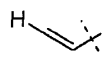
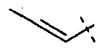
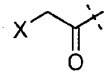
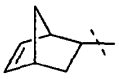
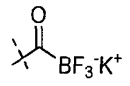
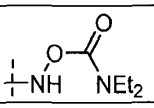
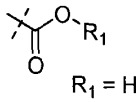
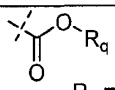
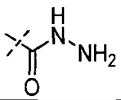
According to Formula (I), R^{10} is a reactive functional group that is suitable for orthogonal coupling reactions. Suitable reactive functional groups are those that readily undergo orthogonal reactions. Exemplary and non-limiting orthogonal chemical reactions include functional groups shown in Table 2. For a given row in Table 2, a functional group X (left column) is suitable for a coupling reaction with a functional group Y (right column). Coupling reactions may be covalent bonds or intermolecular complexes. In most embodiments, the coupling reaction results in a covalent bond. In other reactions, such as adamantane with cyclodextran, the coupling is a non-covalent molecular association. In one aspect, the functional group is selected from azidyl, alkynyl, substituted or unsubstituted C_6-C_{12} cycloalkynyl, C_6-C_{12} heterocycloalkynyl, C_6-C_{12} cycloalkenyl, norbornyl, vinyl carboxyl, vinyl sulfonyl, C_2-C_8 alkenyl, substituted or unsubstituted C_1-C_8 alkoxy, thiol, C_1-C_8 carboxyl, C_1-C_8 carbonyl, oxyamine, carbohydrazide, maleimide, alpha-halo carbonyl, furan, substituted or unsubstituted tetrazinyl, lysine, glutamine, cyclodextrin, and adamantanyl. In another aspect, the functional group is a substituted C_6-C_{12} cycloalkynyl, wherein the substitution includes a fused cyclopropyl group. In another aspect, the functional group is bicyclo[6.1.0]non-4-yn-9-yl. In another aspect, the functional group is azidyl.

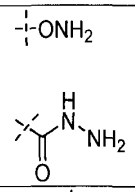
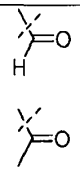
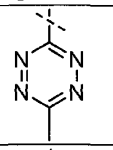
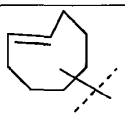
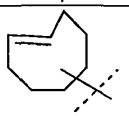
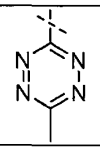
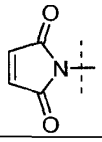
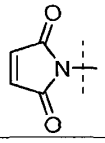
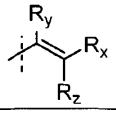
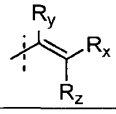
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Table 2. Exemplary Orthogonal Chemistry

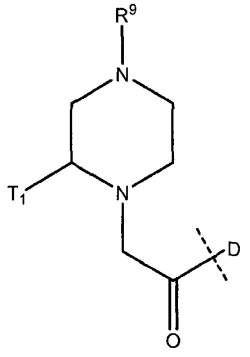
Compound (or polymer) 1-X X:	Compound (or polymer) 2-Y Y:
	
	
	
	

	       X = halogen

       X = halogen	
	
	 R_1 = H or activated ester
 R_q = H or activated ester	
 	

	
	
	
	
	
	
	
Lys	Gln
Gln	Lys
cyclodextrin or other host molecule	adamantane or other host molecule
adamantane or other host molecule	cyclodextrin or other host molecule

In some embodiments, the traceless linker, R, comprises Formula (II):

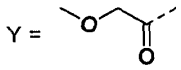
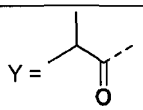
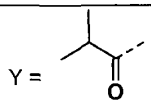
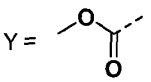
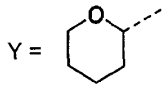
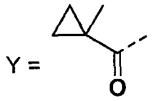
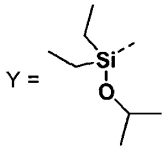
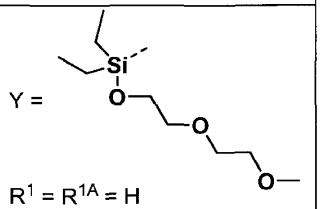
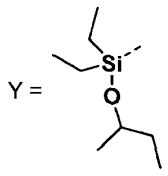
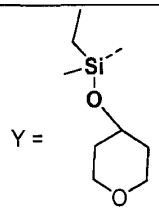
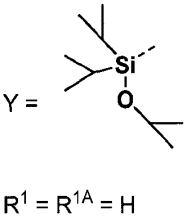
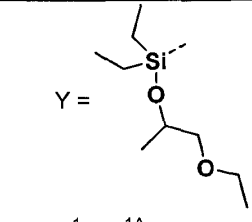


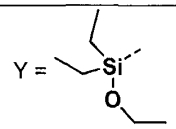
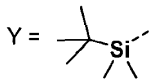
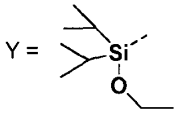
(II)

wherein, R^9 and D are both as defined for Formula (I) above and T_1 comprises a substituted or unsubstituted C_2-C_{10} ester, a substituted or unsubstituted C_3-C_{10} silyl ether containing one or more heteroatoms selected from nitrogen or oxygen.

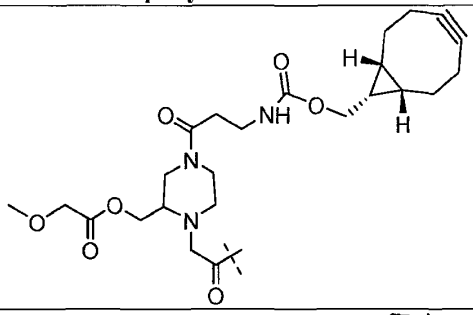
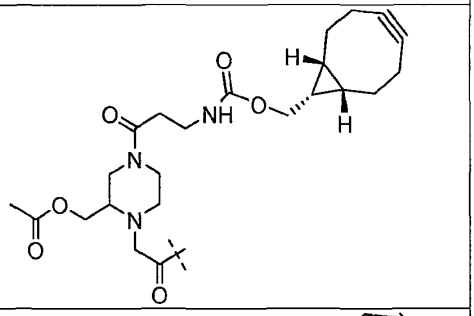
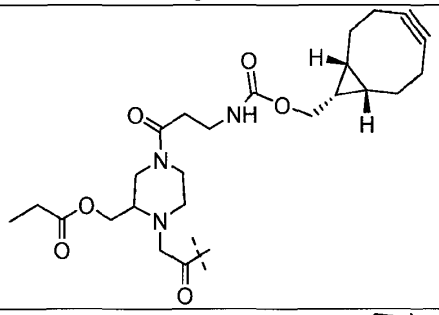
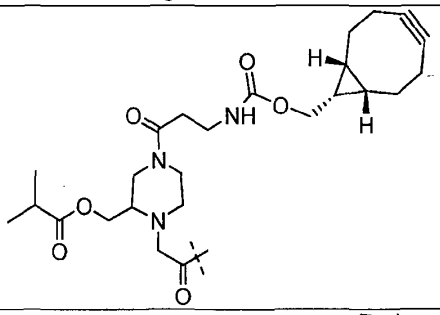
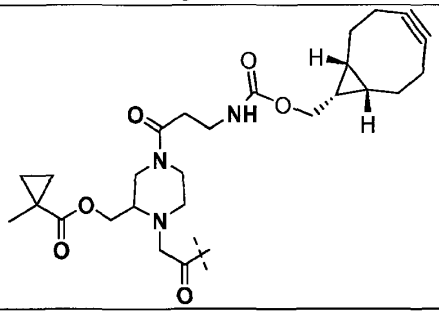
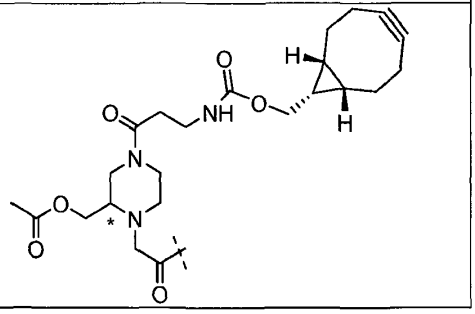
In some aspects, T_1 is $-CR^1R^{1a}OY$, which contains a trigger moiety and comprises one of the following structures in Table 3.

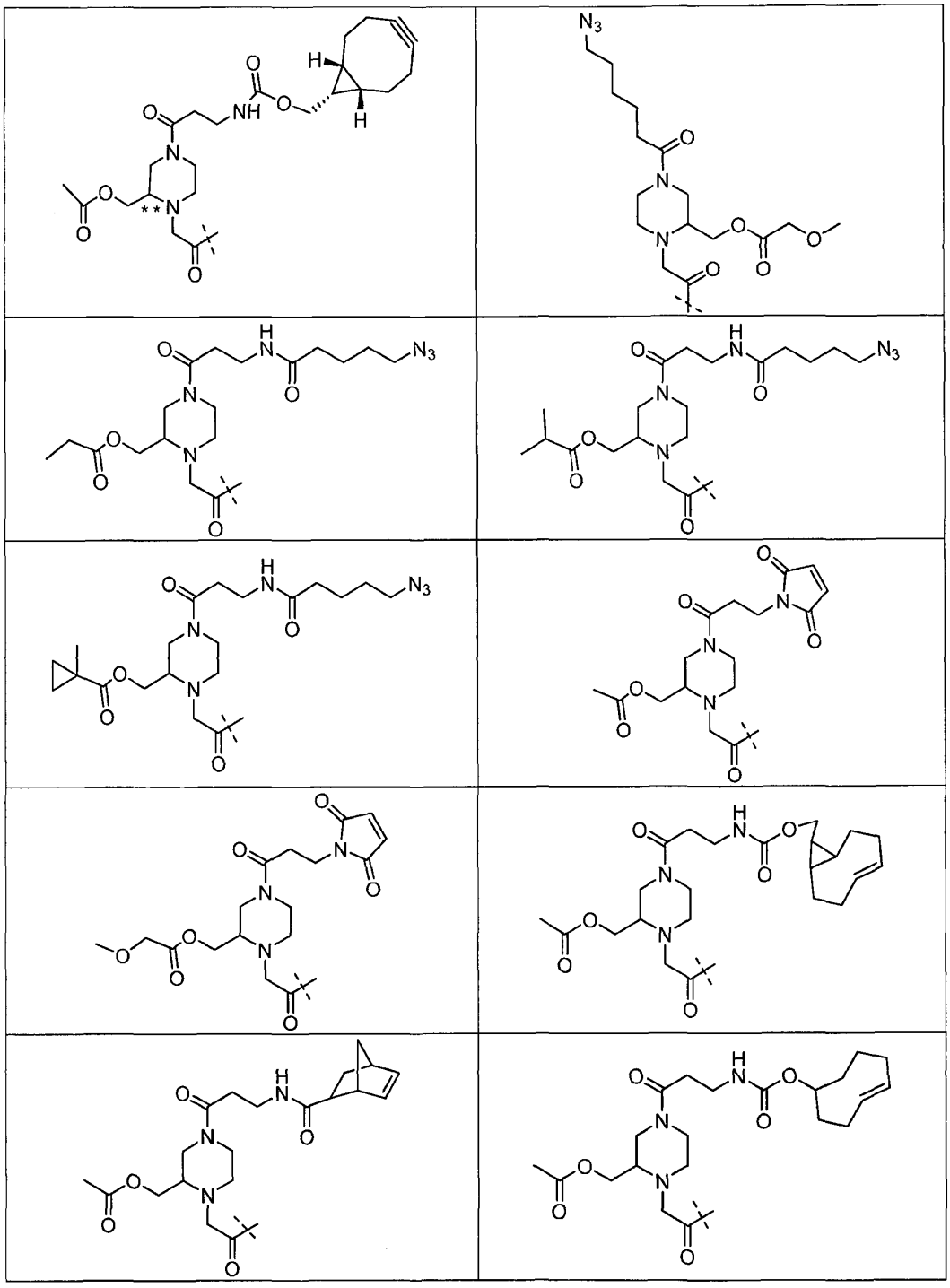
Table 3. Trigger Moieties

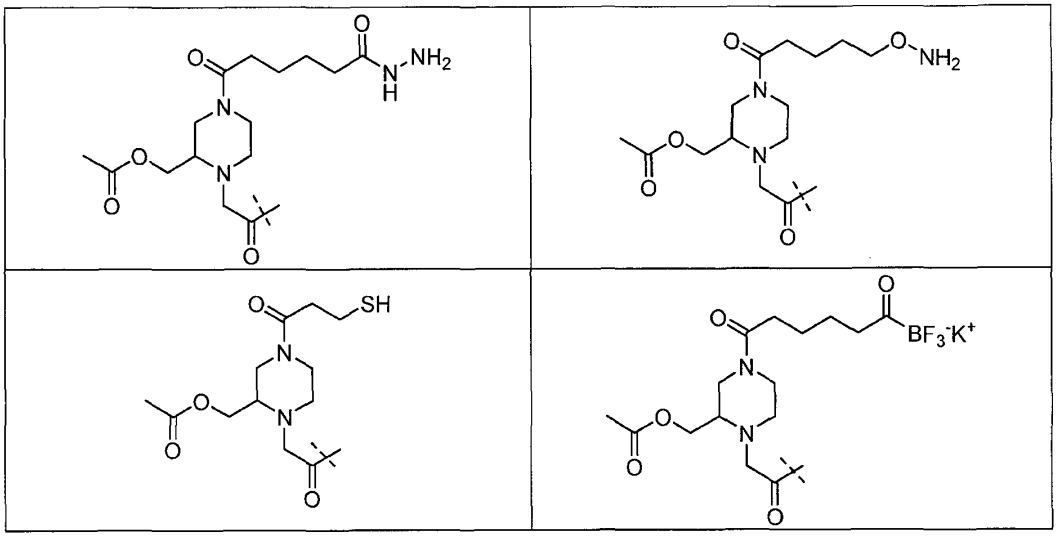
Y =  $R^1 = R^{1A} = H$	Y =  $R^1 = H$; $R^{1A} = CH_3$	Y =  $R^1 = R^{1A} = H$	Y =  $R^1 = R^{1A} = H$
Y =  $R^1 = R^{1A} = H$	Y =  $R^1 = H$; $R^{1A} = CH_3$	Y =  $R^1 = R^{1A} = H$	Y =  $R^1 = R^{1A} = H$
Y =  $R^1 = R^{1A} = H$	Y =  $R^1 = R^{1A} = H$	Y =  $R^1 = R^{1A} = H$	Y =  $R^1 = R^{1A} = H$
Y =  $R^1 = R^{1A} = H$	Y =  $R^1 = R^{1A} = H$	Y =  $R^1 = R^{1A} = H$	Y =  $R^1 = R^{1A} = H$

 $R^1 = R^{1A} = H$	 $R^1 = R^{1A} = H$	 $R^1 = R^{1A} = H$	-

In some embodiments, the traceless linker, R, according to either Formula I or II comprises any one of the following structures in Table 4.

Table 4. Exemplary Traceless Linkers	
	
	
	





In another embodiment, the traceless linker, R, of Formula (I) or Formula (II) is conjugated to a drug or bioactive moeity. In one aspect, the traceless linker, R, of Formula (I) or Formula (II) is conjugated to an ANGPTL3 polypeptide. In another aspect, the ANGPTL3 polypeptide comprises SEQ ID NOs:1, or 3–45. In another aspect, the ANGPTL3 polypeptide comprises D1 (SEQ ID NO:19).

Synthesis of a Traceless Linker, R

Another embodiment is a method for manufacturing a traceless linker, R, of Formula (I) comprising any one of the steps: (A) reacting the N4 nitrogen of a piperazine compound containing a nucleophilic group at the C2 position with a functionalized acyl linker compound to form an N4-acylpiperazine; (B) carboxymethylating the N1 nitrogen of the piperazine ring of the N4-acylpiperazine, wherein the carboxyl group is covalently attached to a suitable protecting group; (C) reacting the nucleophilic group of the N4-acylpiperazine with a trigger compound described herein; (D) if necessary, elaborating the functionalized acyl linker on N4 to contain a functional group suitable for attaching the traceless linker to a carrier compound. In one aspect, the nucleophilic group at the C2 position of the piperazine compound comprises a hydroxyl. In another aspect, the nucleophilic group at the C2 position of the piperazine compound is a primary alcohol. In another aspect, the primary alcohol present in the piperazine compound of step (A) or (D) is conjugated to a suitable protecting group or trigger group. In another aspect, the protecting

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group or trigger group comprises an ester, silyl ether, acetal, carbamate, carbonate, or a disiloxane containing compound. In one aspect, the N4 carboxymethyl ester protecting group is deprotected to form a carboxylic acid, which is suitable for forming an amide bond to a biologically active agent comprising at least one primary or secondary amine or a ring nitrogen atom of an azaheteroaryl ring. In another aspect, in the carboxymethylation step, the carboxyl group of the carboxymethylating reagent is covalently attached via an amide bond to a biologically active agent comprising at least one primary or secondary amine or a ring nitrogen atom of an azaheteroaryl ring. In one aspect, the functional group of step A is suitable for attaching the traceless linker to a carrier compound.

Another embodiment is a method for manufacturing a traceless linker, R, of Formula (I) comprising any one of the steps: (A) carboxymethylating the N1 nitrogen of a piperazine compound containing a nucleophilic group at the C2 position , wherein the carboxyl group is covalently attached to a suitable protecting group; (B) reacting the N4 nitrogen of the piperazine compound with a functionalized alkyl linker to form an N4-alkylpiperazine; (C) reacting the nucleophilic group of the N4-alkylpiperazine with a trigger compound described herein; (D) if necessary, elaborating the functionalized alkyl linker on N4 to contain functional group suitable for attaching the traceless linker to a carrier compound. In one aspect, the reactive group on the piperazine compound comprises a hydroxyl. In another aspect, the nucleophilic group at the C2 position of the piperazine compound is a primary alcohol. In another aspect, the primary alcohol present on the piperazine compound of step (A) or (D) is conjugated to a suitable protecting group or trigger group. In another aspect, the trigger compound comprises an ester, silyl ether, acetal, carbamate, carbonate, or a disiloxane containing compound. In one aspect, the N4 carboxymethyl ester protecting group is deprotected to form a carboxylic acid, which is suitable for forming an amide bond to a biologically active agent comprising at least one primary or secondary amine or a ring nitrogen atom of an azaheteroaryl ring. In another aspect, in the carboxymethylation step, the carboxyl group of the carboxymethylating reagent is covalently attached via an amide bond to a biologically active agent comprising at least one primary or secondary amine or a ring nitrogen atom of an azaheteroaryl ring. In one aspect, the functional group of step A is suitable for attaching the traceless linker to a carrier compound.

Another embodiment is a method for manufacturing a traceless linker, R, of Formula (I) comprising any one of the steps: (A) introducing a functionalized linker at the C4-position of a

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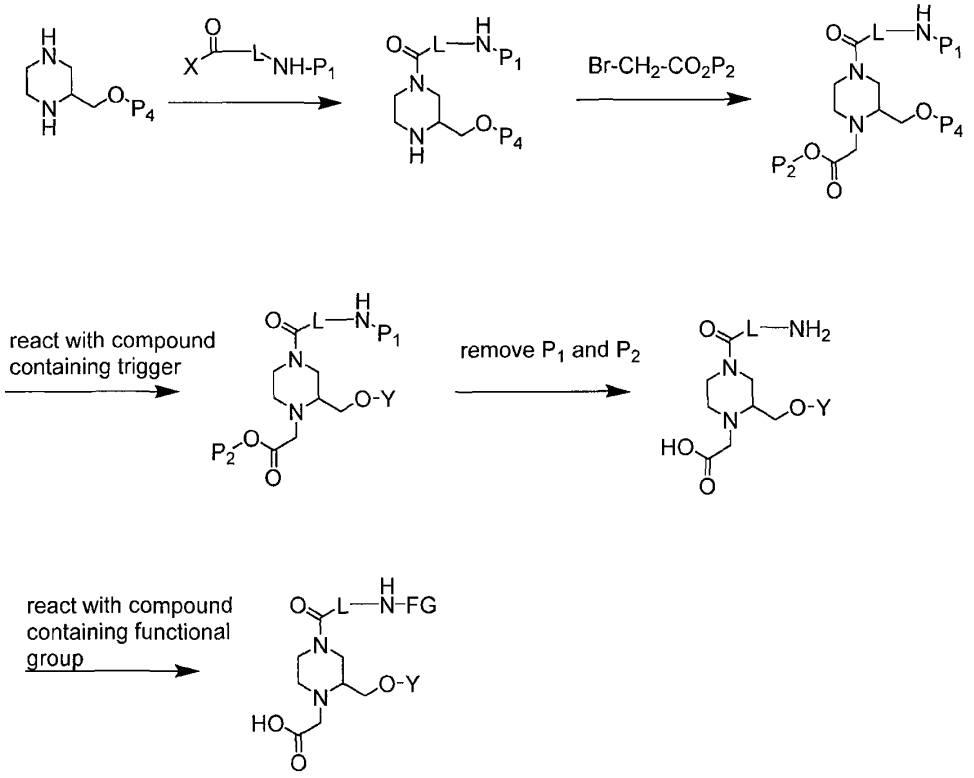
pyridine compound containing a nucleophilic group at the C2 position via a cross coupling or substitution reaction; (B) hydrogenating the 4-substituted pyridine compound to form a 4-substituted piperidine compound; (C) carboxymethylating the N1 nitrogen of the 4-substituted piperidine compound, wherein the carboxyl group is covalently attached to a suitable protecting group; (D) reacting the nucleophilic group of the 4-substituted piperidine compound with a trigger compound described herein; (E) if necessary, elaborating the functionalized alkyl linker on C4 to contain a functional group suitable for attaching the traceless linker to a carrier compound. In one aspect, the nucleophilic group on the pyridine or piperidine compounds comprises a hydroxyl. In another aspect, the nucleophilic group at the C2 position of the pyridine or piperidine compounds is a primary alcohol. In another aspect, the trigger compound comprises an ester, silyl ether, acetal, carbamate, carbonate, or a disiloxane containing compound. In one aspect, the N4 carboxymethyl ester protecting group is deprotected to form a carboxylic acid, which is suitable for forming an amide bond to a biologically active agent comprising at least one primary or secondary amine or a ring nitrogen atom of an azaheteroaryl ring. In another aspect, in the carboxymethylation step, the carboxyl group of the carboxymethylating reagent is covalently attached via an amide bond to a biologically active agent comprising at least one primary or secondary amine or a ring nitrogen atom of an azaheteroaryl ring. In one aspect, the functional group of step A is suitable for attaching the traceless linker to a carrier compound.

Suitable protecting groups are moieties that are reversibly connected to reactive functional groups or chemical functional groups to render them incapable of reacting with for example other chemical functional groups. Exemplary and non-limiting amino protecting groups include a fluorenylmethylenoxy group (FMOC), tert-butyloxycarbonyl (BOC), carboxybenzyl (Cbz), and the like. Exemplary and non-limiting alcohol protecting groups include t-butyl ether, allyl ether, benzyl ether, tert-butyldimethylsilyl ethers (TBDMS), and the like. Deprotecting groups may be added or removed as needed throughout the synthesis to block and expose particular moieties.

In one embodiment, the method for manufacturing a traceless linker is according to reaction **Schemes 1–3**. The following schemes are general and non-limiting schemes for manufacturing traceless linkers. As shown in the Examples herein some synthesis routes do not specifically conform to these general schemes. In another aspect, the method for manufacturing a traceless linker according to the reaction schemes provided in Example 1.

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Scheme 1



wherein one or more of the steps shown above may be performed in a different sequential order or omitted depending on the reagents utilized;

X is an activating group comprising Cl, O-NHS, O(C=O)-R^{2a}, or X-OH and the reaction includes standard peptide coupling reagents such as 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU);

R^{2a} is C_1-C_8 alkyl or aryl;

P_4 is a suitable protecting group or H;

L is an optionally substituted bivalent linker;

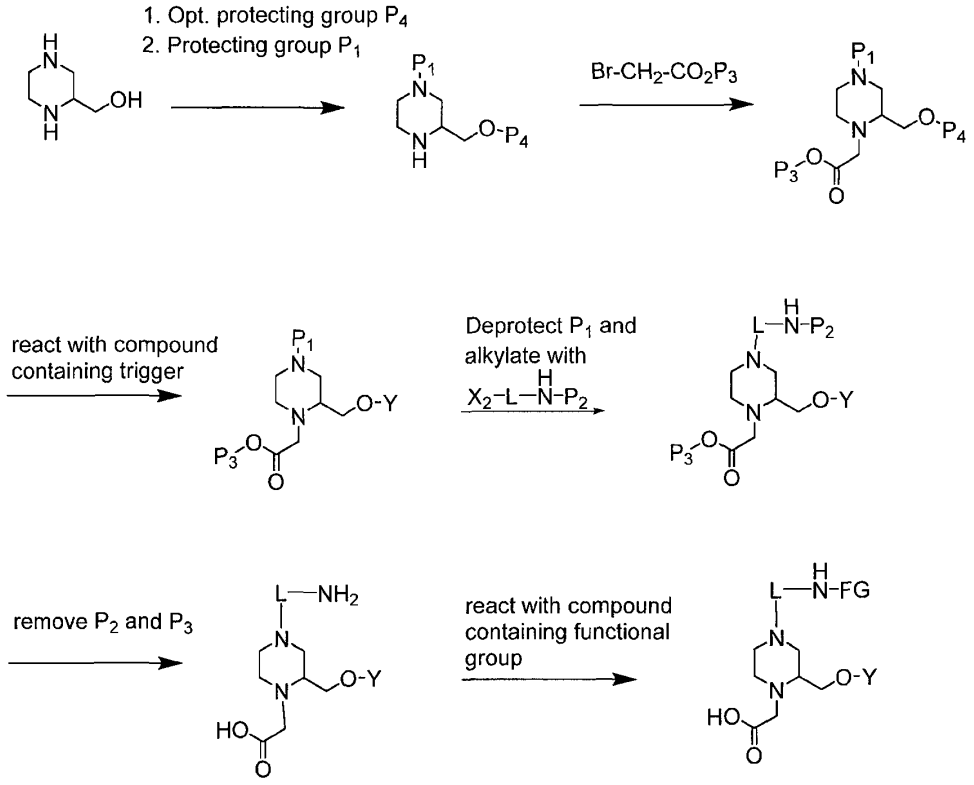
P_1 and P_2 are protecting groups that may be identical;

Y is a suitable trigger provided in Table 3; and

FG comprises a suitable functional group(s) capable of conjugating to a carrier described herein.

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Scheme 2



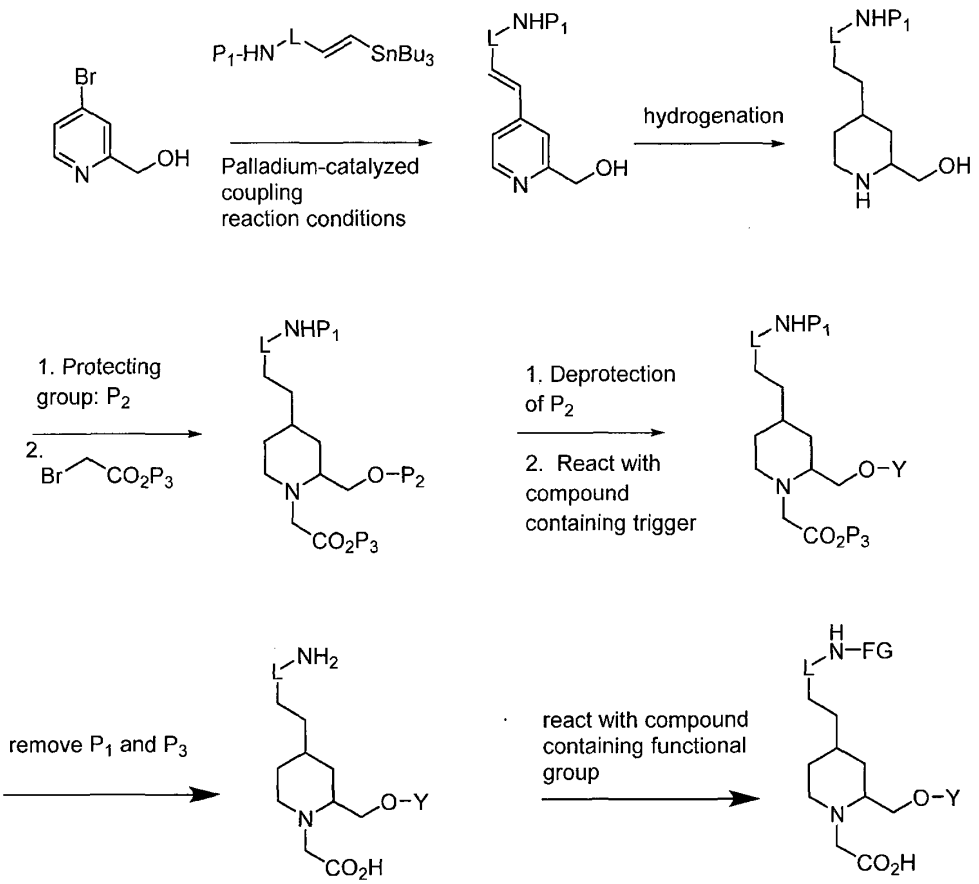
wherein one or more of the steps shown above may be performed in a different sequential order or omitted depending on the reagents utilized;

where, X_2 is an activating group such as Cl, Br, I, or triflate;

Y, L, P_1, P_2, P_4, and FG are as described in Scheme 1;

P_3 is a protecting group that may be identical to P_1 and P_2.

Scheme 3



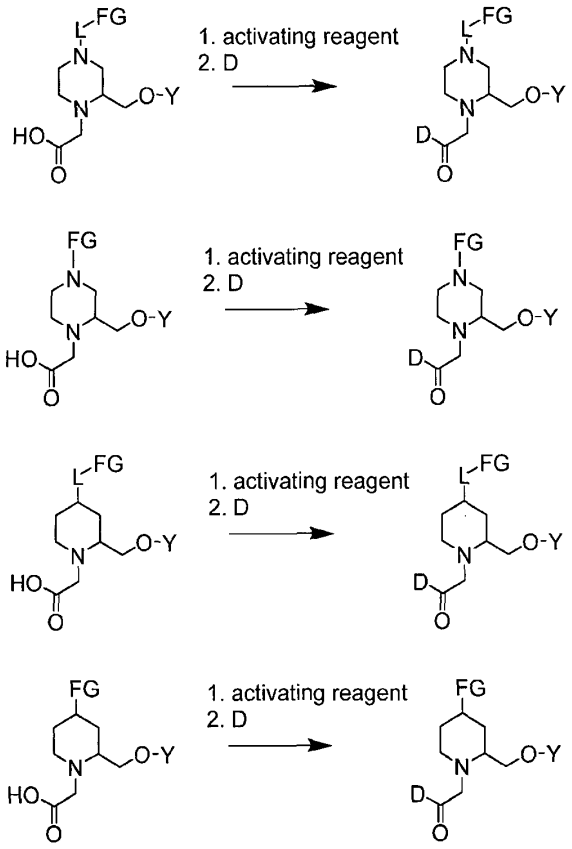
wherein one or more of the steps shown above may be performed in a different sequential order or omitted depending on the reagents utilized; and

L, P_1, P_2, P_3, Y, and FG are as described in Scheme 2.

As described herein, the adduct D–R of Formula (I) or Formula (II) is prepared by reacting the carboxylic acid form of traceless linker R with an amino group of a biologically active agent comprising at least one primary or secondary amine or a ring nitrogen atom of an azaheteroaryl ring to form an amide bond. This process is described in Scheme 4. A suitable carboxylic acid activating agent is used to promote the amide bond forming reaction. As an example, the carboxylic acid may be converted to an amino-reactive form by the action of disuccinimidyl carbonate to form an NHS ester; the NHS ester of traceless linker R is then reacted with amino-containing drug D to form adduct D–R.

In Scheme 4, L, Y, and FG are as described in Schemes 1–3; D is a biologically active agent comprising at least one primary or secondary amine or a ring nitrogen atom of an azaheteroaryl ring.

Scheme 4



One embodiment described herein is a traceless linker, R, of Formula (I) or Formula (II) attached to a carrier composition through a linker having one or more functional groups. In one aspect, the carrier composition comprises R^{11} of a traceless linker as described herein. In one aspect, R^{11} comprises a polymer, biopolymer, or polyethylene glycol connected to R^8 or R^9 through a linker. In one aspect, the carrier composition is a hydrogel. In one aspect, the hydrogel composition comprises hyaluronic acid, polyethylene glycol, polypropylene glycol, polyethylene

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oxide, polyglutamate, polylysine, polysialic acid, polyvinyl alcohol, polyacrylate, polymethacrylate, polyacrylate/polymethacrylate copolymers, polyacrylamide, polymethacrylamide, polyvinylpyrrolidone, polyoxazoline, polyiminocarbonate, polyamino acid, hydrophilic polyester, polyamide, polyurethane, polyurea, dextran, agarose, xylan, mannan, carrageenan, alginate, gelatin, collagen, albumin, cellulose, methylcellulose, ethylcellulose, hydroxypropylmethylcellulose, hydroxyethyl starch, chitosan, nucleic acids, derivatives thereof, co-polymers thereof, or combinations thereof. In another aspect, R^{11} comprises a nanoparticle or a molecular surface. In one aspect, the hydrogel comprises hyaluronic acid or polyethylene glycol. In another aspect, R^{11} comprises a cross-linked hydrogel of hyaluronic acid or polyethylene glycol. In one aspect, where the drug delivery system is utilized in the eye or in the synovial joints, R^{11} comprises hyaluronic acid or cross-linked hyaluronic acid.

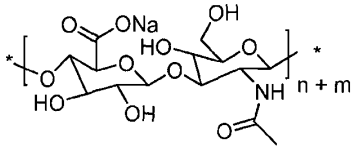
As described herein, R^{11} carrier compositions can be cross-linked to join multiple molecules together and facilitate hydrogel formation. Cross-linking can be accomplished using any means known in the art. *See,* for example, Liu et al., "Solution processable, cross-linked sulfur polymers as solid electrolytes in dye-sensitized solar cells," *Chem. Commun.* 51: 14660-14662 (2015). In one embodiment described herein, hyaluronic acid or polyethylene glycol are functionalized with one or more functional groups shown in Table 2 to provide reactive functional groups for cross-linking. In another aspect, the hyaluronic acid or polyethylene glycol are functionalized with a functional group selected from azidyl, alkynyl, substituted or unsubstituted C_7-C_{12} cycloalkynyl, C_7-C_{12} cycloalkenyl, substituted or unsubstituted C_7-C_{12} heterocycloalkynyl, vinyl carboxyl, vinyl sulfonyl, C_2-C_8 alkenyl, amino, thiol, C_1-C_8 carboxyl, C_1-C_8 carbonyl, oxyamine, carbohydrazide, maleimide, alpha-halo carbonyl, furan, substituted or unsubstituted tetrazinyl, lysine, glutamine, cyclodextrin, and adamantanyl. In another aspect, the hyaluronic acid is functionalized with an azidyl group.

The degree of functionalization can determine the porosity of the hydrogel. In one aspect, about 5% to about 50% of the carrier polymer is functionalized, including all integers within the specified range. In one aspect, the carrier polymer is functionalized about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, or even greater.

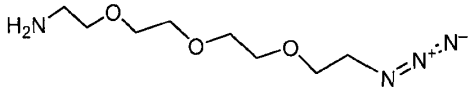
In one aspect, the carrier is hyaluronic acid. In another aspect, hyaluronic acid is reacted with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholin-4-ium chloride (CAS number

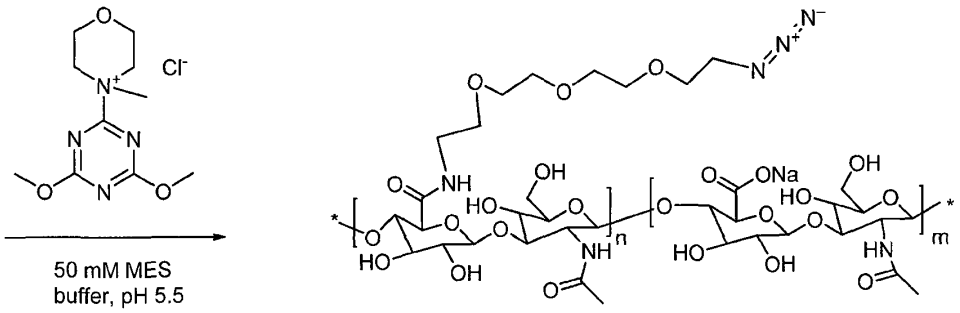
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3945-69-5) and 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethanamine (CAS Number 134179-38-7) to form an azide functionalized hyaluronic acid ([HA-N_3]). In one aspect, the reaction is:



Hyaluronic acid, sodium salt (HA)





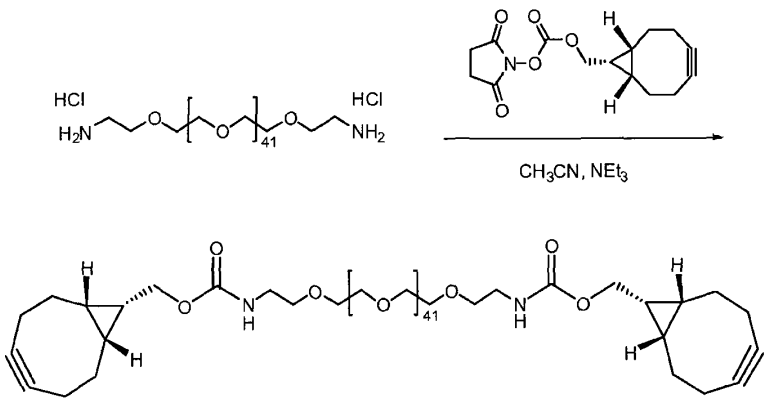
[HA-N_3]

Experimental conditions are described in Example 3. In one aspect, about 5% to about 50% of the hyaluronic acid is functionalized, including all integers within the specified range. In one aspect, the hyaluronic acid is functionalized about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, or even greater.

In another embodiment, polyethylene glycol is functionalized with various reagents to form cros-linkers for linking the functionalized hyaluronic acid monomers as discussed above.

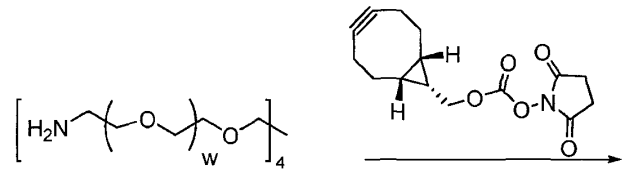
Synthesis of 2kDa 2-arm PEG-BCN crosslinker

In one aspect, M_n ~2 kDa polyethylene glycol diamine hydrochloride is reacted as shown. Reaction conditions are described in Example 4.

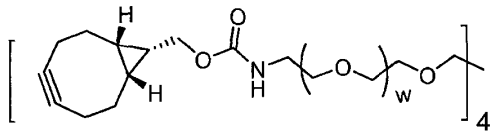


Synthesis of 10kDa 4-arm PEG-BCN crosslinker

In another aspect, M_n ~10 kDa 4-arm polyethylene glycol amine hydrochloride (pentaerythritol core, JenKem Technology) is reacted as shown. Reaction conditions are described in Example 4.

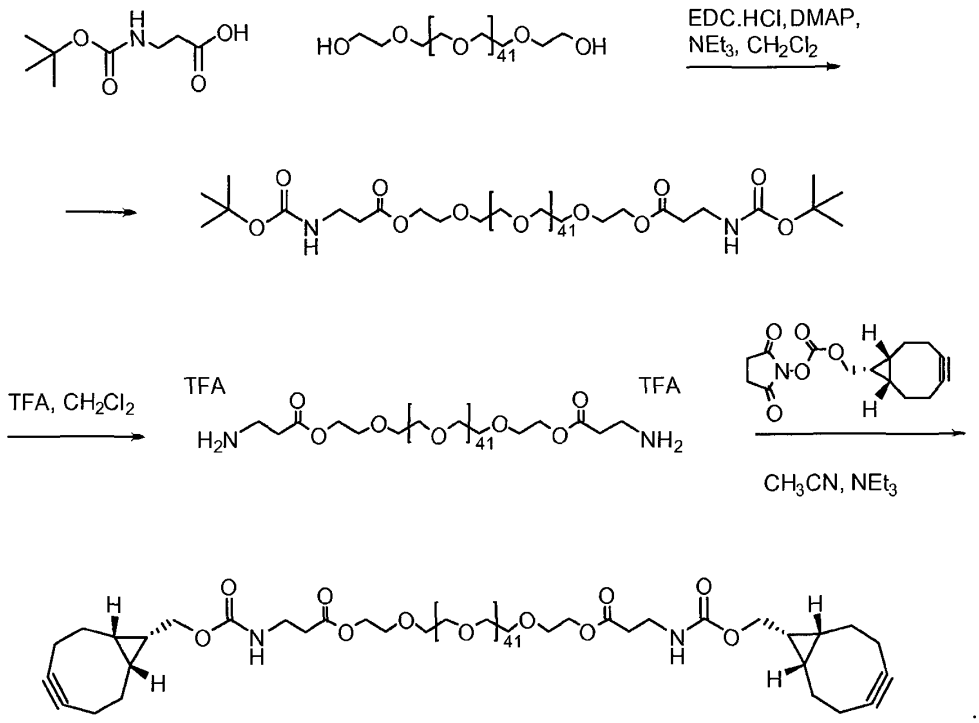


HCl salt, w ~ 56,
pentaerythritol core
JenKem



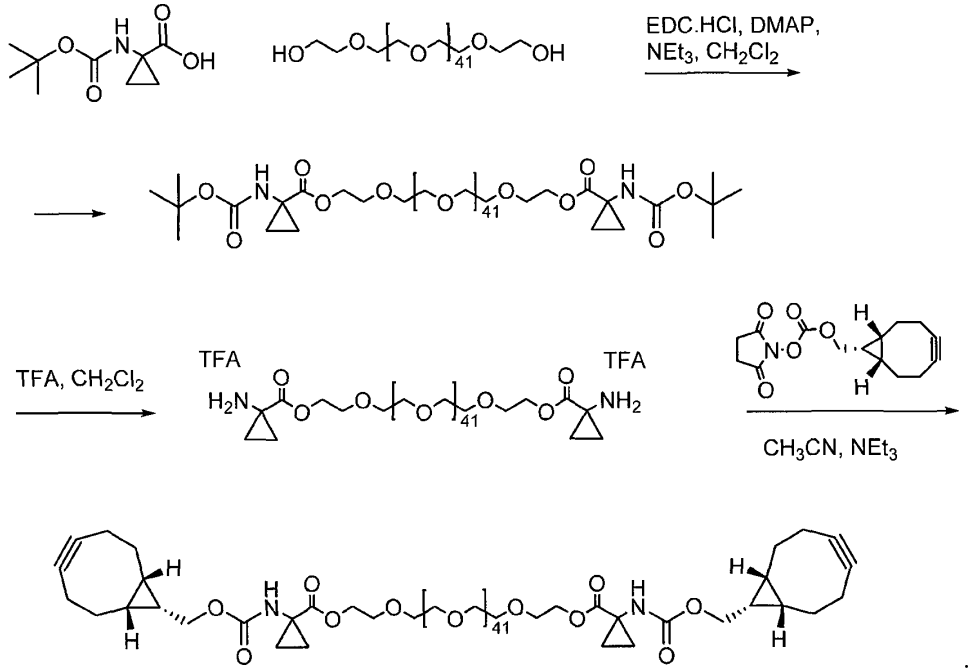
Synthesis of 2kDa 2-arm Beta-alanine PEG-BCN Crosslinker

In another aspect, M_n ~2 kDa polyethylene glycol and 3-((tert-butoxycarbonyl)amino)propanoic acid are reacted as shown. Reaction conditions are described in Example 4.

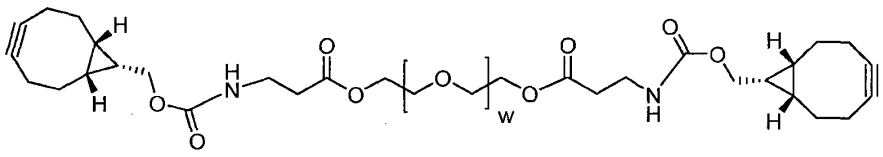


Synthesis of 2kDa 2-arm aminocyclopropanecarboxylic acid PEG-BCN crosslinker

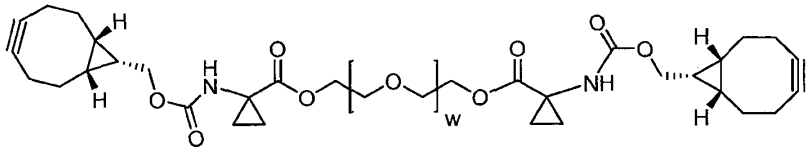
In another aspect, M_n ~2 kDa polyethylene glycol and 1-((tert-butoxycarbonyl)amino)cyclopropane-1-carboxylic acid are reacted as shown. Reaction conditions are described in Example 4.



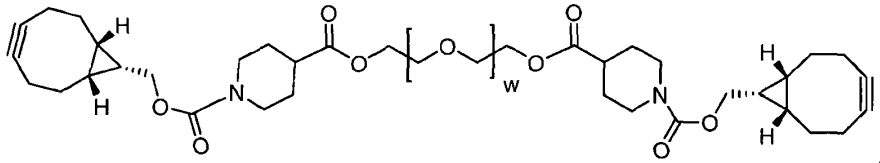
Another embodiment described herein is one or more cross-linking agents. In one aspect, the cross-linking agent comprises Formula V:



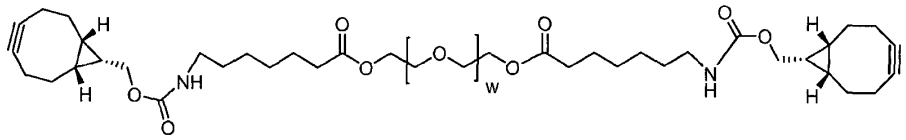
In another aspect, the cross-linking agent comprises Formula VI:



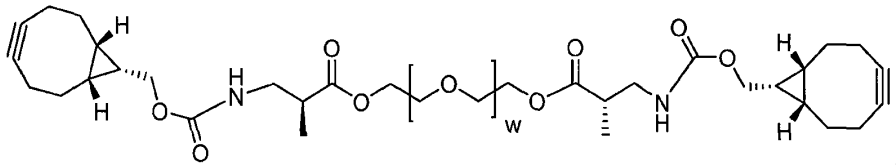
In another aspect, the cross-linking agent comprises Formula VIa:



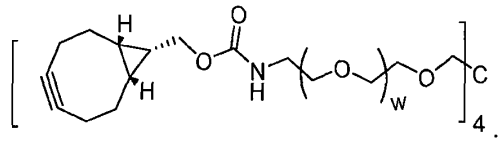
In another aspect, the cross-linking agent comprises Formula VIb:



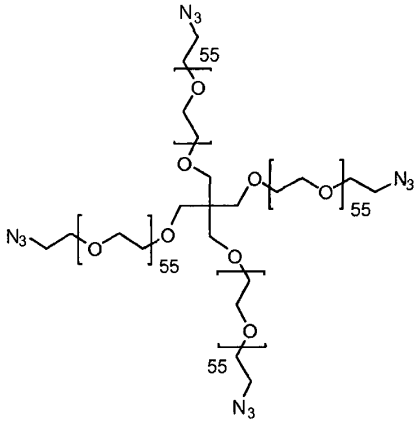
In another aspect, the cross-linking agent comprises Formula VIc:



In another aspect, the cross-linking agent comprises Formula VII:

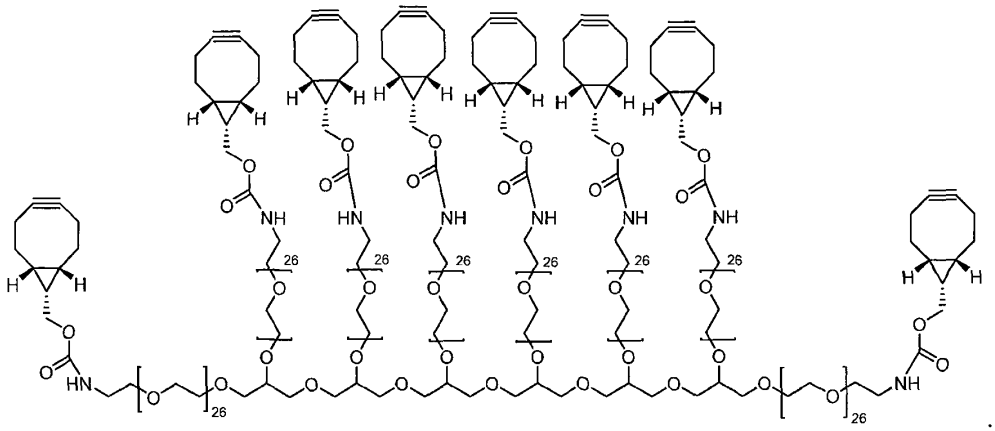


In another aspect, the cross-linking agent comprises Formula VIII:



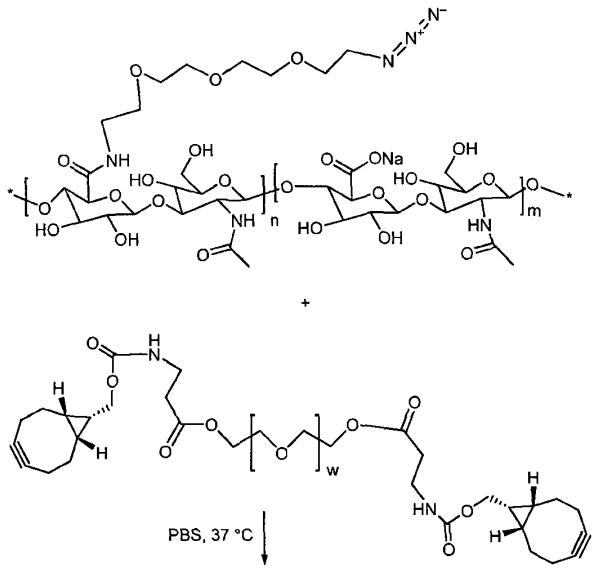
In another aspect, the cross-linking agent comprises Formula IX:

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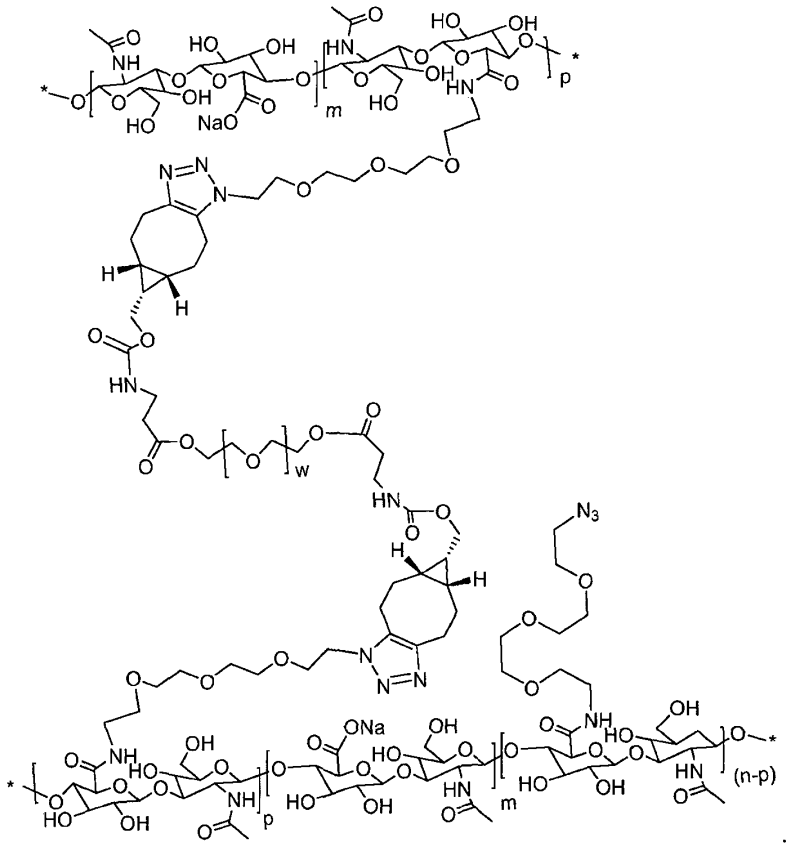


In another embodiment described herein, R^{11} comprises a functionalized carrier that has been reacted with one or more cross-linking agents to form a cross-linked carrier. In one embodiment, the cross-linked carrier forms a hydrogel. In one aspect, the cross-linked carrier is hyaluronic acid that has been functionalized and cross-linked with one or more polyethylene glycol cross linkers as described herein to form a hydrogel.

In one embodiment, a cross-linked carrier comprising hyaluronic acid can be prepared by reacting appropriately functionalized polymers as shown:



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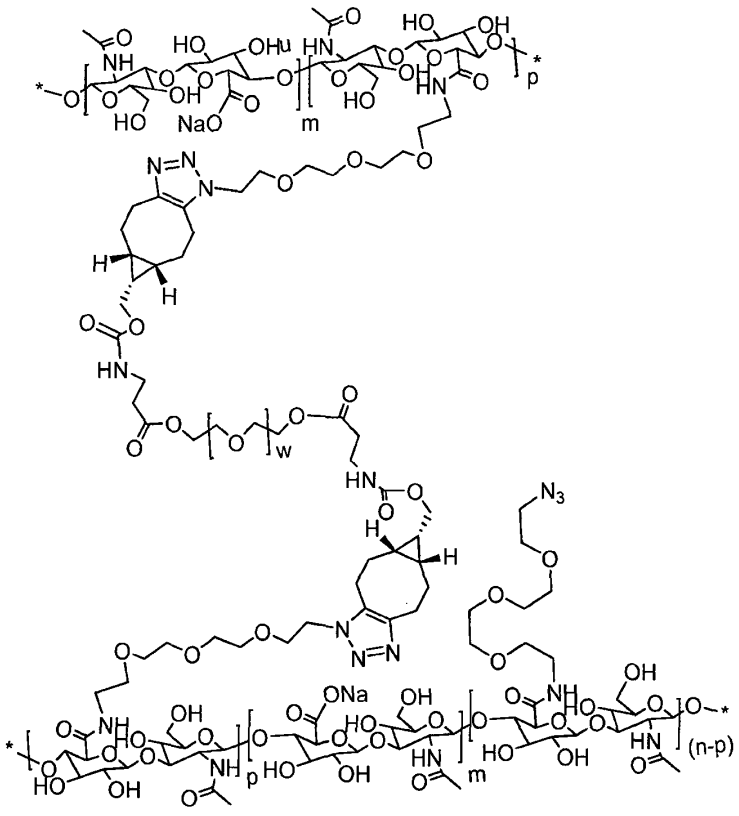
While not being bound by any particular example and by way of illustration, in one embodiment, hyaluronic acid sodium salt labeled by the supplier, Lifecore Biomedical (HA200K, Chaska, MN) as having a nominal average molecular weight varying from batch to batch in the range of 151–300 kDa, as determined by viscometry. For the purposes of this illustration, a molecule of hyaluronic acid sodium salt with an assumed nominal average molecular weight of 200 kDa would consist of an average of approximately 500 monomer units. In this and following structures the unmodified monomer unit is defined as "m," the monomer unit modified with an azido group as "n," the azido-monomer unit conjugated to the crosslinking molecule as "p" and the azido monomer unit conjugated to the traceless linker-drug adduct as "q." For a polymer chain comprising ≈500 monomer units, (n + m + p + q ≈ 500). If the percent modification of the hyaluronic acid sodium salt molecule is 25%, then m = 75% and (n + p + q) = 25 %.

Similarly, the PEG unit present in one embodiment of the cross linker is derived from a starting PEG described as having a nominal average molecular weight of 1 kDa, 2 kDa, 4 kDa, 6

kDa, 8 kDa, or 10 kDa, and would consist of approximately 22, 45, 91, 136, 181, or 226 repeating oxyethylene monomer units, respectively, described in these depicted structures as "w." In some aspects, w can be between about 5 and about 250. In other aspects, w comprises about 5, about 10, about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90, about 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, about 190, about 200, about 210, about 220, about 230, about 240, or about 250 oxyethylene monomer units. In other aspects, w is about 20, about 45, about 90, about 140, about 180, or about 225.

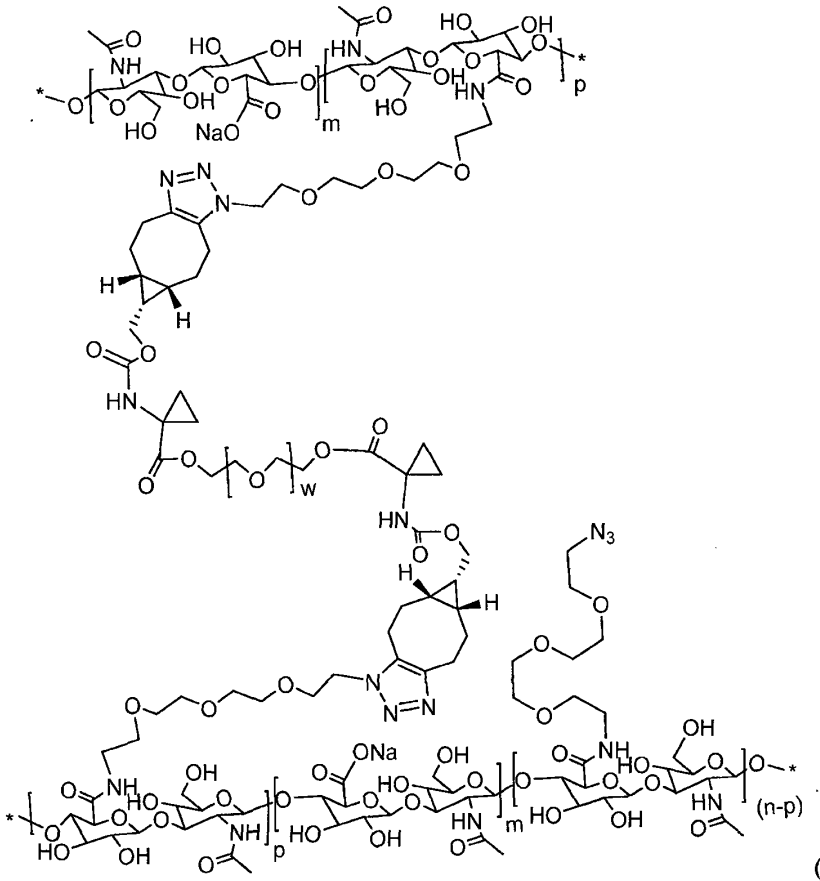
In the following Formulae, in one embodiment, the sum of the unmodified disaccharide repeating unit of hyaluronic acid (m) plus the modified disaccharide repeating unit of hyaluronic acid (n + p + q) in a random distribution (= m + n + p + q) may comprise about 500 units for a nominal average molecular weight as determined by viscometry of approximately 200 kDa. This applies to Formulae X to XXII.

In one aspect, the cross-linked carrier comprises Formula X:



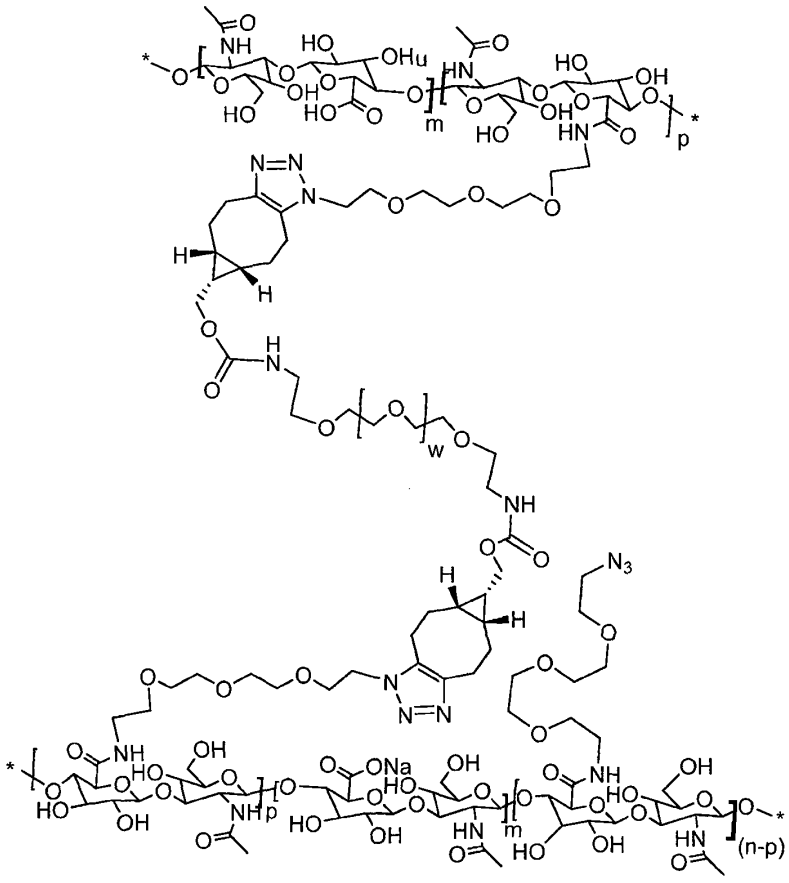
(X).

In another aspect, the cross-linked carrier comprises Formula XI:



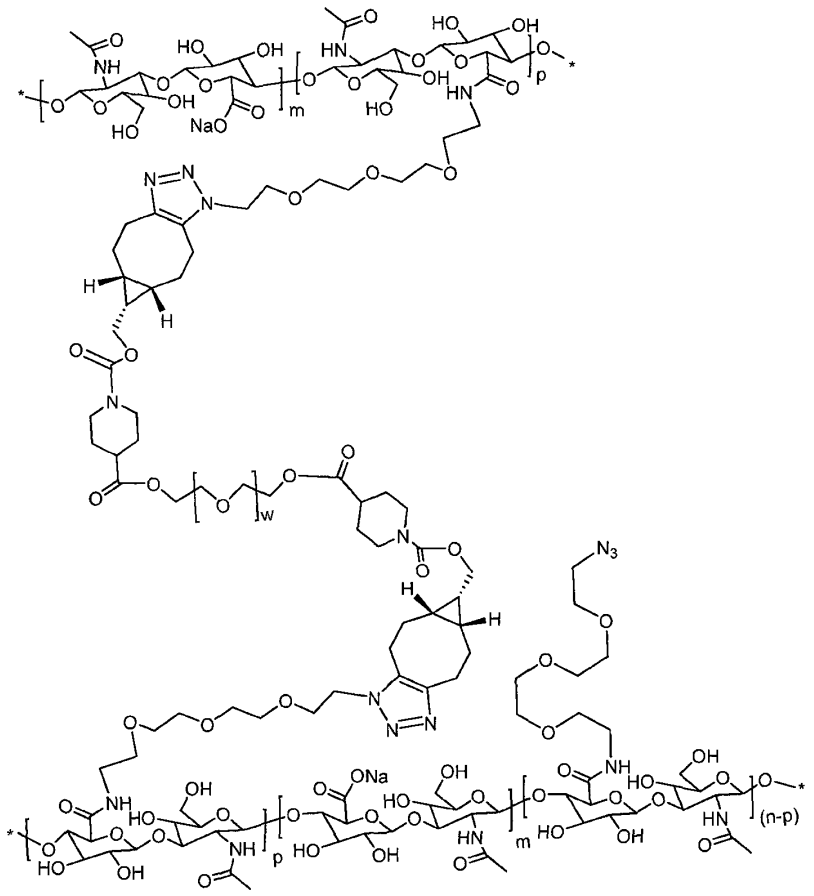
(XI).

In another aspect, the cross-linked carrier comprises Formula XII:



(XII).

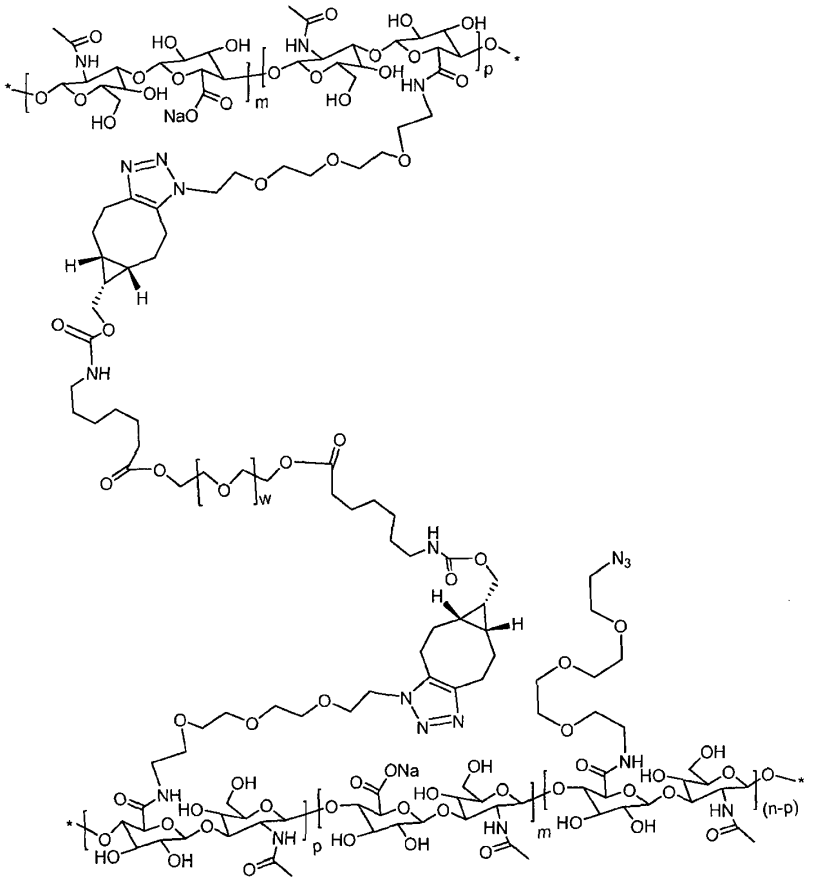
In another aspect, the cross-linked carrier comprises Formula XIIa:



(XIIa).

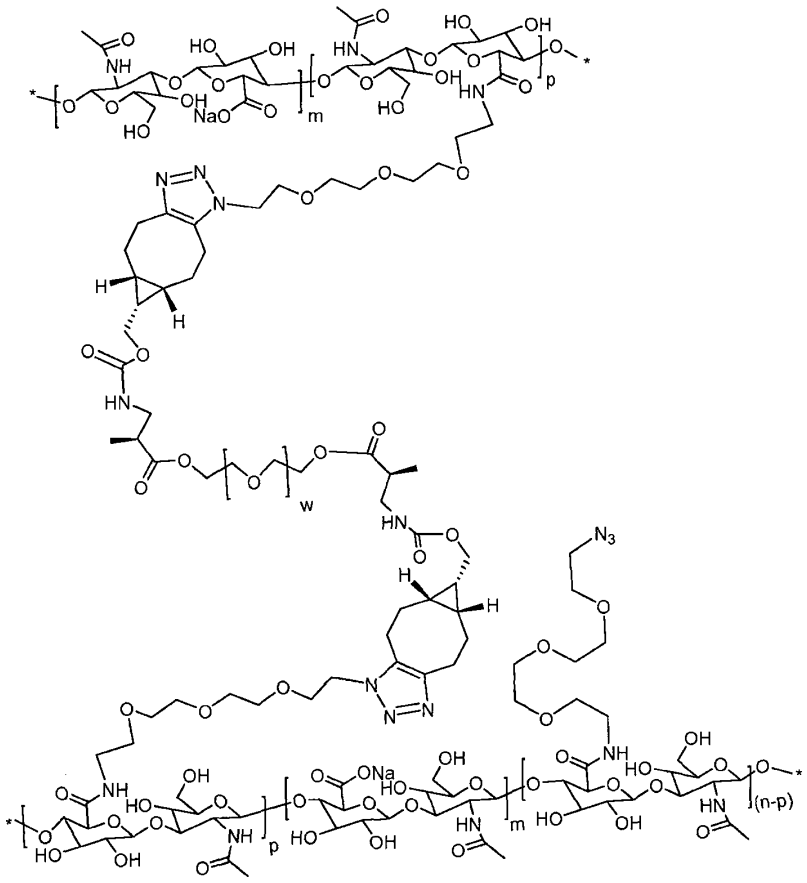
5

In another aspect, the cross-linked carrier comprises Formula XIIb:



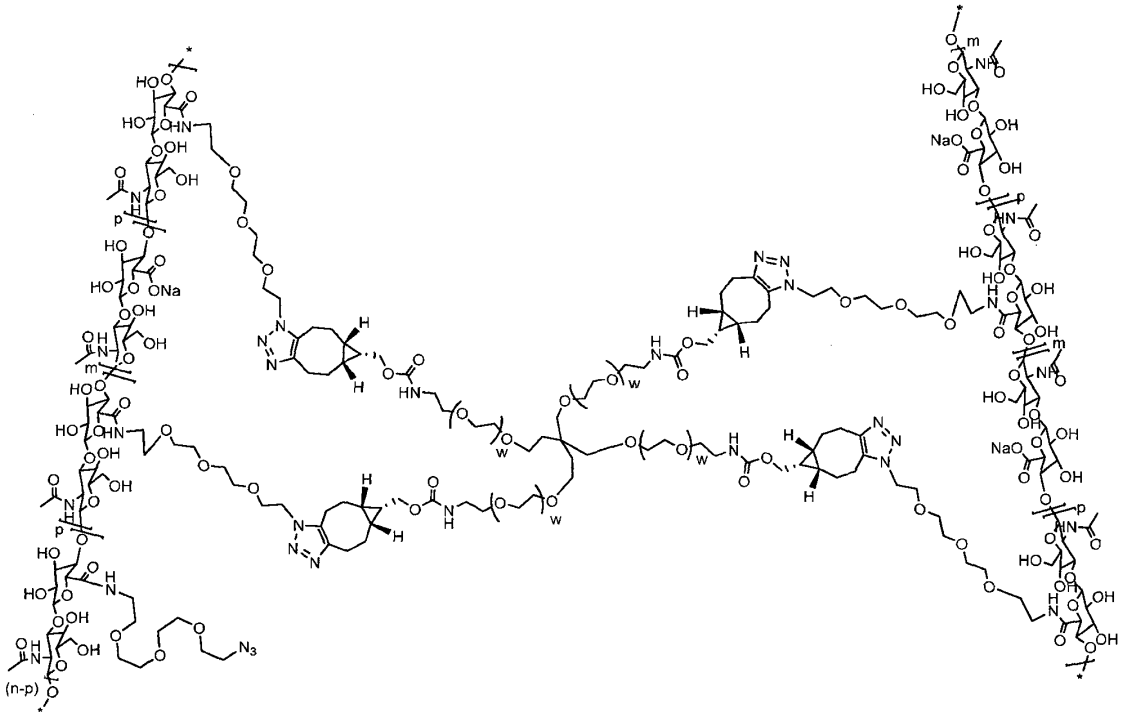
(XIIb).

In another aspect, the cross-linked carrier comprises Formula XIIc:



(XIIc).

In another aspect, the cross-linked carrier comprises Formula XIII:



(XIII).

Another embodiment described herein is a drug adduct, D–R, wherein D is a biologically active moiety comprising at least one primary amine, secondary amine, or ring nitrogen atom of an azaheteroaryl ring; and R is a linker suitable for release of a biologically active moiety. In one aspect, D comprises a protein, nucleic acid, carbohydrate, peptide, nucleotide, oligosaccharide, or small molecule each of which has at least one primary or secondary amine and the small molecule has a molecular weight of between about 100 g/mol and about 2000 g/mol.

One embodiment described herein is a drug adduct, D–R, wherein D comprises an ANGPTL3 polypeptide. In one aspect, D comprises an ANGPTL3 polypeptide having at least 95% identity to any one of SEQ ID NO: 1 or 3–45. In another aspect, D comprises any one of SEQ ID NO:1 or 3–45. In another aspect, D comprises an ANGPTL3 polypeptide having a K423Q substitution or a K423 deletion. In another aspect, D comprises an ANGPTL3 polypeptide comprising amino acid residues 201–460; 207–460; 225–455; 225–455; 225–460; 225–460; 226–455; 226–455; 226–460; 226–460; 228–455; 228–455; 228–460; 228–460; 233–455; 233–455; 233–460; 233–460; 241–455; 241–455; 241–460; 241–460; 242–455; 242–455; 242–460; or 242–

460, each in reference to SEQ ID NO:1. In another aspect, D comprises an ANGPTL3 polypeptide comprising at least 95% identity to amino acid residues 242–460 in reference to SEQ ID NO:1 and a K423Q substitution. In another aspect, D comprises an ANGPTL3 polypeptide comprising amino acid residues 242–460 in reference to SEQ ID NO:1 and a K423Q substitution, D1 (SEQ ID NO:19).

Another embodiment described herein is a drug delivery system comprising a carrier-traceless linker biologically-active agent conjugate, D–R–R^{11}, wherein D comprises a biologically active moiety comprising at least one primary amine, secondary amine, or ring nitrogen atom of an azaheteroaryl ring as described herein, R comprises a traceless linker as described herein attached to R^{11}, comprising a carrier polymer or hydrogel as described herein. In one aspect, R^{11} comprises hyaluronic acid, cross-linked hyaluronic acid, polyethylene glycol, cross-linked polyethylene glycol or other suitable polymer as described herein. In another aspect, R comprises a traceless linker as described herein. In another aspect, D comprises an ANGPTL3 polypeptide as described herein. In one aspect, D comprises an ANGPTL3 polypeptide having at least 95% identity to any one of SEQ ID NO: 1 or 3–45. In another aspect, D comprises any one of SEQ ID NO:1 or 3–45. In another aspect, D comprises an ANGPTL3 polypeptide having a K423Q substitution or a K423 deletion. In another aspect, D comprises an ANGPTL3 polypeptide comprising amino acid residues 201–460; 207–460; 225–455; 225–455; 225–460; 225–460; 226–455; 226–455; 226–460; 226–460; 228–455; 228–455; 228–460; 228–460; 233–455; 233–455; 233–460; 233–460; 241–455; 241–455; 241–460; 241–460; 242–455; 242–455; 242–460; or 242–460, each in reference to SEQ ID NO:1. In another aspect, D comprises an ANGPTL3 polypeptide comprising at least 95% identity to amino acid residues 242–460 in reference to SEQ ID NO:1 and a K423Q substitution. In another aspect, D comprises an ANGPTL3 polypeptide comprising amino acid residues 242–460 in reference to SEQ ID NO:1 and a K423Q substitution, D1 (SEQ ID NO:19).

In another embodiment, D–R–R^{11} comprises Formula XIV, wherein "Drug" comprises an ANGPTL3 polypeptide comprising one or more of SEQ ID NO:1 or 3–45 or polypeptides having at least 90% identity thereto.

In some embodiments, D–R–R^{11} comprises any of the species shown in Formulas XV–XXIII.

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In one embodiment, the drug or biologically active agent D, comprises an ANGPTL3 polypeptide comprising one or more of SEQ ID NO:1 or 3–45.

In one embodiment, the drug or biologically active agent D, comprises D1, which has the sequence of SEQ ID NO:19.

One embodiment described herein is a peptide compositions for repairing cartilage. In particular ANGPTL3 peptides as described herein have increased protease-resistance as compared to a wildtype ANGPTL3 polypeptide.

Another embodiment described herein is a composition and method for administration of ANGPTL3 polypeptides to prevent or ameliorate arthritis or joint injury by administering a polypeptide of the invention into a joint, a cartilage tissue or a cartilage proximal tissue, or systemically. Also described are compositions and methods for induction of mesenchymal stem cell differentiation into chondrocytes.

Another embodiment described herein is a process for assembling a drug delivery system; the process may comprise one of the following sequence of steps:

(a) preparing a carrier molecule, R^{11}, where R^{11} is a cross-linked hydrogel, then this step comprises the process used to prepare that hydrogel; the carrier molecule may optionally be purified at this stage;

(b) separately conjugating the traceless linker, R, to a biologically active agent, D, comprising a primary amine, secondary amine, or a ring nitrogen atom of an azaheteroaryl ring, thereby forming the traceless linker-D adduct; the traceless linker-D adduct may optionally be purified at this stage,

(c) conjugating the carrier molecule, R^{11}, with the traceless linker-D adduct; and

(d) purifying the drug delivery system from the reagents. This process is shown in **Scheme 5A** below.

Another embodiment described herein is a process for assembling a drug delivery system comprising the following steps:

(a) preparing a carrier molecule, R^{11}, where R^{11} is a cross-linked hydrogel, then this step comprises the process used to prepare that hydrogel; the carrier molecule may optionally be purified at this stage;

(b) conjugating the traceless linker, R, to the carrier molecule, R^{11}, thereby forming the carrier molecule-traceless linker adduct, which may optionally be purified at this stage;

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(c) conjugating the biologically active agent, D, comprising a primary amine, secondary amine, or a ring nitrogen atom of an azaheteroaryl ring, to the carrier molecule-traceless linker adduct; and

(d) purifying the drug delivery system from the reagents. This process is shown in **Scheme 5B** below.

Another embodiment described herein is a process for assembling a drug delivery system comprising the following steps:

(a) preparing a non-cross-linked carrier molecule, R^{11}, the carrier molecule may optionally be purified at this stage;

(b) separately conjugating the traceless linker, R, to a biologically active agent, D, comprising a primary amine, secondary amine, or a ring nitrogen atom of an azaheteroaryl ring, thereby forming the traceless linker-D adduct; the traceless linker-D adduct may optionally be purified at this stage;

(c) conjugating the carrier molecule, R^{11}, with the traceless linker-D adduct, which may optionally be purified at this stage;

(d) preparing the cross-linked hydrogel by incubating the non-cross-linked carrier molecule-traceless linker-biolgically active agent, R^{11}–R–D, with the appropriate cross-linking reagent to form the hydrogel; and

(e) purifying the drug delivery system from the reagents. This process is shown in **Scheme 5C** below.

Another embodiment described herein is a process for assembling a drug delivery system comprising the following steps:

(a) preparing a non-cross-linked carrier molecule, R^{11}, the carrier molecule may optionally be purified at this stage;

(b) conjugating the traceless linker, R, to the carrier molecule, R^{11}, thereby forming the carrier molecule-traceless linker adduct, which may optionally be purified at this stage; and

(c) conjugating the biologically active agent, D, comprising a primary amine, secondary amine, or a ring nitrogen atom of an azaheteroaryl ring, to the carrier molecule-traceless linker adduct;

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(d) preparing the cross-linked hydrogel by incubating the non-cross-linked carrier molecule-traceless linker-biolgically active agent, R^{11}–R–D, with the appropriate cross-linking reagent to form the hydrogel; and

(e) purifying the drug delivery system from the reagents. This process is shown in **Scheme 5D** below.

Another embodiment described herein is a process for assembling a drug delivery system comprising the following steps:

(a) preparing a non-cross-linked carrier molecule, R^{11}, the carrier molecule may optionally be purified at this stage;

(b) conjugating the traceless linker, R, to the carrier molecule, R^{11}, thereby forming the carrier molecule-traceless linker adduct, which may optionally be purified at this stage;

(c) preparing the cross-linked hydrogel by incubating the non-cross-linked carrier molecule-traceless linker-adduct, R^{11}–R, with the appropriate cross-linking reagent to form the hydrogel, which may optionally be purified at this stage;

(d) conjugating the biologically active agent, D, comprising a primary amine, secondary amine, or a ring nitrogen atom of an azaheteroaryl ring, to the cross-linked carrier molecule-traceless linker adduct, which may optionally be purified at this stage; and

(e) purifying the drug delivery system from the reagents. This process is shown in **Scheme 5E** below.

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Schemes 5A–E. Scheme 5 shows five processes, A–E, for assembling drug delivery systems as described herein.

Legend:



Carrier molecule, R^{11}, non-crosslinked

crosslinker



Carrier molecule, R^{11}, crosslinked hydrogel

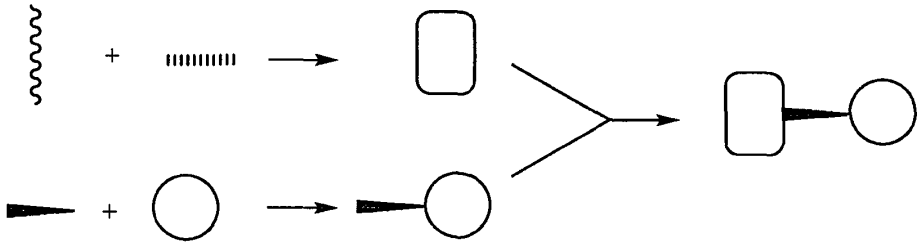


traceless linker, R

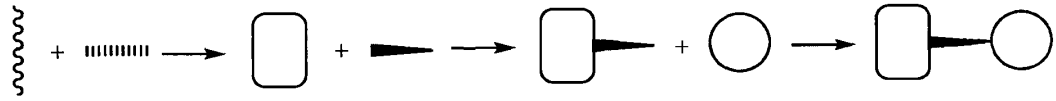


biologically active agent, D

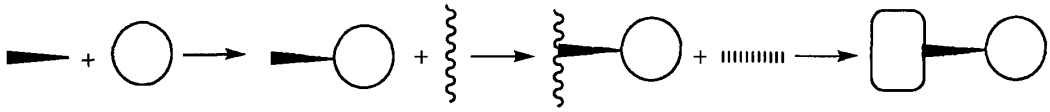
Scheme 5A



Scheme 5B

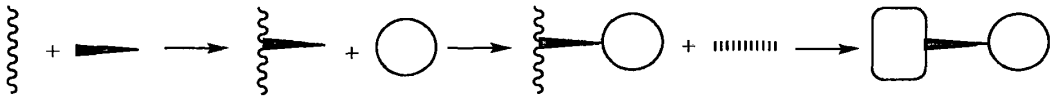


Scheme 5C

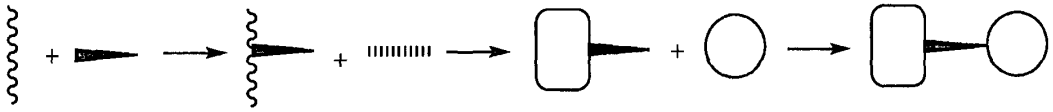


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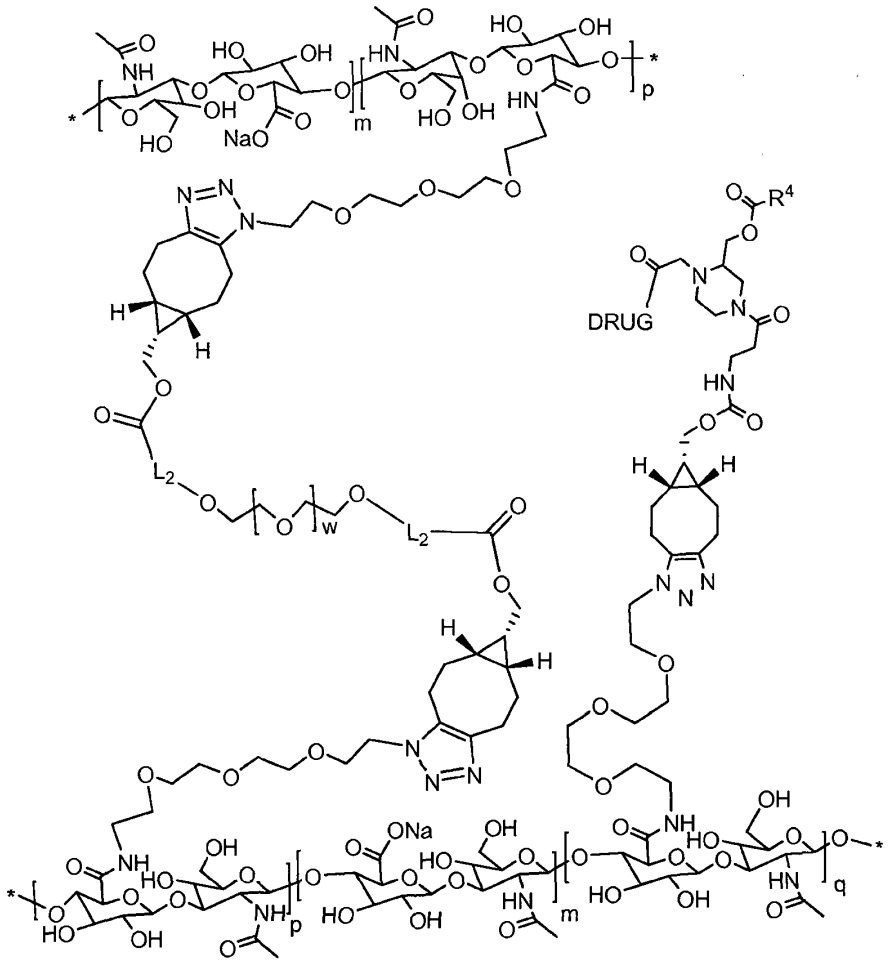
Scheme 5D



Scheme 5E



In one embodiment, the drug delivery system can comprise Formula (XIV):



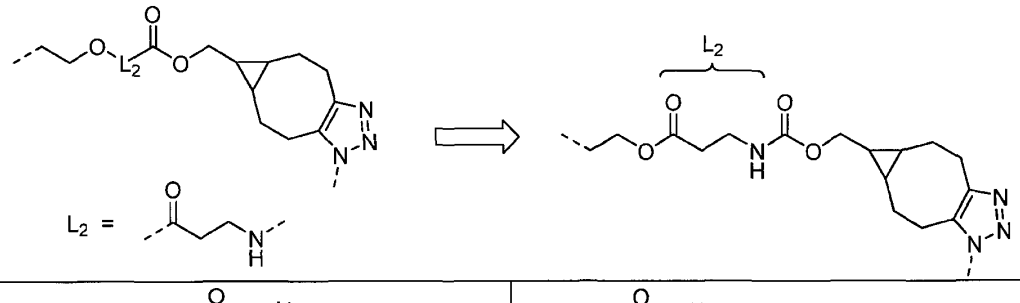
(XIV),

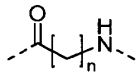
wherein L_2 represents a spacer group, which can be specific to a particular cross-linker, and "DRUG" represents a biologically active molecule comprising at least one primary amine, secondary amine, or ring nitrogen atom of an azaheteroaryl ring that modulates at least one biologically relevant target in a therapeutically beneficial manner. In one aspect, "DRUG" comprises an ANGPTL3 polypeptide as described herein. In another aspect, "DRUG" comprises an ANGPTL3 polypeptide having at least 95% identity to any one of SEQ ID NO: 1 or 3–45. In another aspect, "DRUG" comprises any one of SEQ ID NO:1 or 3–45. In another aspect, "Drug" comprises an ANGPTL3 polypeptide having a K423Q substitution or a K423 deletion. In another aspect, "DRUG" comprises an ANGPTL3 polypeptide comprising amino acid residues 201–460; 207–460; 225–455; 225–455; 225–460; 225–460; 226–455; 226–455; 226–460; 226–460; 228–455; 228–455; 228–460; 228–460; 233–455; 233–455; 233–460; 233–460; 241–455; 241–455; 241–460; 241–460; 242–455; 242–455; 242–460; or 242–460, each in reference to SEQ ID NO:1. In another aspect, "DRUG" comprises an ANGPTL3 polypeptide comprising at least 95% identity to amino acid residues 242–460 in reference to SEQ ID NO:1 and a K423Q substitution. In another aspect, "DRUG" comprises an ANGPTL3 polypeptide comprising amino acid residues 242–460 in reference to SEQ ID NO:1 and a K423Q substitution, e.g., D1 (SEQ ID NO:19).

In one embodiment, spacers, L_2, comprise any species shown in Table 5.

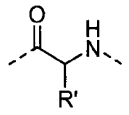
Table 5. Exemplary L_2 Spacers

In the table below, L_2 represents the spacer between O and C(O)O. For example:

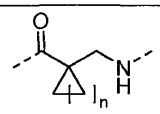
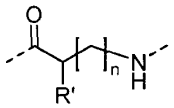
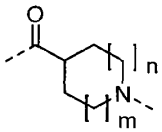
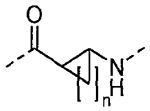
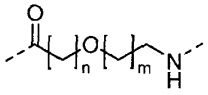
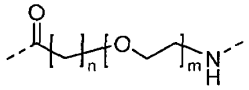
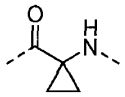




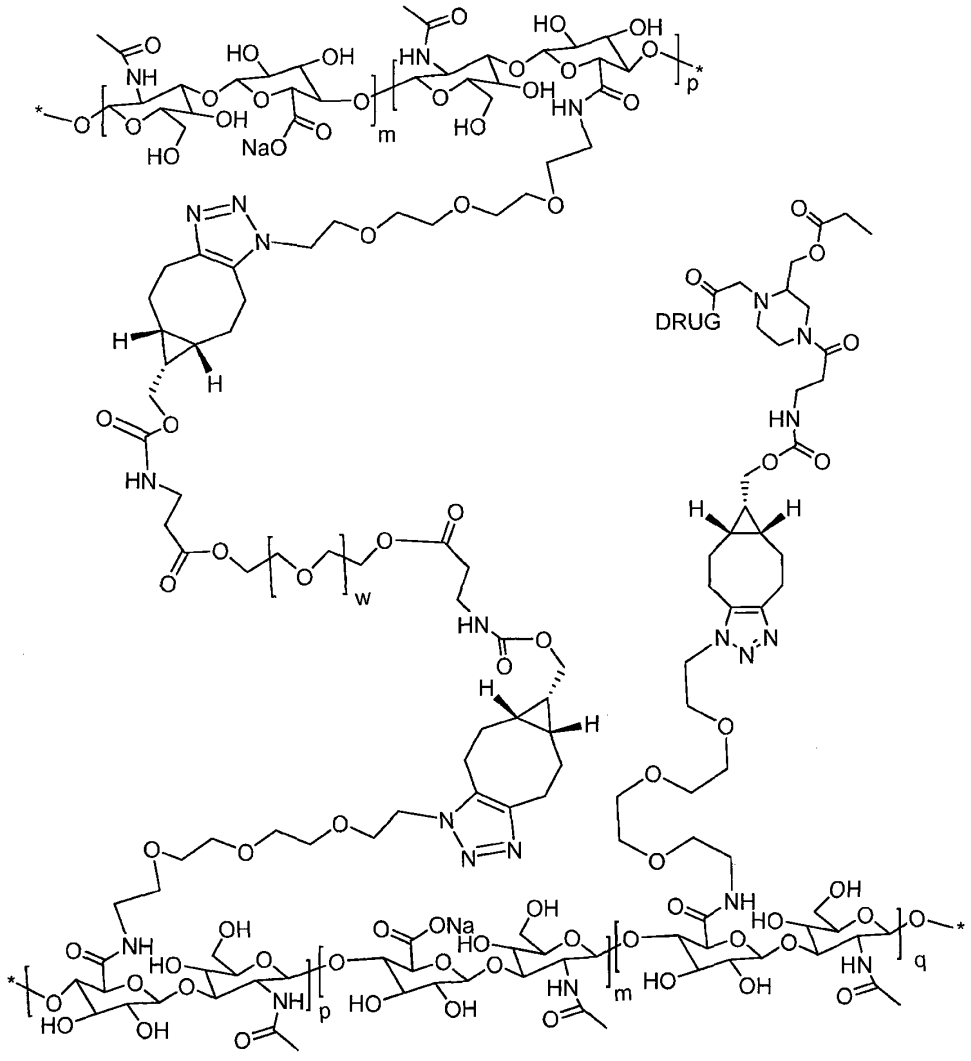
n = 1-10



R' = Me, Et, Pr, iPr, C1-C6 alky, C1-C6 cycloalkyll

 n = 1-6	 R' = Me, Et, Pr, iPr, C1-C6 alkyl, C1-C6 cycloalkyl n = 1-5
 n=0-2 m=0-2	 n = 1-5
 n = 1-6 m = 1-6	 n = 1-6 m = 1-6
 Cp-Glycine	

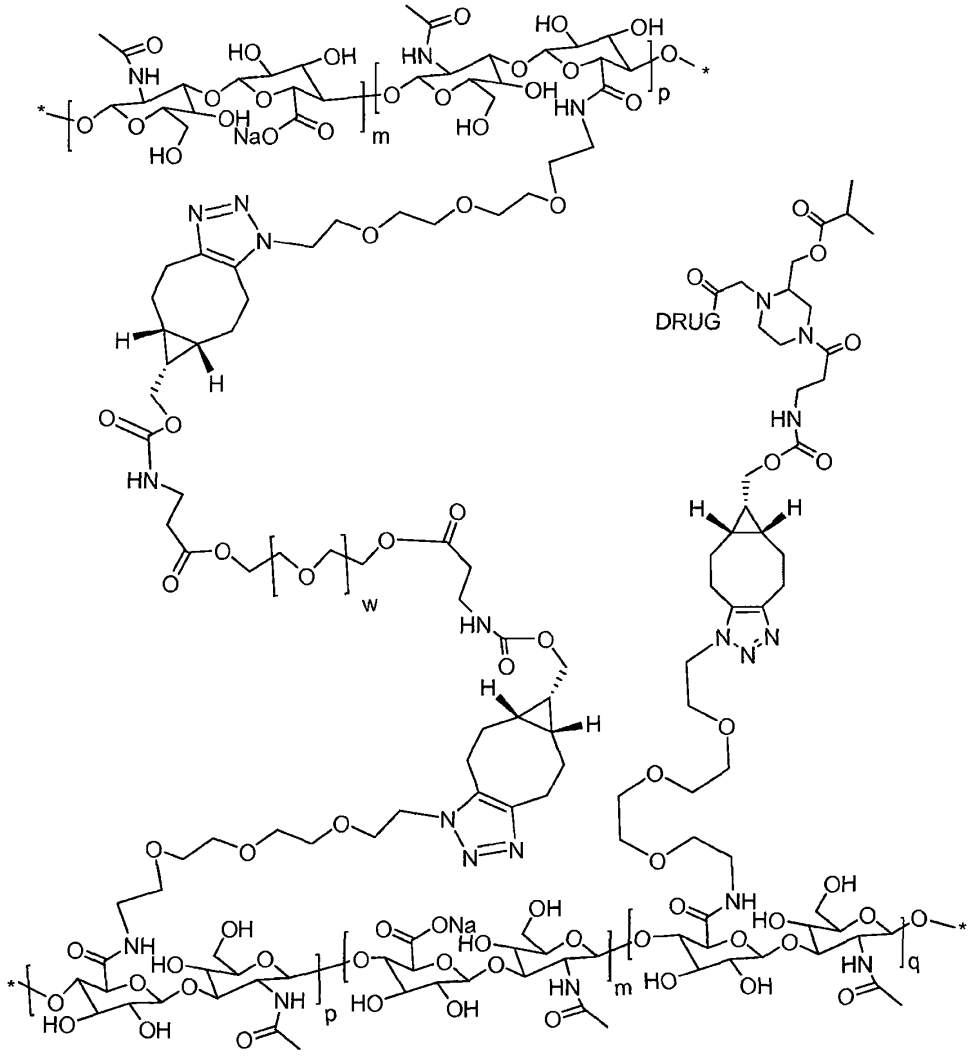
In another embodiment, the drug delivery system can comprise Formula (XV) with the DRUG being an ANGPTL3 polypeptide as described herein:



(XV).

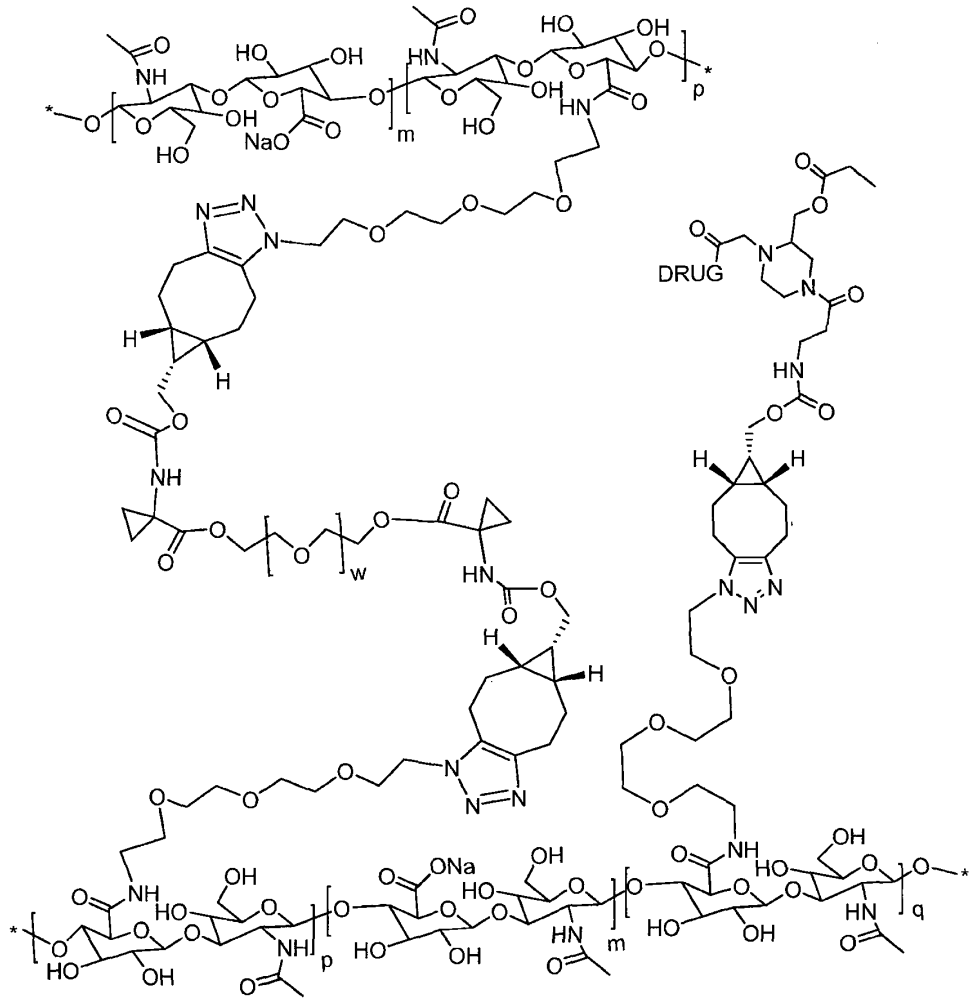
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In another embodiment, the drug delivery system can comprise Formula (XVI) with DRUG being an ANGPTL3 polypeptide as described herein:



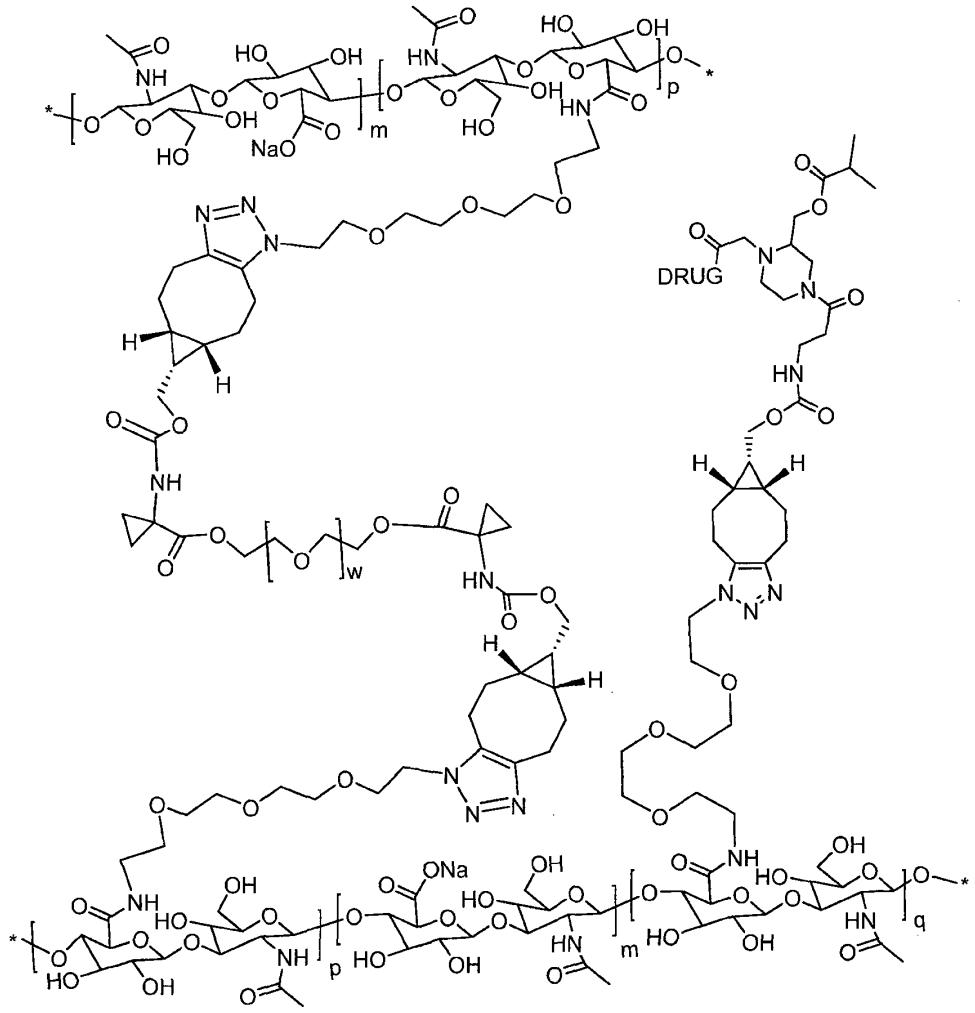
(XVI).

In another embodiment, the drug delivery system can comprise Formula (XVII) with DRUG being an ANGPTL3 polypeptide as described herein:



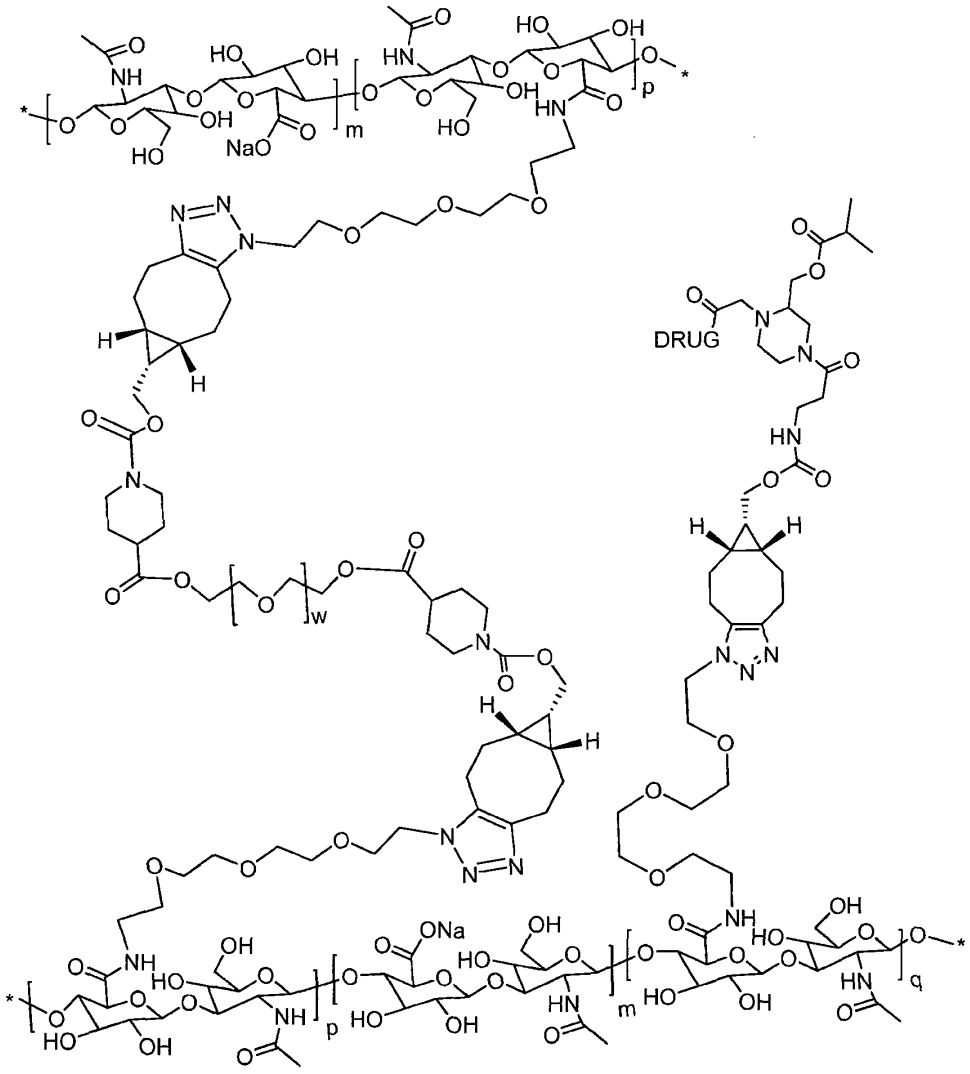
(XVII).

In another embodiment, the drug delivery system can comprise Formula (XVIII) with DRUG being an ANGPTL3 polypeptide as described herein:



(XVIII).

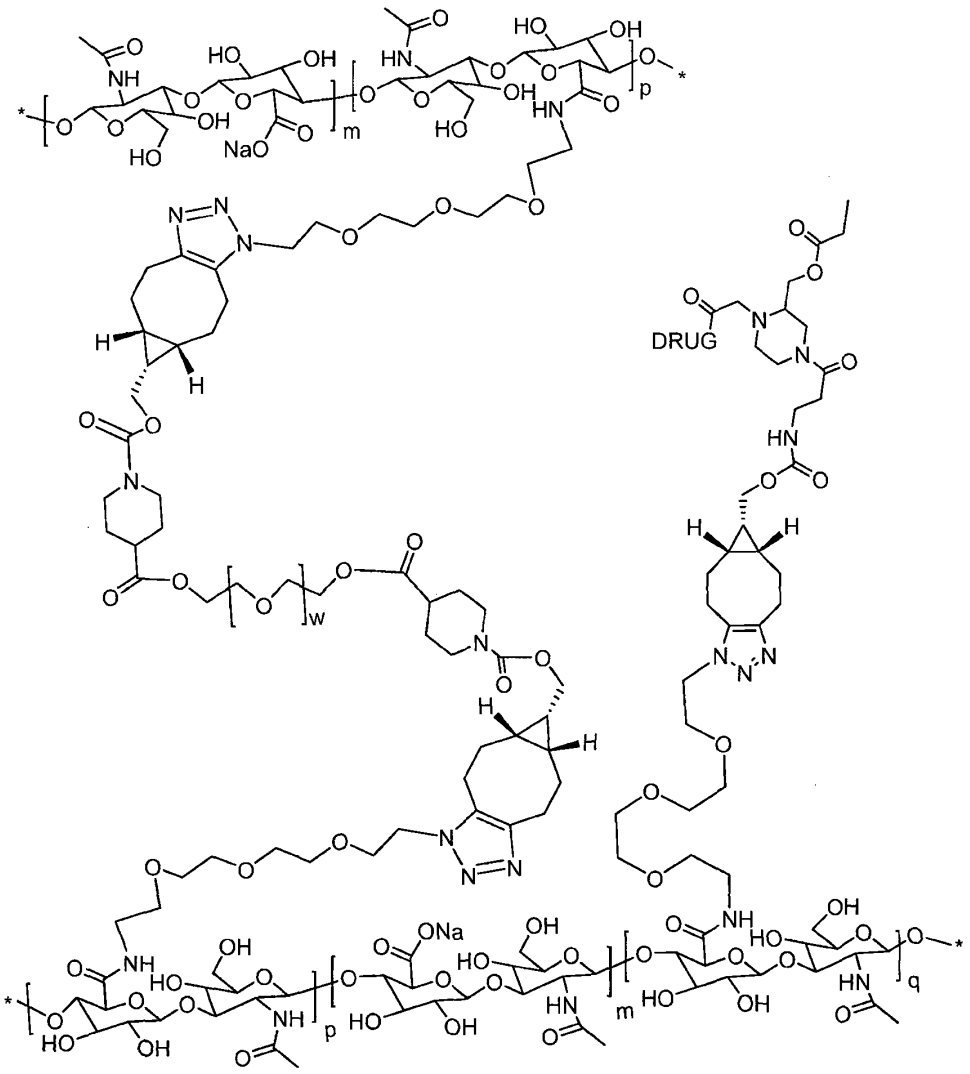
In another embodiment, the drug delivery system can comprise Formula (XVIIIa) with DRUG being an ANGPTL3 polypeptide as described herein:



(XVIIIa).

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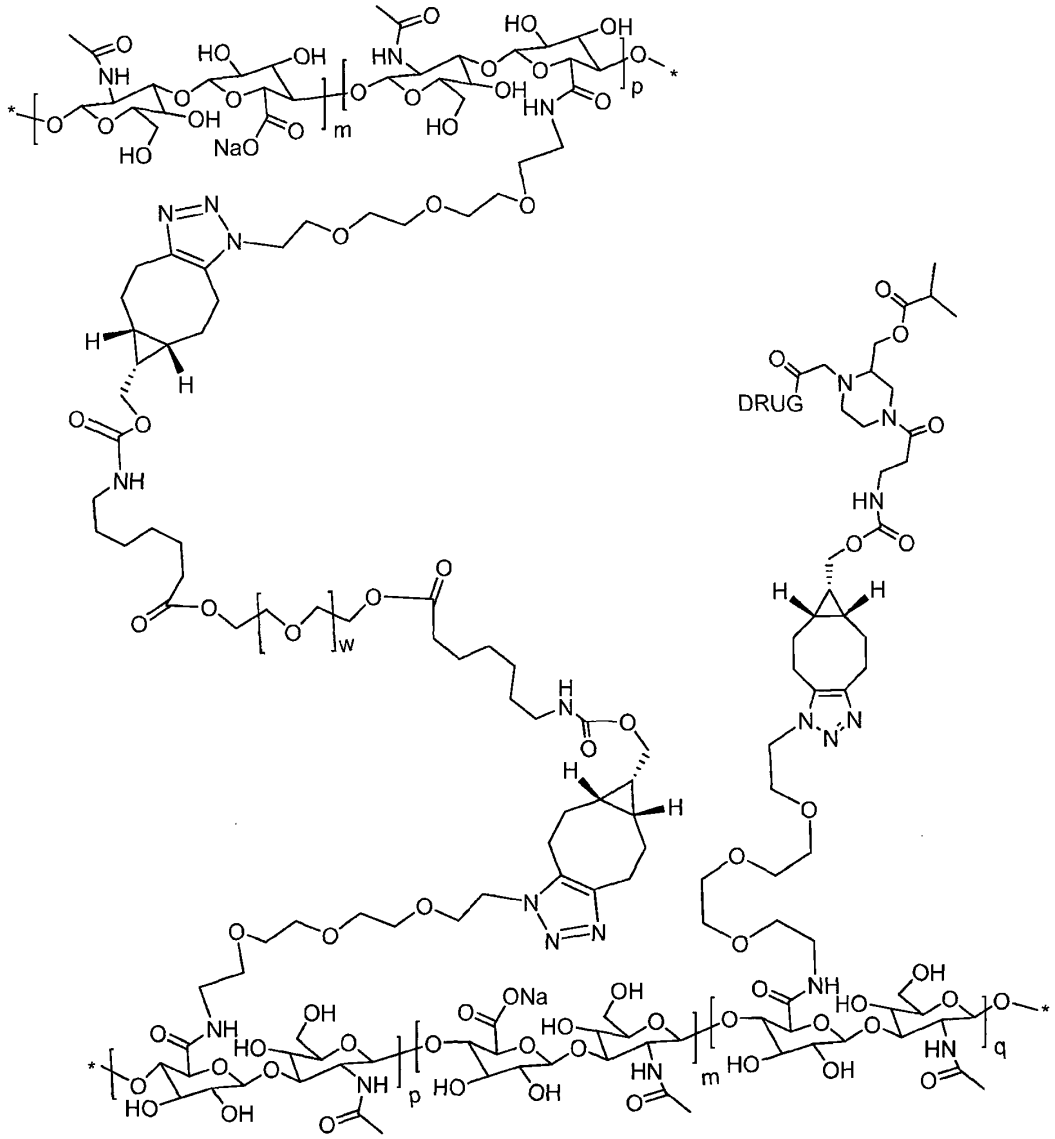
In another embodiment, the drug delivery system can comprise Formula (XVIIIb) with DRUG being an ANGPTL3 polypeptide as described herein:



(XVIIIb).

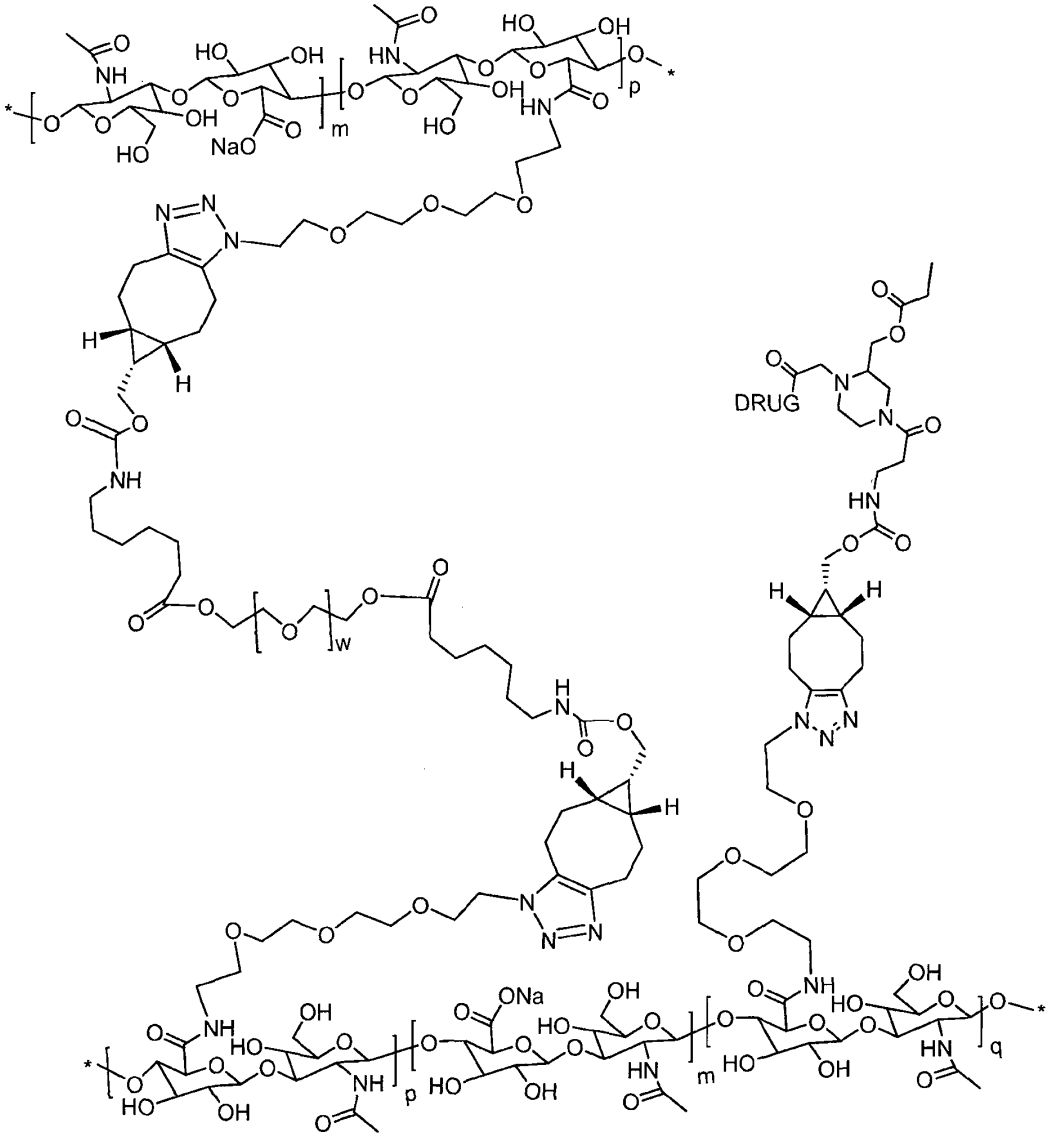
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In another embodiment, the drug delivery system can comprise Formula (XVIIIc) with DRUG being an ANGPTL3 polypeptide as described herein:



(XVIIIc).

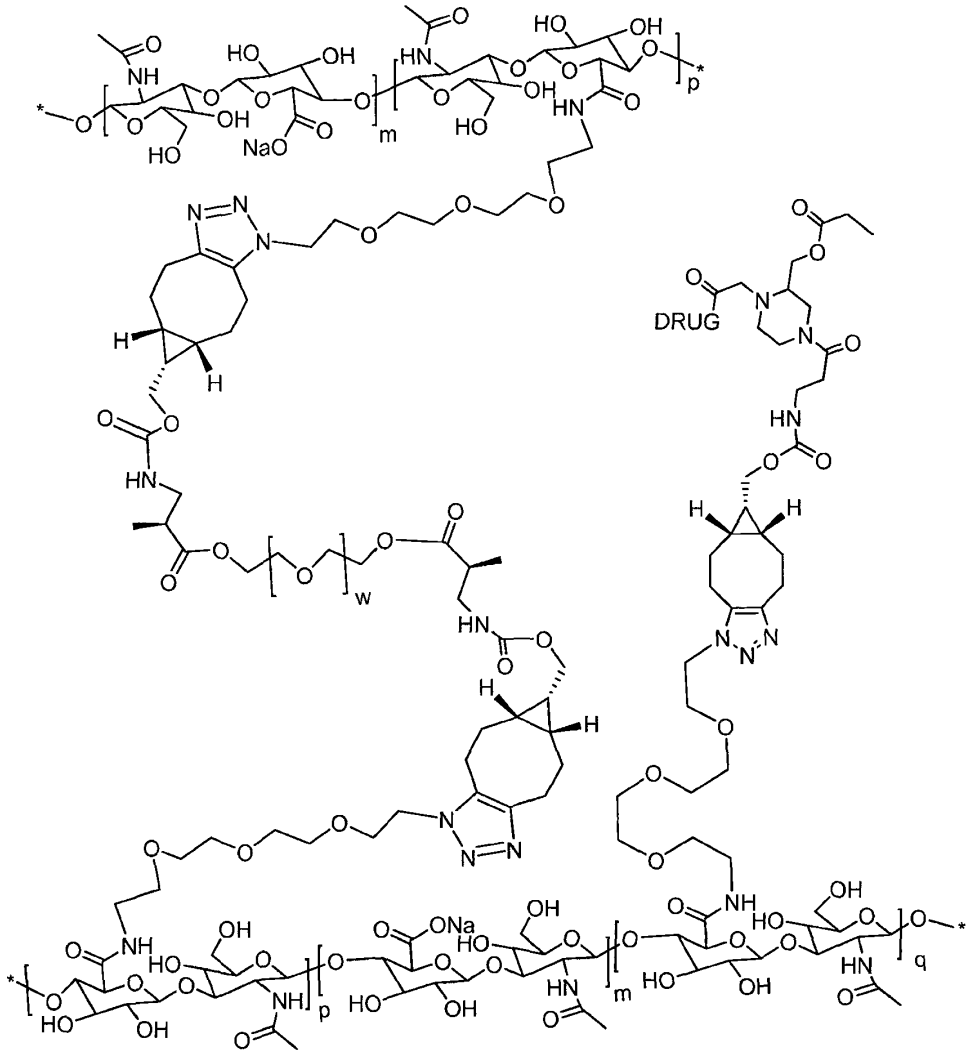
In another embodiment, the drug delivery system can comprise Formula (XVIIId) with DRUG being an ANGPTL3 polypeptide as described herein:



(XVIIId).

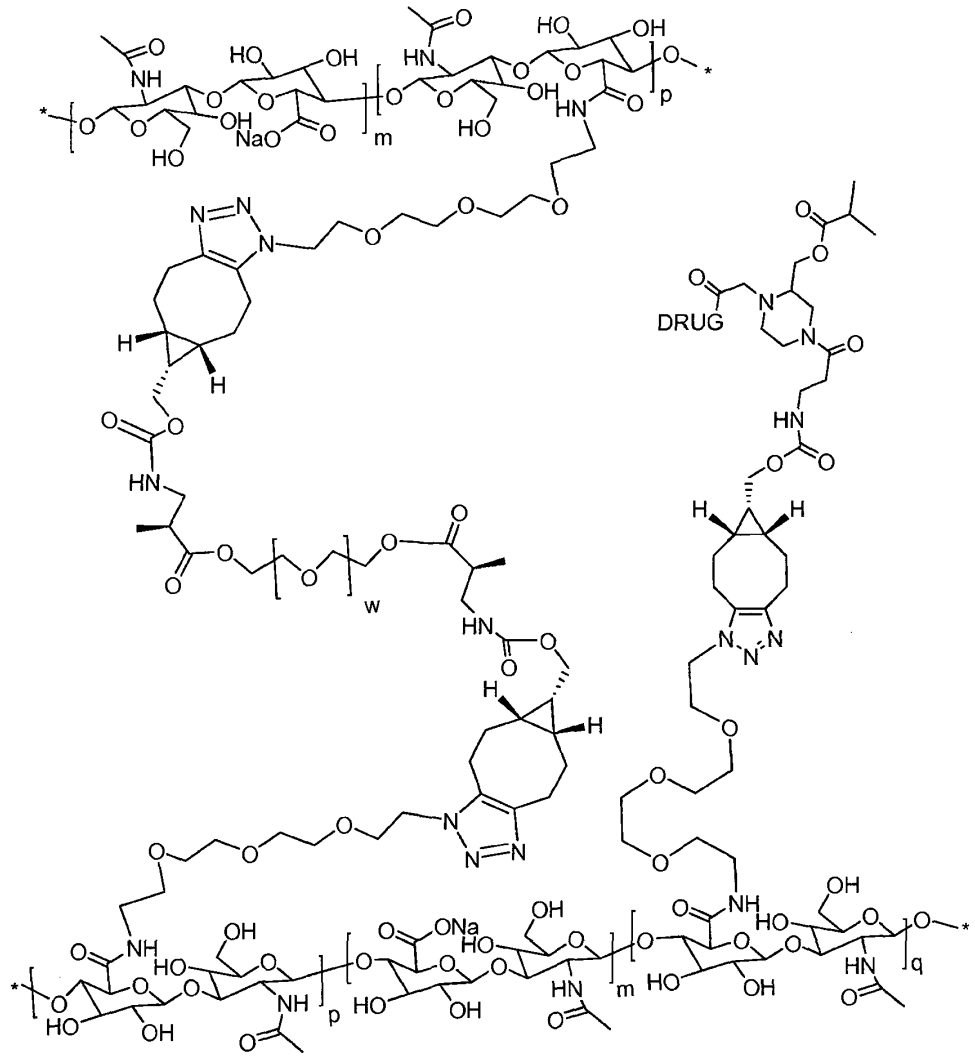
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In another embodiment, the drug delivery system can comprise Formula (XVIIIe) with DRUG being an ANGPTL3 polypeptide as described herein:



(XVIIIe).

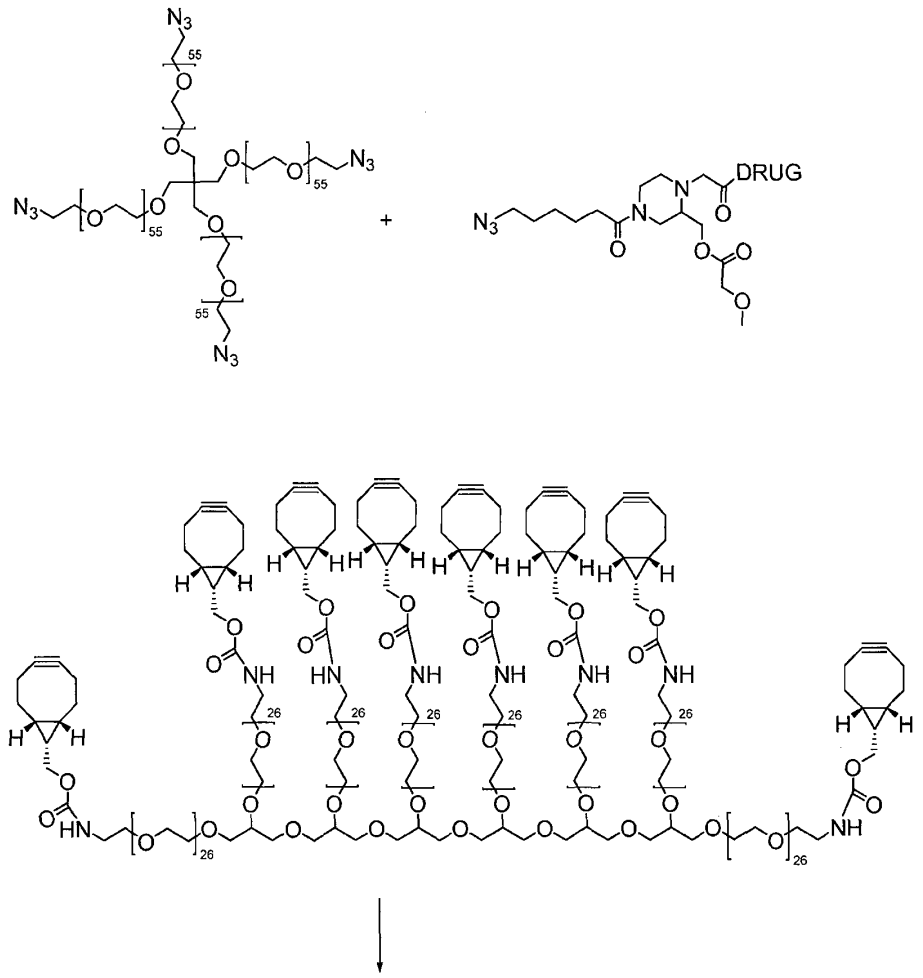
In another embodiment, the drug delivery system can comprise Formula (XVIIIf) with DRUG being an ANGPTL3 polypeptide as described herein:



(XVIIIf).

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In another embodiment, the drug delivery system can comprise Formula (XXIII) with the drug being an ANGPTL3 polypeptide as described herein:



(XXIII).

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Another embodiment described herein is a method for treating diseases or disorders using the drug delivery systems described herein. Another embodiment described herein relates to the drug delivery system or a pharmaceutically acceptable salt thereof comprising D–R represented by Formula (I) for use as a medicament. Another embodiment is the use of the drug delivery system or a pharmaceutically acceptable salt thereof comprising D–R represented by Formula (I) for the manufacture of a medicament for the treatment of joint damage or disease including arthritis, osteoarthritis, traumatic arthritis, or acute joint injury or trauma. In one aspect, pharmaceutical compositions of the drug delivery systems described herein can be administered to a subject in need thereof by injection. In one aspect, administration may be made by injection or surgical implantation.

Another embodiment described herein relates to the drug delivery system or a pharmaceutically acceptable salt thereof comprising D–R represented by Formula (I), wherein D comprises an ANGPTL3 polypeptide comprising at least 95% sequence identity to one or more of SEQ ID NOs:1, 3–45 for use in the treatment of joint damage or disease including arthritis, osteoarthritis, traumatic arthritis, acute joint injury, or trauma. In one aspect, D comprises D1 (SEQ ID NO:19). In one aspect, the composition is a solution or suspension that is injected into the joint. In another aspect, the composition is a gel or semi-solid composition that is implanted in the joint using surgical means or by large-bore injection. In another aspect, the composition is in the form of particles that are injected into a joint or in the proximity of a joint. In another aspect, the composition is implanted in or around the joint as a biodegradable mesh or gauze that is eventually absorbed or processed in situ. In another aspect, the composition is impregnated into sutures, staples, plates, meshes, or similar articles that are utilized during surgery to reattach or repair tendons, ligaments, cartilage, bone, or other joint components following trauma or disease. In another aspect, the composition is a solution or suspension that is delivered intravenously, intraarterially, subcutaneously, intramuscularly, or intraperitoneally. In another aspect, the composition is a gel or semi-solid composition that is implanted using surgical means or large-bore injection. In another aspect, the composition is a gel or semi-solid composition that is applied topically or directly to cutaneous wounds.

In one embodiment, the drug delivery system as described herein releases the biologically active agent at a particular release rate. In one aspect, the release rate can be tuned or modulated by the "trigger" component of the traceless linker, R. Without being bound by any theory, it is

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believed that reaction of the trigger under physiological conditions generates a nucleophilic functional group, for example a hydroxyl functional group that in an intramolecular fashion cleaves the amide bond linking the drug to the drug delivery system. In one embodiment, the reaction of the trigger is a hydrolysis reaction resulting in the formation of a hydroxyl functional group. Without being bound by any theory, it is believed that steric hindrance of the trigger moiety is correlated with slower reaction of the trigger and the presence and proximity of electron withdrawing groups is correlated with faster reaction of the trigger.

In one embodiment the half-life for the release of the biologically active moiety is about 0.5 hours, about 1 hour, about 2 hours, about 4 hours, about 6 hours, about 8 hours, about 10 hours, about 12 hours, about 14 hours, about 16 hours, about 18 hours, about 20 hours, about 22 hours, about 24 hours, about 1 day, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 7 days, about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, about 12 months, about 1 year, about 1.5 years, about 2 years, about 2.5 years, about 3 years, about 3.5 years, about 4, or greater than 4 years. In one aspect, the biologically active moiety release half-life is about 2.5 days, about 4.5 days, about 7 days, about 10 days, about 11 days, about 12 days, about 14 days, about 15 days, about 21 days, about 28 days, about 30 days, about 31 days, about 32 days, about 40 days, about 58 days, about 60 days, about 65 days, about 70 days, about 80 days, about 125 days, about 165 days, about 380 days, about 940 days, or even greater.

In one embodiment the half-life for the traceless linker trigger ester hydrolysis is about 0.5 hours, about 1 hour, about 2 hours, about 4 hours, about 6 hours, about 8 hours, about 10 hours, about 12 hours, about 14 hours, about 16 hours, about 18 hours, about 20 hours, about 22 hours, about 24 hours, about 1 day, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 7 days, about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, about 12 months, about 1 year, about 1.5 years, about 2 years, about 2.5 years, about 3 years, about 3.5 years, about 4, or greater than 4 years. In one aspect, the traceless linker trigger ester hydrolysis half-life is about 1 day, about 1.5 days, about 2 days, about 2.5 days, about 4 days, about 5 days, about 10 days, about 12 days, about 15 days, about 20 days, about 30 days, about 32 days, about 35 days, about 40 days,

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about 55 days, about 60 days, about 90 days, about 120 days, about 150 days, about 180 days, about 200 days, about 300 days, about 400 days, or even longer.

In another embodiment the half-life for the clearance of the drug delivery system, $R–R^{11}$, following release of the drug, D (e.g., $D–R–R^{11} \rightarrow R–R^{11} + D$), from the tissue, organ, or compartment into which the drug delivery system was dosed is about 0.5 hours, about 1 hour, about 2 hours, about 4 hours, about 6 hours, about 8 hours, about 10 hours, about 12 hours, about 14 hours, about 16 hours, about 18 hours, about 20 hours, about 22 hours, about 24 hours, about 1 day, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 7 days, about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, about 12 months, about 1 year, about 1.5 years, about 2 years, about 2.5 years, about 3 years, about 3.5 years, about 4, or greater than 4 years.

Other embodiments described herein are pharmaceutical compositions comprising the drug delivery system, $D–R–R^{11}$, as described herein. In one aspect, the pharmaceutical compositions are suitable for injection or implantation in a subject in need thereof.

Pharmaceutical compositions suitable for administration by injection or implantation include sterile aqueous solutions, suspensions, or dispersions and sterile powders or lyophilisates for the extemporaneous preparation of sterile injectable solutions or dispersion.

For intravenous administration, suitable carriers include phosphate buffered saline (PBS), physiological saline, Ringer's solution, or water for injection. In all cases, the composition should be sterile and should be fluid to the extent that easy syringability exists. Preferred pharmaceutical formulations are stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. In general, the relevant carrier can be a solvent or dispersion medium containing, for example, water, buffers, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, amino acids, sorbitol, sodium

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chloride, or combinations thereof in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, aluminum monostearate and gelatin.

Certain injectable compositions are aqueous isotonic solutions or suspensions, and suppositories are advantageously prepared from fatty emulsions or suspensions. Said compositions may be sterilized and/or contain adjuvants, such as preservatives, stabilizers, wetting agents, emulsifying agents, solution promoters, salts for regulating the osmotic pressure, buffers, or combinations thereof. In addition, they may also contain other therapeutically valuable substances. Said compositions are prepared according to conventional mixing, granulating or coating methods, respectively, and contain about 0.1–75%, or contain about 1–50%, of the active ingredient.

Sterile injectable solutions or suspensions can be prepared by incorporating the drug delivery system in the required amount in an appropriate solvent with one or a combination of ingredients, as required, followed by filtration sterilization. Generally, solutions or suspensions are prepared by incorporating the active compound into a sterile vehicle such as sterile PBS and any excipients. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred preparation methods are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Transmucosal or transdermal administration means are also possible. Suitable compositions for transdermal application include an effective amount of a biologically active agent with a suitable carrier. Carriers suitable for transdermal delivery include absorbable pharmacologically acceptable solvents to assist passage through the skin of the host. For example, transdermal devices are in the form of a bandage comprising a backing member, a reservoir containing the compound optionally with carriers, optionally a rate controlling barrier to deliver the compound of the skin of the host at a controlled and predetermined rate over a prolonged period of time, and means to secure the device to the skin.

Suitable compositions for topical application, e.g., to the skin, eyes, or joints, include aqueous solutions, suspensions, ointments, creams, gels or sprayable formulations, e.g., for delivery by aerosol or the like. Such topical delivery systems will in particular be appropriate for dermal application. They are thus particularly suited for use in topical, including cosmetic,

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formulations well known in the art. Such may contain solubilizers, stabilizers, tonicity enhancing agents, buffers, or preservatives.

As used herein, a topical application may also pertain to an inhalation or to an intranasal application. They may be conveniently delivered in the form of a dry powder (either alone, as a mixture, for example a dry blend with lactose, or a mixed component particle, for example with phospholipids) from a dry powder inhaler or an aerosol spray presentation from a pressurised container, pump, spray, atomizer or nebuliser, with or without the use of a suitable propellant.

Also described herein are pharmaceutical compositions and dosage forms comprising one or more agents that reduce the rate by which the compositions described herein as active ingredients will decompose. Such agents, which are referred to herein as "stabilizers," include, but are not limited to, antioxidants such as ascorbic acid, pH buffers, or salt buffers, etc.

The effective amount of a pharmaceutical composition to be employed therapeutically will depend, for example, upon the therapeutic context and objectives. One skilled in the art will appreciate that the appropriate dosage levels for treatment will thus vary depending, in part, upon the therapeutic agent incorporated into the drug delivery system, the indication for which the drug delivery system is being used, the route of administration, and the size (body weight, body surface, or organ size) and condition (the age and general health) of the patient. Accordingly, the clinician can titer the dosage and modify the route of administration to obtain the optimal therapeutic effect.

The frequency of dosing will depend upon the pharmacokinetic parameters of the therapeutic agent incorporated into the drug delivery system being used. Typically, a clinician will administer the composition until a dosage is reached that achieves the desired effect. The composition can therefore be administered as a single dose, as two or more doses (which may or may not contain the same amount of the desired molecule) over time, or as a continuous infusion via an implantation device or catheter. Further refinement of the appropriate dosage is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them. Appropriate dosages can be ascertained through use of appropriate dose-response data.

The drug delivery system can be prepared as solutions or suspensions of micro-particles. In one aspect, the carrier is formed into microparticulate which can be administered by a standard syringe to the target tissue, e.g., administration subcutaneously, intra-articularly, intra tendon, or intramuscularly. Such particles may have a mean particle size distribution of between 1 μm and

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5000 μm. Other aspects comprise biodegradable gauze, mesh, or sutures that have been impregnated with drug delivery systems as described herein.

In some embodiments, microparticles of the drug delivery systems described herein can be produced by emulsion polymerization, lithography, spinning, molding, spray drying, milling, extrusion, mechanical comminution, or similar procedures known in the art. In one embodiment, the drug delivery systems, carrier polymers, or hydrogels as described herein can be divided into microparticles by extrusion through mesh or screens. In one aspect, the extrusion can be repeated multiple times and/or through successively smaller meshes to achieve the desired particle distribution size.

In one embodiment, based on laser diffraction to measure particle size, the drug delivery system has a mean particle size distribution of between 1 μm and 5000 μm when suspended in an isotonic aqueous formulation buffer. In some aspects, the drug delivery system has a mean particle size distribution of between 10 μm and 1000 μm when suspended in isotonic buffer. In another aspect, the drug delivery system has a mean particle size distribution of between 50 μm and 500 μm when suspended in an isotonic aqueous buffer. In another aspect, the drug delivery system has a mean particle size distribution of between 100 μm and 300 μm when suspended in an isotonic aqueous buffer. In another aspect, the drug delivery system has a mean particle size distribution of between 200 μm and 300 μm when suspended in an isotonic aqueous buffer. In some embodiments, the mean particle size distribution comprises about 10 μm, about 50 μm, about 100 μm, about 150 μm, about 200 μm, about 250 μm, about 300 μm, about 350 μm, about 400 μm, about 450 μm, about 500 μm, about 750 μm, about 1000 μm, about 1500 μm, about 2000 μm, about 2500 μm, or about 5000 μm.

The particle sizes may be determined using standard techniques known to one of ordinary skill in the art. The exemplary techniques that can be used for measuring the particle size distributions of drug delivery system particles may include laser diffraction analysis, light scattering (e.g., dynamic light scattering), microscopic particle image analysis, elutriation, or aerosol mass spectrometry. The sample of drug delivery system particles may be measured as a dry sample or a wet sample. Any commercially available instrument for measuring particle sizes may be used, including instruments from Cilas; Brookhaven Instruments Corporation; Malvern Instruments; Horiba Scientific; or Wyatt following the recommended operating procedures according to the manufacturer's instructions.

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The measured particle sizes using the techniques described herein may be expressed as a derived diameter with a normal distribution or non-normal distribution with a mean, median (e.g., mass median diameter), and mode of particle diameter sizes. The particle size distribution may be expressed as a diameter number distribution, a surface area distribution, or a particle volume distribution. The mean of the particle size distribution may be calculated and expressed in various ways, such as the volume mean diameter ($D[4,3]$ or d_{43}), mean surface area diameter ($D[3,2]$ or d_{32}) or the mean number particle diameter ($D[1,0]$ or d_{10}). Because the particle size distribution values vary depending on the measurement methodology and how the distribution is expressed, the comparison of different mean particle size distributions must be calculated by the same methodology in order to yield an accurate comparison. For example, a sample with a measured and calculated volume mean diameter must be compared with a second sample having a measured and calculated volume mean diameter, ideally measured using the same measuring instrument under the same conditions. Thus, the specific particle size distributions described herein are not intended to be limited to any one type of method for measuring or calculating a particle size distribution (e.g., a diameter number distribution, a surface area distribution, or a particle volume distribution), but rather indicate particle size values and distributions thereof for each method of measuring particle sizes described herein.

In one embodiment drug delivery systems can be administered by injection through a needle smaller than 0.6 mm inner diameter (e.g., 20 gauge), preferably through a needle smaller than 0.3 mm inner diameter (e.g., 25 gauge), more preferably through a needle smaller than 0.25 mm inner diameter (e.g., 27 gauge), even more preferably through a needle smaller than 0.2 mm inner diameter (e.g., 28 gauge), and most preferably through a needle smaller than 0.16 mm inner diameter (e.g., 30 gauge). For example when a 100 μm to 300 μm particle size distribution of a drug delivery system is injected a 20 gauge needle may be optimal for delivery. Because the particle morphology is flexible, however, needle sizes narrower than the drug delivery system particle size may be used successfully.

The phrases and terms "can be administered by injection," "injectable," or "injectability" refer to a combination of factors such as a certain force applied to a plunger of a syringe containing the drug delivery systems described herein swollen in a liquid at a certain concentration (w/v) and at a certain temperature, a needle of a given inner diameter connected to the outlet of such syringe,

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and the time required to extrude a certain volume of the drug delivery systems from the syringe through the needle.

In one embodiment, an injectability measurement is carried out for the drug delivery system suspended in PBS or physiological saline to a concentration of about 0.1% to about 20% (w/v) including all integers within the specified percentage range.

Consequently, the drug delivery systems show the beneficial effect of a controlled release rate in respect of the released drug D–H. Preferably, a sustained release rate is obtained. Sustained release means that the administration intervals of the respective drug delivery systems described herein are expanded compared to administration of the drug in the absence of the drug delivery system. For example, drug delivery systems that are based on drugs commonly administered once or several times a day provide therapeutically effective levels for at least three days, at least one week, for at least one month, for several months, or for years.

Another embodiment described herein is a pharmaceutical composition of the drug delivery systems described herein. The pharmaceutical compositions can comprise one or more excipients, such as:

(i) Buffering agents: physiologically tolerated buffers to maintain pH in a desired range, such as sodium phosphate, bicarbonate, succinate, histidine, citrate and acetate, sulphate, nitrate, chloride, pyruvate. Antacids such as $Mg(OH)_2$ or $ZnCO_3$ may be also used. Buffering capacity may be adjusted to match the conditions most sensitive to pH stability.

(ii) Isotonicity modifiers: to minimize pain that can result from cell damage due to osmotic pressure differences at the injection depot. Glycerin and sodium chloride are examples. Effective concentrations can be determined by osmometry using an assumed osmolality of 285–315 mOsmol/kg for serum.

(iii) Preservatives and/or antimicrobials: multidose parenteral preparations may require the addition of preservatives at a sufficient concentration to minimize the risk of subjects becoming infected upon injection and corresponding regulatory requirements have been established. Typical preservatives include m-cresol, phenol, methylparaben, ethylparaben, propylparaben, butylparaben, chlorobutanol, benzyl alcohol, phenylmercuric nitrate, thimerosol, sorbic acid, potassium sorbate, benzoic acid, chlorocresol, and benzalkonium chloride.

(iv) Stabilizers: Stabilization is achieved by strengthening of the protein-stabilising forces, by destabilization of the denatured state, or by direct binding of excipients to the protein. Stabilizers

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may be amino acids such as alanine, arginine, aspartic acid, glycine, histidine, lysine, proline, sugars such as glucose, sucrose, trehalose, polyols such as glycerol, mannitol, sorbitol, salts such as potassium phosphate, sodium sulphate, chelating agents such as EDTA, hexaphosphate, ligands such as divalent metal ions (zinc, calcium, etc.), other salts or organic molecules such as phenolic derivatives. In addition, oligomers or polymers such as cyclodextrins, dextran, dendrimers, polyethylene glycol, polyvinylpyrrolidone, protamine, or human serum albumin may be used.

(v) Anti-adsorption agents: Mainly ionic or ion-ionic surfactants or other proteins or soluble polymers are used to coat or adsorb competitively to the inner surface of the composition's container, e.g., poloxamer (Pluronic F-68), PEG dodecyl ether (Brij 35), polysorbate 20 and 80, dextran, polyethylene glycol, PEG-polyhistidine, BSA and HSA and gelatines. Chosen concentration and type of excipient depends on the effect to be avoided but typically, a monolayer of surfactant is formed at the interface just above the CMC value.

(vi) Lyophilization or cryoprotectants: During freeze- or spray drying, excipients may counteract the destabilising effects caused by hydrogen bond breaking and water removal. For this purpose, sugars and polyols may be used, but corresponding positive effects have also been observed for surfactants, amino acids, non-aqueous solvents, and other peptides. Trehalose is particularly efficient at reducing moisture-induced aggregation and also improves thermal stability potentially caused by exposure of protein hydrophobic groups to water. Mannitol and sucrose may also be used, either as sole lyo/cryoprotectant or in combination with each other where higher ratios of mannitol or sucrose are known to enhance physical stability of a lyophilized cake. Mannitol may also be combined with trehalose. Trehalose may also be combined with sorbitol or sorbitol may be used as the sole protectant. Starch or starch derivatives may also be used.

(vii) Oxidation protection agents: antioxidants such as ascorbic acid, ectoine, methionine, glutathione, monothioglycerol, morin, polyethylenimine (PEI), propyl gallate, vitamin E, chelating agents such aus citric acid, EDTA, hexaphosphate, thioglycolic acid.

(viii) Viscosifiers or viscosity enhancers: retard settling of the particles in the vial and syringe and are used in order to facilitate mixing and resuspension of the particles and to make the suspension easier to inject (i.e., low force on the syringe plunger). Suitable viscosifiers or viscosity enhancers are, for example, carbomer viscosifiers like Carbopol 940, Carbopol Ultrez 10, cellulose derivatives like hydroxypropylmethylcellulose (hypromellose, HPMC) or diethylaminoethyl cellulose (DEAE or DEAE-C), colloidal magnesium silicate (Veegum) or sodium silicate,

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hydroxyapatite gel, tricalcium phosphate gel, xanthans, carrageenans like Satiagum UTC 30, aliphatic poly(hydroxy acids), such as poly(D,L- or L-lactic acid) (PLA) and poly(glycolic acid) (PGA) and their copolymers (PLGA), terpolymers of D,L-lactide, glycolide and caprolactone, poloxamers, hydrophilic poly(oxyethylene) blocks and hydrophobic poly(oxypropylene) blocks to make up a triblock of poly(oxyethylene)-poly(oxypropylene)-poly(oxyethylene) (e.g., Pluronic.™), polyetherester copolymer, such as a polyethylene glycol terephthalate/polybutylene terephthalate copolymer, sucrose acetate isobutyrate (SAIB), dextran or derivatives thereof, combinations of dextrans and PEG, polydimethylsiloxane, collagen, chitosan, polyvinyl alcohol (PVA) and derivatives, polyalkylimides, poly (acrylamide-co-diallyldimethyl ammonium (DADMA)), polyvinylpyrrolidone (PVP), glycosaminoglycans (GAGs) such as dermatan sulfate, chondroitin sulfate, keratan sulfate, heparin, heparan sulfate, hyaluronan, ABA triblock or AB block copolymers composed of hydrophobic A-blocks, such as polylactide (PLA) or poly(lactide-co-glycolide) (PLGA), and hydrophilic B-blocks, such as polyethylene glycol (PEG) or polyvinyl pyrrolidone. Such block copolymers as well as the abovementioned poloxamers may exhibit reverse thermal gelation behavior (fluid state at room temperature to facilitate administration and gel state above sol-gel transition temperature at body temperature after injection).

(ix) Diffusion agents: modifies the permeability of connective tissue through the hydrolysis of components of the extracellular matrix in the interstitial space such as, but not limited to, hyaluronic acid, a polysaccharide found in the intercellular space of connective tissue. A spreading agent such as, but not limited to, hyaluronidase temporarily decreases the viscosity of the extracellular matrix and promotes diffusion of injected drugs.

(x) Other auxiliary agents: such as wetting agents, viscosity modifiers, antibiotics, hyaluronidase. Acids and bases such as hydrochloric acid and sodium hydroxide are auxiliary agents necessary for pH adjustment during manufacture.

The drug delivery system may be provided as a liquid, a suspension, or as a dry composition.

In one embodiment, the drug delivery system is a dry composition. Suitable methods of drying are, for example, spray drying and lyophilization (freeze-drying). In one aspect, the drug delivery system is dried by lyophilization.

In one embodiment, the drug delivery system is sufficiently dosed in the composition to provide therapeutically effective amounts of biologically active agent for at least 12 hours in one

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application. In one aspect, one application of drug delivery system is sufficient for about 1 day, about 2 days, about 3 days, about 4 days, about 5 days, about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, one month, 2 months, 3 months, 4 months, 6 months, 9 months, one year, 2 years, 3 years, 4 years, or even longer.

In one embodiment, the drug delivery system is provided as a single dose, meaning that the container in which it is supplied contains one pharmaceutical dose.

In another embodiment, the composition is provided as a multiple dose composition, meaning that it contains more than one therapeutic dose. Preferably, a multiple dose composition contains at least 2 doses. Such multiple dose drug delivery systems can either be used for different subjects in need thereof or is intended for use in one subject, wherein the remaining doses are stored after the application of the first dose until needed.

In another embodiment, the drug delivery system is comprised in one or more containers. For liquid or suspension compositions, the container is preferably a single chamber syringe. For dry compositions, preferably the container is a dual-chamber syringe. The dry composition is provided in a first chamber of the dual-chamber syringe and reconstitution solution is provided in a second chamber of the dual-chamber syringe.

Prior to applying the dry drug delivery system to a subject in need thereof, the dry composition is reconstituted. Reconstitution can take place in the container in which the dry drug delivery system is provided, such as in a vial, syringe, dual-chamber syringe, ampoule, and cartridge. Reconstitution is done by adding a predefined amount of reconstitution solution to the dry composition. Reconstitution solutions are sterile liquids, such as phosphate buffered saline, isotonic saline, water for injection, or other buffers, which may contain further excipients, such as preservatives and/or antimicrobials, such as, for example, benzylalcohol and cresol. Preferably, the reconstitution solution is sterile phosphate buffered saline (PBS) or physiological saline. Alternatively, the reconstitution solution is sterile water for injection.

Another embodiment is a method of preparing a reconstituted composition comprising a therapeutically effective amount of a drug delivery system, and optionally one or more pharmaceutically acceptable excipients, the method comprising the step of contacting the composition with a volume of reconstitution vehicle. The reconstituted drug delivery system may then be administered by injection or other routes.

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Another embodiment is a reconstituted composition comprising a therapeutically effective amount of a drug delivery system, a reconstitution vehicle, and optionally one or more pharmaceutically acceptable excipients.

Another embodiment is a pre-filled syringe comprising a solution or a suspension comprising a therapeutically effective amount of a drug delivery system, and optionally one or more pharmaceutically acceptable excipients. In one aspect, the syringe is filled with between about 0.01 mL and about 5 mL of a drug delivery system as described herein. In one aspect, the syringe is filled with between about 0.05 mL and about 5 mL, between about 1 mL and about 2 mL, between about 0.1 mL and about 0.15 mL, between about 0.1 mL, about 0.5 mL, between about 0.15 mL and about 0.175 mL, or about 0.5 to about 5 mL. In one embodiment, the syringe is filled with 0.165 mL of a drug delivery system as described herein. In some aspects, a syringe is filled with about 0.01 mL, about 0.02 mL, about 0.03 mL, about 0.04 mL, about 0.05 mL, about 0.06 mL, about 0.07 mL, about 0.08 mL, about 0.09 mL, about 0.1 mL, about 0.2 mL, about 0.3 mL, about 0.4 mL, about 0.5 mL, about 0.6 mL, about 0.7 mL, about 0.8 mL, about 0.9 mL, about 1 mL, about 1.2 mL, about 1.5 mL, about 1.75 mL, about 2 mL, about 2.5 mL, about 3 mL, about 4 mL, or about 5 mL of a drug delivery system as described herein. A syringe is often filled with more than the desired dose to be administered to the patient, to take into account wastage due to "dead space" within the syringe and needle. There may also be a pre-determined amount of waste when the syringe is primed by the physician, so that it is ready to inject the patient.

In one embodiment, a syringe is filled with a dosage volume (i.e., the volume of medicament intended for delivery to the patient) of between about 0.01 mL and about 5 mL depending on the route of injection (e.g., between about 0.01 mL and about 0.1 mL, between about 0.1 mL and about 0.5 mL, between about 0.2 mL and about 2 mL, between about 0.5 mL and about 5 mL, or between about 1 mL and about 5 mL) of a drug delivery system as described herein. In one embodiment intended for intra-articular injection, a syringe is filled with a dosage volume of between about 0.05 mL and about 5.0 mL of a drug delivery system solution or suspension with a drug concentration of 1 mg/mL to 40 mg/mL as described herein. In one embodiment intended for subcutaneous injection, a syringe is filled with a dosage volume of between about 0.1 mL and about 5.0 mL of a drug delivery system solution or suspension with a drug concentration of 0.1 mg/mL to 40 mg/mL as described herein. In other embodiments intended for injection by other routes, a syringe is filled with a dosage volume of between about 0.01 mL and about 5.0 mL of a

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drug delivery system solution or suspension with a drug concentration of 0.1 mg/mL to 40 mg/mL as described herein. In some aspects, a syringe is filled with about 0.01 mL, about 0.02 mL, about 0.03 mL, about 0.04 mL, about 0.05 mL, about 0.06 mL, about 0.07 mL, about 0.08 mL, about 0.09 mL, about 0.1 mL, about 0.2 mL, about 0.3 mL, about 0.4 mL, about 0.5 mL, about 0.6 mL, about 0.7 mL, about 0.8 mL, about 0.9 mL, about 1 mL, about 1.2 mL, about 1.5 mL, about 1.75 mL, about 2 mL, about 2.5 mL, about 3 mL, about 4 mL, or about 5 mL of a drug delivery system as described herein for delivery to a patient in need thereof.

As the syringe contains a medicament solution, the outlet may be reversibly sealed to maintain sterility of the medicament. This sealing may be achieved by a sealing device as is known in the art, such as a luer lock or a tamper resistant seal.

Another embodiment is a kit comprising one or more pre-filled syringes comprising a solution or suspension of one or more drug delivery systems as described herein. In one embodiment, such a kit comprises a pre-filled syringe comprising drug delivery systems as described herein in a blister pack or a sealed sleeve. The blister pack or sleeve may be sterile on the inside. In one aspect, pre-filled syringes as described herein may be placed inside such blister packs or sleeves prior to undergoing sterilization, for example terminal sterilization.

Such a kit may further comprise one or more needles for administration of drug delivery systems as described herein. Such kits may further comprise instructions for use, a drug label, contraindications, warnings, or other relevant information. One embodiment described herein is a carton or package comprising one or more pre-filled syringes comprising one or more drug delivery systems as described herein contained within a blister pack, a needle, and optionally instructions for administration, a drug label, contraindications, warnings, or other relevant information.

A terminal sterilization process may be used to sterilize the syringe and such a process may use a known process such as an ethylene oxide or a hydrogen peroxide (H_2O_2) sterilization process. Needles to be used with the syringe may be sterilised by the same method, as may kits described herein. In one aspect, a package is exposed to the sterilising gas until the outside of the syringe is sterile. Following such a process, the outer surface of the syringe may remain sterile (whilst in its blister pack) for up to 6 months, 9 months, 12 months, 15 months, 18 months, 24 months or longer. Thus, in one embodiment, a pre-filed syringe as described herein (in its blister pack) may have a shelf life of up to 6 months, 9 months, 12 months, 15 months, 18 months, 24 months, or even

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longer. In one embodiment, less than one syringe in a million has detectable microbial presence on the outside of the syringe after 18 months of storage. In one aspect, the pre-filled syringe has been sterilised using ethylene oxide with a Sterility Assurance Level of at least 10^{-6}. In another aspect, the pre-filled syringe has been sterilised using hydrogen peroxide with a Sterility Assurance Level of at least 10^{-6}. Significant amounts of the sterilising gas should not enter the variable volume chamber of the syringe. The term "significant amounts" As used herein, refers to an amount of gas that would cause unacceptable modification of the drug delivery system solution or suspension within the variable volume chamber. In one embodiment, the sterilization process causes ≤10% (preferably ≤5%, ≤3%, ≤1%) alkylation of the drug delivery system. In one embodiment, the pre-filled syringe has been sterilised using ethylene oxide, but the outer surface of the syringe has ≤1 ppm, preferably ≤0.2 ppm ethylene oxide residue. In one embodiment, the pre-filled syringe has been sterilised using hydrogen peroxide, but the outer surface of the syringe has ≤1 ppm, preferably ≤0.2 ppm hydrogen peroxide residue. In another embodiment, the pre-filled syringe has been sterilised using ethylene oxide, and the total ethylene oxide residue found on the outside of the syringe and inside of the blister pack is ≤0.1 mg. In another embodiment, the pre-filled syringe has been sterilised using hydrogen peroxide, and the total hydrogen peroxide residue found on the outside of the syringe and inside of the blister pack is ≤0.1 mg.

Another aspect is a kit of parts. For liquid and suspension compositions, and when the administration device is simply a hypodermic syringe, the kit may comprise the syringe, a needle and a container comprising the drug delivery system composition for use with the syringe. In case of a dry composition, the container may have one chamber containing the dry drug delivery system composition, and a second chamber comprising a reconstitution solution. In one embodiment, the injection device is a hypodermic syringe adapted so the separate container with drug delivery system composition can engage with the injection device such that in use the liquid or suspension or reconstituted dry composition in the container is in fluid connection with the outlet of the injection device. Examples of administration devices include but are not limited to hypodermic syringes and pen injector devices. Particularly preferred injection devices are the pen injectors, in which case the container is a cartridge, preferably a disposable cartridge.

Another embodiment comprises a kit comprising a needle and a container containing the drug delivery system composition and optionally further containing a reconstitution solution, the

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container being adapted for use with the needle. In one aspect, the container is a pre-filled syringe. In another aspect, the container is dual chambered syringe.

Another embodiment is a cartridge containing a composition of drug delivery system as hereinbefore described for use with a pen injector device. The cartridge may contain a single dose or plurality of doses of drug delivery system.

In another embodiment the drug delivery system solution or suspension comprises a drug delivery system and one or more excipients, and also other biologically active agents, either in their free form or as drugs or combined with other drug delivery systems such as pegylated drugs or hydrogel linked drugs. In one aspect, such additional one or more biologically active agents is a free form drug or a second drug delivery system.

In another embodiment, one or more drug delivery systems are simultaneously administered, with each drug delivery system having either separate or related biological activities.

In an alternative embodiment, the drug delivery system is combined with a second biologically active compound in such way that the drug delivery system is administered to a subject in need thereof first, followed by the administration of the second compound. Alternatively, the drug delivery system composition is administered to a subject in need thereof after another compound has been administered to the same subject.

Another embodiment is a drug delivery system or a pharmaceutically acceptable salt thereof comprising D–R represented by Formula (I) for use as a medicament.

Another embodiment is a drug delivery system or pharmaceutically acceptable salt thereof comprising D–R represented by Formula (I) for use in the treatment of a musculoskeletal disorder.

Another embodiment is the use of drug delivery system or a pharmaceutically acceptable salt thereof comprising D–R represented by Formula (I) for the manufacture of a medicament for the treatment of a musculoskeletal disorders.

Another embodiment is a drug delivery system or a pharmaceutical composition for use in a method of treating or preventing diseases or disorders which can be treated by the biologically active moiety released from the drug delivery system.

Another embodiment is a method of manufacturing a solution or suspension composition of drug delivery system. In one embodiment, such composition is made by:

(i) admixing the drug delivery system with one or more excipients;

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(ii) transferring amounts of the liquid or suspension composition equivalent to single or multiple doses into suitable containers; and

(iii) sealing the containers.

Another embodiment is the method of manufacturing a dry composition of a drug delivery system. In one embodiment, such composition is made by:

(i) admixing the drug delivery system with one or more excipients;

(ii) transferring amounts equivalent to single or multiple doses into suitable containers;

(iii) drying the composition in said containers; and

(iv) sealing the containers.

Suitable containers are vials, syringes, dual-chamber syringes, ampoules, and cartridges.

Another embodiment is a method for the synthesis of a drug delivery system or a pharmaceutically acceptable salt thereof as defined above. Drug delivery systems or precursors of drug delivery systems may be prepared by known methods or in accordance with the reaction sequences as described below. The starting materials used in the preparation (synthesis) of drug delivery systems or precursors thereof are known or commercially available, or can be prepared by known methods or as described below.

It will be readily apparent to one of ordinary skill in the relevant arts that suitable modifications and adaptations to the compositions, methods, and applications described herein can be made without departing from the scope of any embodiments or aspects thereof. The compositions and methods provided are exemplary and are not intended to limit the scope of any of the specified embodiments. All of the various embodiments, aspects, and options disclosed herein can be combined in any and all variations or iterations. The scope of the compositions, formulations, methods, and processes described herein include all actual or potential combinations of embodiments, aspects, options, examples, and preferences herein described.

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EXAMPLES

Abbreviations

Ac	Acetyl
ACN	Acetonitrile
AcOH	acetic acid
Ac_2O	acetic anhydride
aq.	Aqueous
atm	Atmosphere
Boc, BOC	*tertiary* butyl carboxy
Boc-anhydride	di-*tert*-butyl dicarbonate
$(Boc)_2O$, $(BOC)_2O$	di-*tert*-butyl dicarbonate
br.	Broad
BSA	bovine serum albumin
BuOH	Butanol
CAD	charged aerosol detector
calcd.	Calculated
Cat, cat	Catalytic
CBZ, Cbz	Carbobenzyloxy
$Cu(OTf)_2$	copper(II) trifluoromethane sulfonate
d	Doublet
dd	doublet of doublets
DCM	Dichloromethane
DIAD	diisopropyl azodicarboxylate
DIPEA, DIEA	*N,N*-diisopropylethylamine
DMAP	4,4-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	Dimethylsulfoxide
DOSY-NMR	One dimensional diffusion ordered NMR
DSC	*N,N'*-disuccinimidyl carbonate
ECL	Electrochemiluminescence
Elem. Anal.	Elemental analysis
ELSD	evaporative light scattering detector
EDC·HCl	*N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride
ESI	electrospray ionization
EtOAc, AcOEt	ethyl acetate
Et	Ethyl
EtOH	Ethanol
FCC	flash column chromatography
FITC	fluorescein isothiocyanate

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g	Grams
G	Gauge
h	hour(s)
HATU	2-(1*H*-7-azabenzotriazol-1-yl)--1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium
HC	HPLC condition
HOBt	1-Hydroxybenzotriazole hydrate
HPLC	high performance liquid chromatography
IPA	2-propanol
IR	infrared spectroscopy
i or iso	Iso
K_2CO_3	potassium carbonate
kD, kDa	Kilodalton
L	liter(s)
LCMS	liquid chromatography-mass spectrometry
M	Molar
MHz	mega Hertz
m	Multiplet
Me	Methyl
MeCN	Acetonitrile
MeOH	Methanol
MES	2-(N-morpholino)ethanesulfonic acid
mg	milligram(s)
µg	Microgram
mM	Millimolar
mm	millimeter(s)
min	Minutes
mL	milliliter(s)
mmol	Millimoles
µL	Microliter
µmol	Micromoles
MOPS	3-(*N*-morpholino)propanesulfonic acid
MS	mass spectrometry
MsCl	methanesulfonyl chloride
MsOH	methanesulfonic acid
MWCO	molecular weight cut off
m/z	mass to charge ratio
N	normal
NA	not available
$NaBH_4$	sodium borohydride

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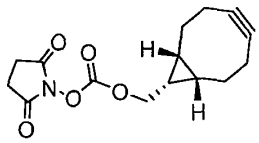
$NaBH_3CN$	sodium cyanoborohydride
$Na(AcO)_3BH$	sodium triacetoxyborohydride
ng	nanogram
NH_4Cl	ammonium chloride
NHS	N-hydroxysuccinimide
nM	nanomolar
NMR	nuclear magnetic resonance
OMe	methoxy
PBS	phosphate buffered saline
1× PBS	phosphate buffered saline, typically about 10 mM PO_4^{3-}
Pd/C	palladium on carbon
$Pd(dppf)Cl_2$	1,1′-bis(diphenylphosphino)ferrocene-palladium(II)dichloride
$Pd(dppf)Cl_2•CH_2Cl_2$ adduct	1,1′-bis(diphenylphosphino)ferrocene-palladium(II)dichloride dichloromethane complex
$Pd(PPh_3)_4$	tetrakis(triphenylphosphine)palladium(0)
Ph	phenyl
ppm	parts per million
psi	pounds per square inch
rac	racemic
rcf	relative centrifugal force
RP	reverse phase
rt, RT	room temperature
s	singlet
sat.	saturated
SDS-page	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SFC	Supercritical Fluid Chromatography
t	triplet
t-Bu, tBu	tertiary-butyl
$t_{½}$	half life
t_r or ret. time	retention time
TBAF	tetra-*n*-butylammonium fluoride
TBSCl, TBDMSCl	*tert*-butyldimethylsilyl chloride
TEA, Et_3N, NEt3	triethylamine
tert-	tertiary
TFA	trifluoroacetic acid
Tf_2O	trifluoromethanesulfonic anhydride
THF	tetrahydrofuran
TLC	Thin Layer Chromatography
TMS	trimethylsilyl

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TMSOTf	trimethylsilyl trifluoromethanesulfonate
Tris	tris(hydroxymethyl)aminomethane
Triton X-100	t-octylphenoxypolyethoxyethanol (CAS 9002-93-1)
Ts	*p*-toluenesulfonyl
TsOH	*p*-toluenesulfonic acid
Tween 20	polysorbate 20, Polyoxyethylene (20) sorbitan monolaurate
UPLC	ultra performance liquid chromatography
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

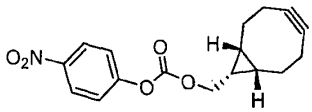
Methods

Synthesis of



((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2,5-dioxopyrrolidin-1-yl) carbonate used in some following examples was described by A. M. Jawalekar, et al; *Molecules*, **2013**, *18*, 7346-7363.

Synthesis of



((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (4-nitrophenyl) carbonate used in some following examples was described by J. Dommerholt, et al; *Angew. Chem. Int. Ed.* **2010**, *49*, 9422-9425.

All other intermediates and reagents not specifically described as synthesized herein are commercially available and were used as delivered.

LCMS Methods

Method 1

Column SunFire C18 3.5µm 3.0 × 30 mm; Column Temperature 40 °C; Flow 2.0 mL/min; Stop Time 2.20 min; pH 2.2; Eluent A1 0.05% TFA in Water; Eluent B1 Acetonitrile; Gradient Time (min)/ %A (Eluent A1): %B (Eluent B1); 0.00/ 95: 5; 1.70/ 5: 95; 2.00 /5 :95; 2.10 /95: 5.

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Method 2

Column AcQuity UPLC BEH C18 1.7 μm 2.1 × 30 mm; Column Temperature 50 °C; Flow 1.0 mL/min; Stop Time 2.00 min; pH 2.6; Eluent A1 0.1% Formic Acid in Water; Eluent B1 0.1% Formic Acid in Acetonitrile; Gradient Time (min) /%A (Eluent A1): %B (Eluent B1) 0.00/ 98: 2; 0.10/ 98:2; 1.50/ 2: 98; 1.80/ 2: 98; 1.90/ 98: 2; 2.00 /98:2.

Method 3

Column: Kinetex C18 100A (2.6 μm 100 × 4.6 mm); mobile phase A (0.1%formic acid in water), B (acetonitrile); gradient (time (min)/%B): 0/5, 1/30, 3/95, 4/95, 4.1/5, 6/5.

Method 4

Column: AcQuity UPLC BEH C18 1.7 μm 2.1 × 30 mm; column temperature 50 °C; Flow 1.0 mL/min; Stop time: 2.00 min; pH 2.6; Eluent A1 0.1% formic acid in Water; Eluent B1 0.1% formic acid in Acetonitrile; Gradient Time (min)/ %A (Eluent A1): %B (Eluent B1); 0.00/ 98: 2; 0.10/ 98:2; 1.50/ 2: 98; 1.80/ 2: 98; 1.90/ 98: 2; 2.00 /98 :2.

Method 5

Column: SunFire C18 3.5 μm 3.0 × 30 mm; Column Temperature 40 °C; Flow 2.0 mL/min; Stop Time 2.20 min; pH 2.2; Eluent A1 5 mM Ammonium Hydroxide in Water; Eluent B1 Acetonitrile; Gradient Time (min)/ %A (Eluent A1) : %B (Eluent B1); 0.00/ 95 :5; 1.70 /5: 95; 2.00/ 5: 95; 2.10/ 95: 5.

Method 6

Column: Kinetex C18 100A, (2.6 μm 100 × 4.60 mm); Gradient / (Time (min)/%B) 0/5, 1/30, 3/95, 4/95, 4.1/5, 6/5; mobile phase: 0.1% Formic acid in Water (A)/ acetonitrile (B); Flow: 1.4 mL/min; Column Temperature: 40 °C.

Method 7

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Column: Synergi MAX-RP 100A Mercury (2.5 μm 100 × 4.6 mm); mobile phase A (0.1% formic acid in water), B (acetonitrile); gradient (time (min)/%B): 0/30, 0.5/30, 1.5/95, 2.4/95, 2.5/30, 3.0/30.

Method 8

Column: Kinetex C18 100A (2.6 μm 100 × 4.6 mm); mobile phase A (0.1% formic acid in water), B (acetonitrile); gradient (time (min)/%B): 0/50, 1/70, 2/100, 4/100, 4.1/50, 6/50. The ESI-MS data was recorded on an Acquity G2 Xevo-QTOF-MS. The positive ion mass spectrum was deconvoluted using MaxEnt 1 program in the MassLynx software package.

Method 9

Column: Acclaim PepMap C4, 5 μm, 300 Å, (300 μm × 15 cm); mobile phase A (0.1% formic acid in water/acetonitrile (95/5%)), B (0.1% formic acid in acetonitrile/isopropanol/water (47.5/47.5/5%); gradient (time (min)/%B): 0/5, 2/5, 10/40, 11/95, 13/95, 14/5, 20/5. Samples were diluted 1/100 in MeOH/H_2O (50/50%) and ESI-MS data was recorded on a Lumos Orbitrap MS system coupled to an Ultimate 3000 CapLC. The positive ion mass spectrum was deconvoluted using BioPharma Finder 3.0 (Thermo Fisher Scientific) and the mass was reported.

Method 10

Column AcQuity UPLC HSS T3 C18 (1.8 μm 2.1 × 50 mm); Column Temperature 60 °C; Flow 1.0 mL/min; Stop Time 2.00 min; Eluent A1 0.05% Formic Acid + 3.75 mM Ammonium acetate in Water; Eluent B1 0.04% Formic Acid in Acetonitrile; Gradient Time (min) /%A (Eluent A1): %B (Eluent B1) 0.00/ 95: 5; 1.40/ 2:98; 1.80/ 2: 98; 1.90/ 95:5; 2.00/ 95:5. The UPLC instrument was coupled to a single-stage quadrupole mass spectrometry using electrospray ionization (ESI) in both positive and negative modes. The deconvoluted *m/z* for $(M+H)^+$ was reported.

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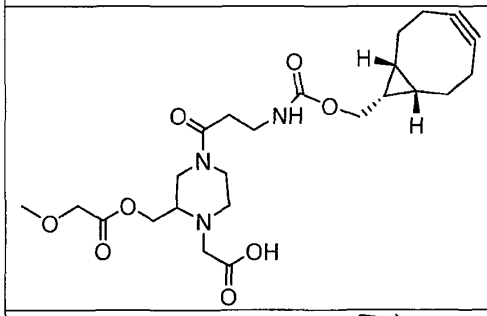
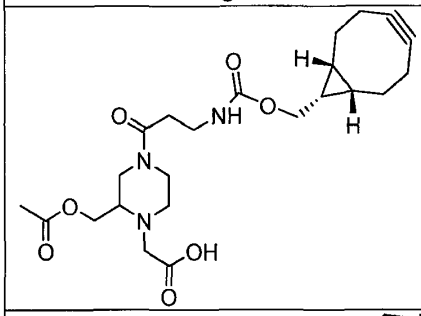
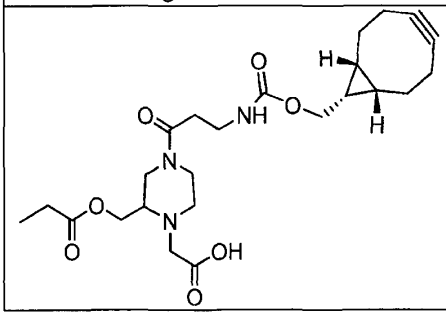
Example 1

Traceless linkers

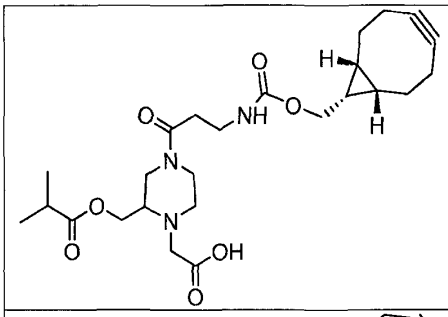
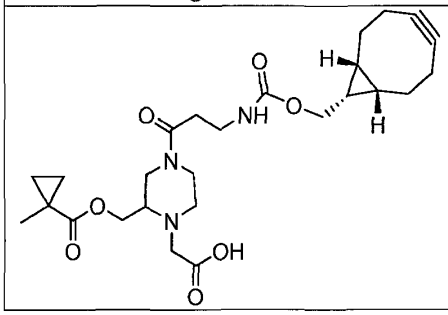
This example describes the synthesis of a number of traceless linkers, capable of being conjugated to both an amine-containing drug and to a carrier.

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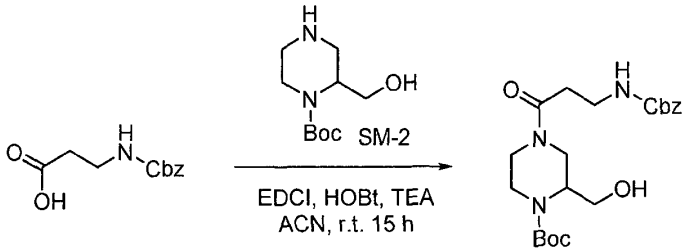
Table 6. Traceless Linkers L1–L5

Structure	Number
	L1
	L2
	L3

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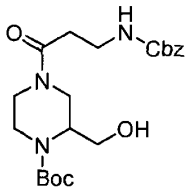
	L4
	L5

Synthesis of Traceless Linkers
Common Intermediate: L-INT-1c



SM-1 L-INT-1c

L-INT-1c. 1-(Boc)-2-(hydroxymethyl)-4-(N-Cbz-beta-alanyl)piperazine

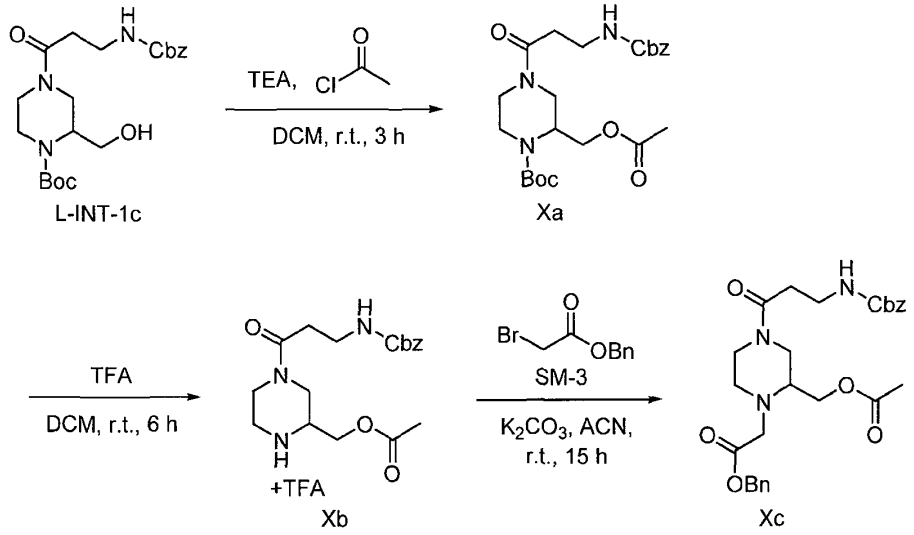


In a 250-mL flask were charged tert-butyl 2-(hydroxymethyl)piperazine-1-carboxylate **SM-2** (5.81 g, 26.9 mmol), 3-(((benzyloxy)carbonyl)amino)propanoic acid **SM-1** (5 g, 22.4 mmol) and acetonitrile (100 mL). To this suspension was successively added triethylamine (9.37 mL,

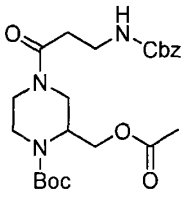
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67.2 mmol), HOBt (0.686 g, 4.48 mmol) and EDC.HCl (6.44 g, 33.6 mmol). The reaction mixture was stirred at room temperature for 15 hr. After this time, the mixture was diluted with water and extracted three times with ethyl acetate. The combined organic layer was successively washed with a solution of 1M HCl, a saturated solution of $NaHCO_3$ and finally with brine. The organic layer was dried over anhydrous magnesium sulfate, filtered and concentrated on vacuo using a rotoevaporator to afford L-INT-1c (8.75 g, 83% yield) as a colorless oil. The material was used in the next step without further purification. MS (ESI+; method 10) *m/z* 422.5 (M+H). ^{1}H NMR (500 MHz, MeOH-*d4*) δ 7.49-7.27 (m, 5H), 5.15-4.98 (m, 2H), 4.56-4.27 (m, 1H), 4.19-3.83 (m, 3H), 3.61-3.37 (m, 4H), 3.29-2.55 (m, 5H), 1.49 (s, 9H).

Linker intermediate Xc

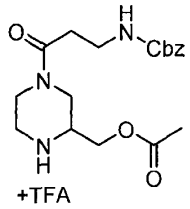


Xa. 1-(Boc)-2-(acetoxymethyl)-4-(N-Cbz-beta-alanyl)piperazine



In a 500 mL flask was prepared a solution of **L-INT-1c** (8.6 g, 20.4 mmol) in dichloromethane (160 mL). The solution was cooled down to 0 °C using an ice bath, then triethylamine (7.11 mL, 51.0 mmol) was added followed by acetyl chloride (1.74 mL, 24.48 mmol). The reaction mixture was stirred at room temperature for 4 hr. After this time, the mixture was quenched with water and extracted with dichloromethane. The combined organic layer was successively washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated on vacuo using a rotoevaporator to afford **Xa** (10.6 g, 95% yield) as a yellow oil. The material was used in the next step without further purification. MS (ESI+; method 10) *m/z* 464.3 (M+H). ^{1}H NMR (500 MHz, MeOH-*d4*) δ 7.48-7.27 (m, 5H), 5.18-5.01 (m, 2H), 4.54-3.84 (m, 6H), 3.47-3.36 (m, 2H), 3.19-2.51 (m, 5H), 2.33-2.01 (m, 3H), 1.49 (s, 9H). Exchangeable protons are not visible in MeOD.

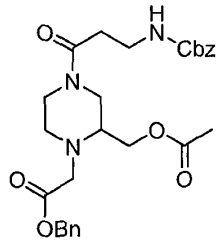
Xb. 1-(N-Cbz-beta-alanyl)-3-(acetoxymethyl)piperazine, trifluoroacetic acid salt



To a 0 °C solution of **Xa** (10.6 g, 19.44 mmol) in dichloromethane (160 mL) was slowly added trifluoroacetic acid (22.46 mL, 292 mmol). The ice bath was removed and the solution was stirred at room temperature for 6 hr. After this time, the mixture was concentrated to dryness to provide the trifluoroacetic acid salt **Xb**, which was used without further purification. MS (ESI+) *m/z* 364.3 (M+H). ^{1}H NMR (500 MHz, MeOH-*d4*) δ 7.52-7.22 (m, 5H), 5.09 (s, 2H), 4.62-4.04 (m, 4H), 3.71-3.38 (m, 5H), 3.17-2.59 (m, 4H), 2.27-2.10 (m, 3H). Exchangeable protons are not visible in MeOD.

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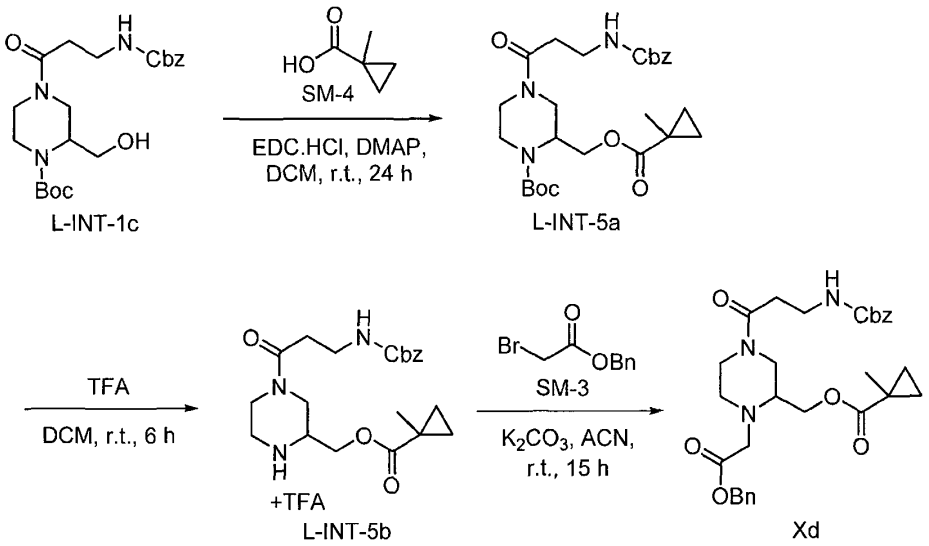
Xc. 2-(acetoxymethyl)-4-(N-Cbz-beta-alanyl)-1-piperazineacetic acid benzyl ester



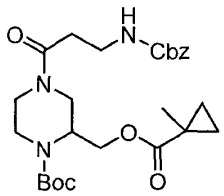
In a 250 mL flask were charged **Xb** (16 g, 18.1 mmol), potassium carbonate (7.50 g, 54.3 mmol) and acetonitrile (160 mL). Then, benzyl 2-bromoacetate **SM-3** (4.26 mL, 27.1 mmol) was added and the reaction mixture was stirred at room temperature for 15 hr. After this time, the reaction mixture was quenched with water and extracted with ethyl acetate. The combined organic layer was successively washed with brine, dried over anhydrous magnesium sulfate, filtered and concentrated on vacuo using a rotoevaporator. The crude mixture was purified by flash chromatography (Biotage isolera, RediSep column: silica 220 g, Eluent A: dichloromethane, Eluent B: dichloromethane + MeOH (9:1), gradient from 0 to 100 % B in 20 min) to afford a mixture, which was not pure enough. A second purification was performed using flash chromatography (Biotage isolera, RediSep column: silica SI KP-NH 44 g, Eluent A: cyclohexane, Eluent B: ethyl acetate, gradient from 0 to 100 %B in 20 min) to obtain **Xc** (6.52 g, 63% yield) as a colorless oil. MS (ESI+) *m/z* 512.3 (M+H). ^{1}H NMR (500 MHz, MeOH-*d4*) δ 7.49-7.15 (m, 10H), 5.17 (s, 2H), 5.08 (s, 2H), 4.19-3.96 (m, 3H), 3.82-3.54 (m, 3H), 3.40 (m, 2H), 3.27-3.08 (m, 2H), 3.06-2.94 (m, 1H), 2.88-2.73 (m, 2H), 2.69-2.52 (m, 2H), 1.99 (d, *J* = 5.1 Hz, 3H). Exchangeable protons are not visible in MeOD.

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Xd. 2-(1'-methylcyclopropylcarbonyloxymethyl)-4-(N-Cbz-beta-alanyl)-1-piperazineacetic acid benzyl ester



L-INT-5a. 1-(Boc)-2-(1'-methylcyclopropylcarbonyloxymethyl)-4-(N-Cbz-beta-alanyl)piperazine

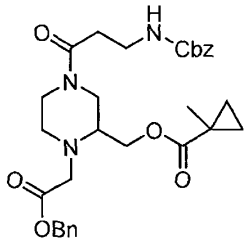


To a solution of 1-methylcyclopropane carboxylic acid **SM-4** (47.5 mg, 0.475 mmol) in dichloromethane (2 mL) was added EDC·HCl (91 mg, 0.475 mmol) and DMAP (58.0 mg, 0.475 mmol). The reaction was stirred for 30 min at room temperature and **L-INT-1c** (100 mg, 0.237 mmol) was added. The reaction mixture was stirred at room temperature for 15 hr. After this time, the reaction mixture was quenched with a saturated solution of ammonium chloride and extracted with ethyl acetate. The combined organic layer was successively dried over anhydrous magnesium sulfate, filtered, and concentrated to dryness using a rotavap. The crude residue was purified by flash chromatography (Teledyne Isco, column: RediSep 4 g. Eluent A: dichloromethane, Eluent B: dichloromethane/MeOH 8:2, gradient from 0 to 100%B in 20 min) to

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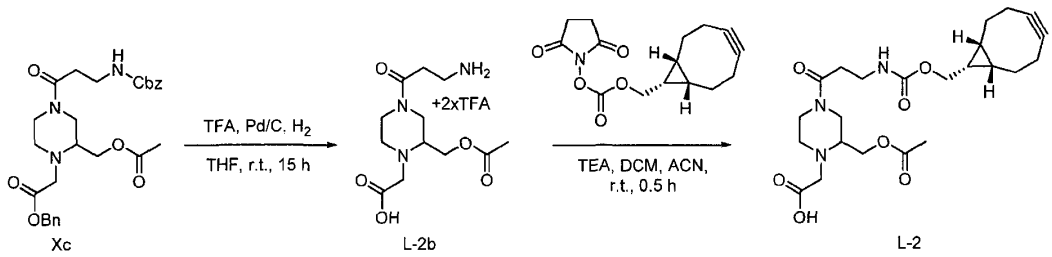
afford the desired compound (97 mg, 77% yield) as a light yellow oil. MS (ESI+; method 10) *m/z* 504.2 (M+H).

Xd. 2-(1'-methylcyclopropylcarbonyloxymethyl)-4-(N-Cbz-beta-alanyl)-1-piperazineacetic acid benzyl ester

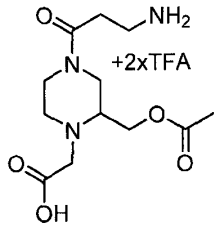


Xd was prepared in two steps from L-INT-5a following the general methods described for the synthesis of **Xb** and **Xc**. **L-INT-5**: UPLC-MS (method 10): retention time = 1.18 min; MS (ESI+) *m/z* 552.4 (M+H). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 7.40-7.30 (m, 10H), 7.19-7.17 (m, 1H), 5.14-5.06 (m, 2H), 5.05-5.00 (m, 2H), 4.07-3.97 (m, 2H), 3.91-3.79 (m, 1H), 3.67-3.56 (m, 3H), 3.23-3.18 (m, 2H), 3.10-2.88 (m, 3H), 2.78-2.57 (m, 3H), 2.45-2.39 (m, 2H), 1.20 (m, 3H), 1.08-1.07 (m, 2H), 0.72-0.71 (m, 2H).

Traceless linkers; L1-L7

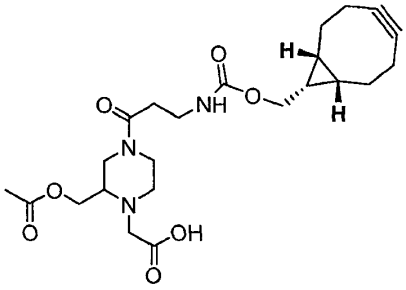


L-2b: 2-(acetoxymethyl)-4-(beta-alanyl)-1-piperazineacetic acid, bis trifluoroacetic acid salt



Xc (3 g, 5.86 mmol) was dissolved in tetrahydrofuran (60 mL) and trifluoroacetic acid (1.04 mL, 13.49 mmol) was added followed by Palladium on charcoal (0.624 g, 5.86 mmol). The flask was successively put under vacuum and backfilled with hydrogen. The operation was repeated three times and the reaction mixture was stirred at room temperature for 15 hr. After this time, the mixture was diluted with a dichloromethane/methanol mixture (9:1, 50 mL) and filtered through a pad of Celite. The Celite was carefully washed with methanol (50 mL) and the combined filtrate was concentrated on vacuo to provide crude title material **L-2b** (3.2 g, 95% yield) as a yellowish foam. MS (ESI+) *m/z* 288.3 (M+H). ^{1}H NMR (500 MHz, MeOH-*d4*) δ 4.50-4.16 (m, 3H), 4.10-3.82 (m, 3H), 3.72-3.39 (m, 4H), 3.29-3.20 (m, 3H), 2.92-2.76 (m, 2H), 2.11 (d, *J* = 5.1 Hz, 3H). Exchangeable protons are not visible in MeOD.

L2. 2-(acetoxymethyl)-4-(N-(((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methoxycarbonyl)-beta-alanyl)-1-piperazineacetic acid



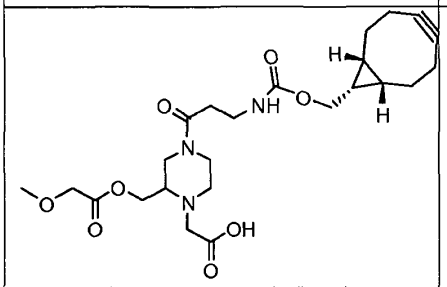
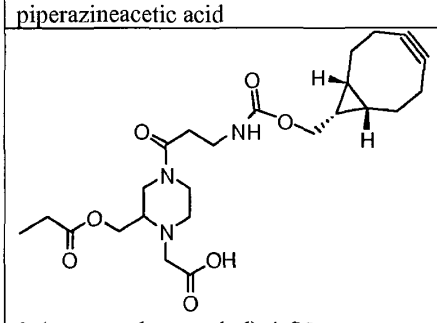
Crude **L-2b** (200 mg, 0.349 mmol) was taken up in dichloromethane (2 mL) and acetonitrile (3 mL) to provide a faintly cloudy mixture. Addition of triethylamine (0.195 mL, 1.397 mmol) and ((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2,5-dioxopyrrolidin-1-yl) carbonate (112 mg, 0.384 mmol) resulted in a solution, which was evaporated after 1 hr. The crude material was either: (i) Dissolved in 6 mL 3:1 acetonitrile : water and purified by HPLC (Column: Waters XBridge BEH 5 µm 19 × 150 mm; method: 15–30% acetonitrile 10 min gradient (10 mM ammonium hydroxide) in water 30 mL/min). The fractions were evaporated, flash frozen and lyophilized to provide **L2**; or (ii) Directly adsorbed on Isolute and purified by reverse phase chromatography (Teledyne Isco, column: RediSep C18 15.5 gold, Eluent A: water, Eluent B: acetonitrile, gradient from 10 to 100% B in 20 min) to afford **L2** after lyophilization. White powder. UPLC-MS (method 10): retention time = 0.71 min; MS (ESI+) *m/z* 464.3 (M+H). ^{1}H NMR (500 MHz, MeOH-*d4*) δ 4.31 - 4.11 (m, 4H), 4.06-3.96 (m, 1H), 3.84-3.64 (m, 1H), 3.55-

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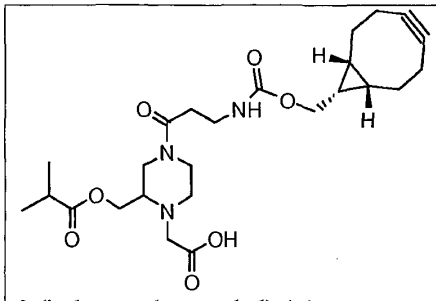
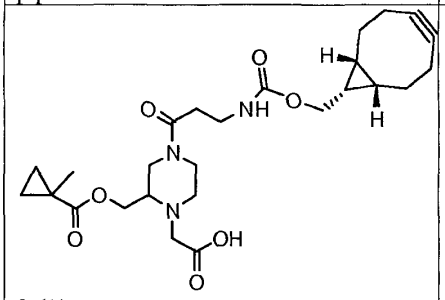
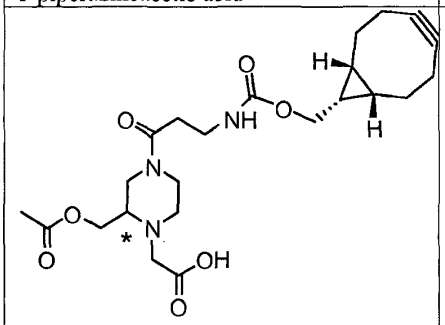
3.36 (m, 5H), 3.29-3.00 (m, 2H), 2.98-2.74 (m, 2H), 2.66-2.54 (m, 2H), 2.32 -2.16 (m, 6H), 2.10-2.03 (m, 3H), 1.69-1.57 (m, 2H), 1.46-1.35 (m, 1H), 1.03-0.89 (m, 2H). Exchangeable protons are not visible in MeOD.

Species shown in Table 7 were prepared using methods analogous to those used in the synthesis of **L2**.

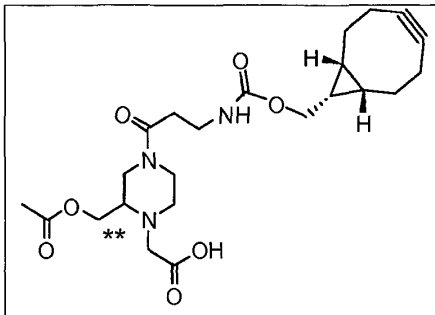
Table 7. Exemplary Traceless Linker Species

Structure	Number (starting intermediate)	LCMS: [M+H]; retention time; method	^{1}H-NMR (400 MHz, CD_3OD)
 2-(2′-methoxyacetoxymethyl)-4-(N-(((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methoxycarbonyl)-beta-alanyl)-1-piperazineacetic acid	**L1 (L-INT-1)**	494.1; 0.98 min; 1	4.25 (m, 2H); 4.14 (m, 4H); 4.0-3.44 (m, 5H), 3.42 (s, 3H), 3.3-3.1 (m, 2H), 2.96 (m, 1H), 2.83 (m, 1H), 2.69 (m, 1H), 2.62 (m, 2H), 2.21 (m, 6H), 1.61 (m, 2H), 1.37 (m, 1H), 0.93 (m, 2H)
 2-(propanoyloxymethyl)-4-(N-(((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methoxycarbonyl)-beta-alanyl)-1-piperazineacetic acid	**L3 (L-INT-3)**	478.2; 1.03 min; 1	4.17 (m, 3H); 3.99 (dd, 1H), 3.76 (dd, 1H), 3.47 (m, 2H), 3.37 (M, 2H), 3.06 (dd, 1H), 2.91 (m, 1H), 2.79 (m, 1H), 2.60 (t, 2H), 2.36 (m, 2H), 2.19 (m, 6H), 1.59 (m, 2H), 1.38 (m, 1H), 1.12 (t, 3H), 0.96 (m, 2H)

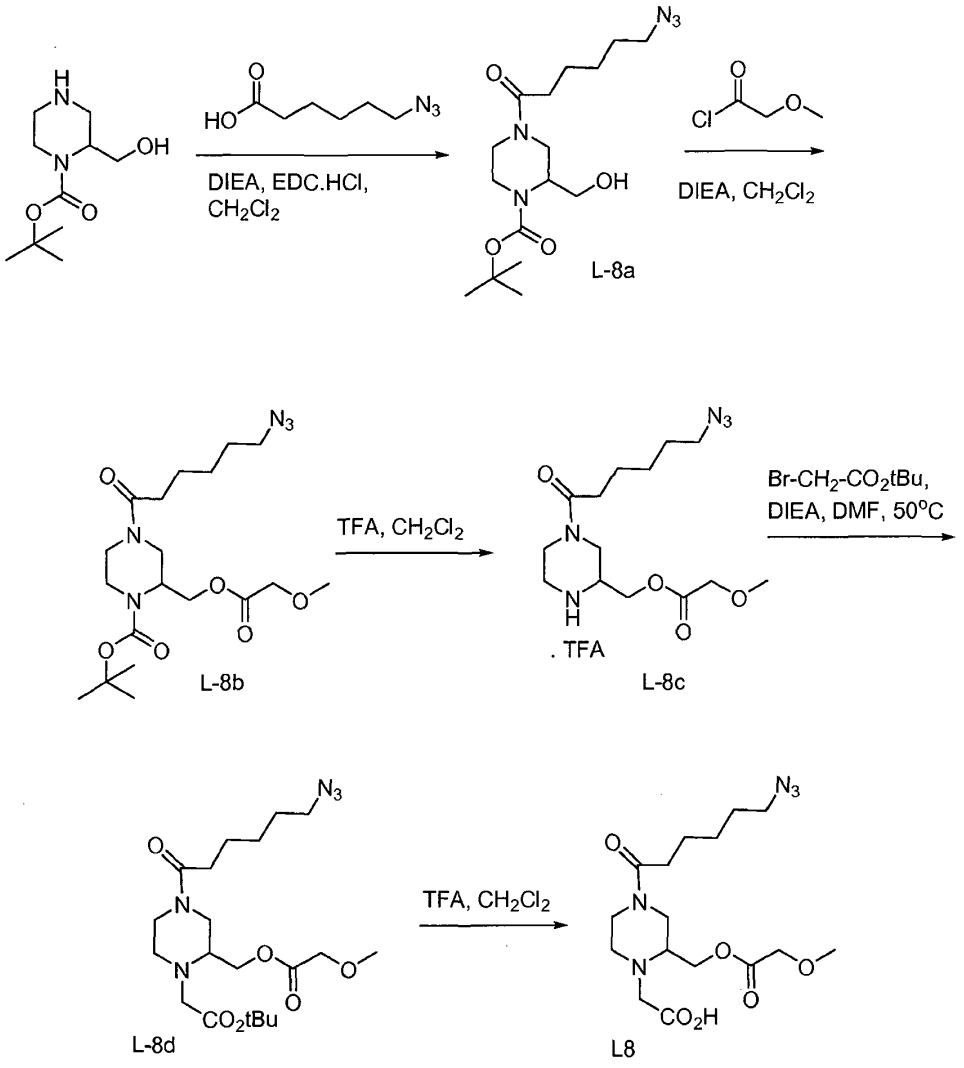
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 2-(isobutanoyloxymethyl)-4-(N-(((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methoxycarbonyl)-beta-alanyl)-1-piperazineacetic acid	**L4** **(L-INT-4)**	492.7; 0.82 min; 2	4.17 (m, 3H); 3.99 (dd, 1H), 3.76 (dd, 1H), 3.41 (m, 4H), 3.06 (dd, 1H), 2.90 (m, 1H), 2.79 (m, 1H), 2.58 (m, 3H), 2.19 (m, 6H), 1.60 (m, 2H), 1.37 (m, 1H), 1.16 (d, 6H), 0.96 (t, 2H)
 2-(1′-methylcyclopropylcarbonyloxymethyl)-4-(N-(((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methoxycarbonyl)-beta-alanyl)-1-piperazineacetic acid	**L5** **(L-INT-5)**	504.2; 1.09 min; 1	4.16 (m, 4H); 3.99 (m, 1H), 3.76 (m, 1H), 3.5-3.35 (m, 5H), 3.06 (m, 1H), 2.93 (m, 1H), 2.88 (dt, 1H), 2.77 (m, 1H), 2.61 (m, 2H), 2.19 (m, 6H), 1.60 (m, 2H), 1.38 (m, 1H), 1.29 (s, 3H), 1.22 (d, 2H), 0.93 (t, 2H), 0.73 (dq, 2H)
 Isomer 1. 2-(acetoxymethyl)-4-(N-(((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methoxycarbonyl)-beta-alanyl)-1-piperazineacetic acid	**L6** **(L-INT-6)**	464.2; 0.95 min; 1	Used as a crude, no NMR

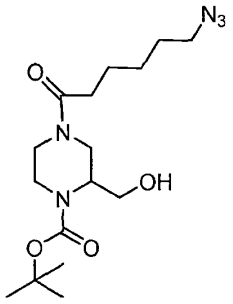
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 Isomer 2. 2-(acetoxymethyl)-4-(N-(((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methoxycarbonyl)-beta-alanyl)-1-piperazineacetic acid	**L7** **(L-INT-7)**	464.2; 0.96 min; 1	Used as a crude, no NMR

Traceless linker L8. 2-(2′-methoxyacetoxymethyl)-4-(6′-azidohexanoyl)-1-piperazineacetic acid

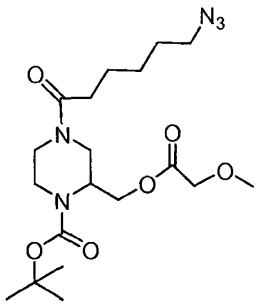


L-8a. 1-(Boc)-2-(hydroxymethyl)-4-(6′-azidohexanoyl)piperazine



6-azidohexanoic acid (743 mg, 4.73 mmol) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (1.0 g, 5.22 mmol) were weighed into a round-bottomed flask and dissolved in dichloromethane (20 mL). After 5 min, solid tert-butyl 2-(hydroxymethyl)piperazine-1-carboxylate (0.98 g, 4.53 mmol) and diisopropylethylamine (1.0 mL, 5.73 mmol) were added and the solution was stored at room temperature overnight. The next day, the reaction mixture was diluted with 100 mL dichloromethane and washed with 1 M HCl (2 × 50 mL), 1 M NaOH (1 × 50 mL), and brine (1 × 50 mL). The organic phase was concentrated to provide **L-8a**. MS (ESI+) *m/z* 356.3 (M+H).

L-8b. 1-(Boc)-2-(2′-methoxyacetoxymethyl)-4-(6″-azidohexanoyl)piperazine

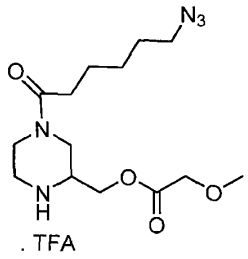


L-8a (959 mg, 2.70 mmol) was dissolved in dichloromethane (12 mL) in a round-bottomed flask. A stir bar and diisopropylethylamine (1 mL, 5.73 mmol) were added, followed by 2-methoxyacetyl chloride (0.32 mL, 3.51 mmol), and the flask was capped. The reaction was stirred at room temperature. After 6 h, 100 µL additional 2-methoxyacetyl chloride was added. After 30 min, the reaction mixture was diluted with 90 mL ethyl acetate and washed with 1 M HCl (2 × 25 mL) and brine (1 × 20 mL). The organic phase was concentrated using a rotoevaporator. The

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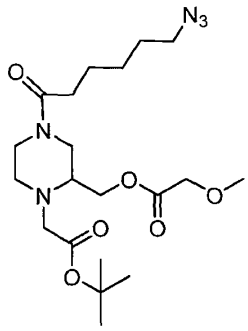
product was purified by flash column chromatography on silica with an ethyl acetate : heptanes gradient. Product containing fractions were combined and concentrated to provide **L-8b**. MS (ESI+) *m/z* = 428.3 (M+H).

L-8c. 1-(6′-azidohexanoyl)-3-(2″-methoxyacetoxymethyl)piperazine, trifluoroacetic acid salt



L-8b (846 mg, 1.979 mmol) was dissolved in trifluoroacetic acid (10 mL, 130 mmol) and dichloromethane (10 mL). The solution was stirred at room temperature. After 1 h, the solvents were removed using a rotoevaporator and the product dried under vacuum to provide **L-8c**. MS (ESI+) *m/z* 328.2 (M+H).

L-8d. 2-(2′-methoxyacetoxymethyl)-4-(6″-azidohexanoyl)-1-piperazineacetic acid t-butyl ester

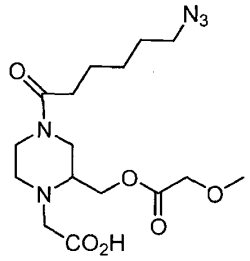


L-8c (439 mg, 0.994 mmol) was dissolved in dimethylformamide (5 mL) in a glass vial with a stirbar. t-Butyl bromoacetate (0.220 mL, 1.491 mmol) and diisopropylethylamine (0.868 mL, 4.97 mmol) were added, and the vial was capped. The reaction was stirred at 50 °C overnight.

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The next day, the reaction mixture was removed from the heat source and stored at −20 °C until purification the following day. The reaction mixture was purified without extractive work up by flash column chromatography on silica with a heptane : ethyl acetate gradient. Product containing fractions were combined, and concentrated to provide **L-8d**. MS (ESI-) *m/z* = 486.5 (M+formate).

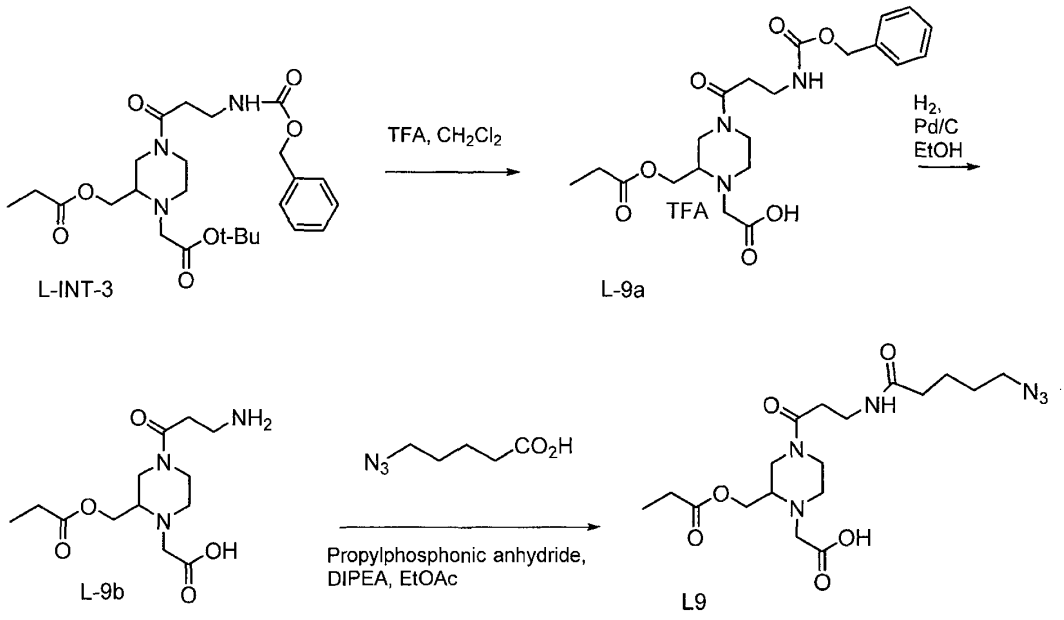
L8. 2-(2′-methoxyacetoxymethyl)-4-(6″-azidohexanoyl)-1-piperazineacetic acid



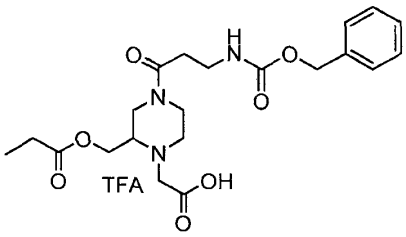
L-8d (150 mg, 0.317 mmol) was dissolved in dichloromethane (5 mL) and trifluoroacetic acid (5 mL) and the reaction was stirred at room temperature overnight. The next day, the solution was concentrated using a rotoevaporator and the residue was redissolved in 4 mL acetonitrile. The solution was filtered and purified by preparative reverse phase HPLC with mass directed fraction collection (method below). Product containing fractions were pooled, frozen, and lyophilized to provide **L8**. MS (ESI+) *m/z* = 386.5 (M+H). Preparative HPLC conditions: Waters Sunfire C18; particle size: 5 μm; column size: 30 × 50 mm; eluent/gradient: 10% CH_3CN/H_2O/0.7 min, 10–30% CH_3CN/H_2O/3.5 min, 30-95% CH_3CN/H_2O 0.5 min (CH_3CN and H_2O containing 0.1% TFA); flow rate: 75 mL/min; column temperature: room temperature; collection *m/z*: +385.

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Traceless linkers: L9-L11



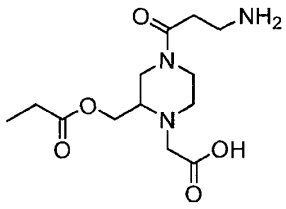
L-9a. 2-(propanoyloxymethyl)-4-(N-Cbz-beta-alanyl)-1-piperazineacetic acid, trifluoroacetic acid salt



L-INT-3 (250 mg, 0.51 mmol) was dissolved in dichloromethane (5 mL) at 0 °C, then treated with trifluoroacetic acid (2.5 mL) and stirred at room temperature 6 h under argon. The reaction was evaporated, washed with pentane and dried to provide crude **L-9a** as a trifluoroacetic acid salt. LCMS (method 5): retention time = 0.262 min; MS (ESI+) *m/z* 436.2 (M+H).

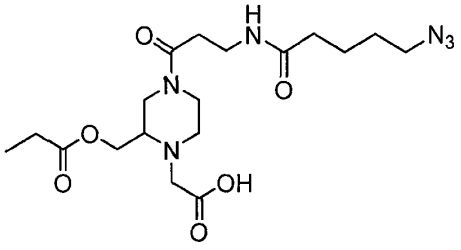
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L-9b. 2-(propanoyloxymethyl)-4-(beta-alanyl)-1-piperazineacetic acid



L-9a (250 mg, 0.57 mmol) was dissolved in ethanol (5 mL), to which was added 10% palladium on carbon (25 mg). The solution was stirred under a balloon of hydrogen gas for 6 h and then filtered through Celite®, washing with ethanol. The combined filtrates were evaporated to provide compound **L-9b**. LCMS (method 5): retention time = 0.774 min; MS (ESI+) *m/z* 302.2 (M+H).

L9.

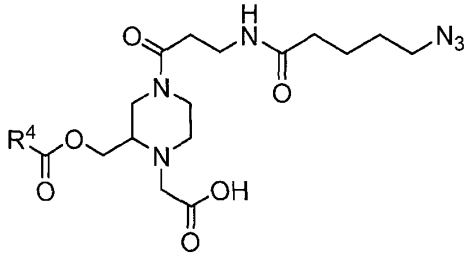


L-9b (100 mg, 0.33 mmol) and 5-azidopentanoic acid (57 mg, 0.40 mmol) in ethyl acetate (5 mL) at 0 °C were treated with diisopropylethylamine (107 mg, 0.83 mmol) and propylphosphonic anhydride (50% solution in ethyl acetate, 158 mg, 0.50 mmol). The reaction was stirred at room temperature 16 h under argon, then quenched with water and evaporated. Initial flash chromatography (SiO_2, 10% methanol : dichloromethane) was followed by preparative HPLC (column: zorbax C-18 4.6 × 150 mm; Mobile phase A = : methanol (1:1) 0.01% TFA in water, mobile phase B=acetonitrile : methanol (1:1); time= 0 min: 30% B; 1 min: 70% B; 6 min: 100% B; 1 mL/min) to provide **L9**. LCMS (method 5): retention time = 0.18 min; MS (ESI+) *m/z* 427.1 (M+H). ^{1}H-NMR (MeOH-*d4*, ppm) (all assignments provisional) 4.23-4.17 (m, 2H), 4.16-3.98 (m, 1H), 3.78-3.62 (m, 1H), 3.54 (s, 2H), 3.46-3.43 (t, J=4, 3H), 3.26-3.20 (m, 1H), 3.18-3.05

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(m, 1H), 3.03-2.76 (m, 2H), 2.64-2.61 (t, J=8,2H), 2.43-2.36 (m, 2H), 2.24-2.21 (t, J=8, 2H), 1.75-1.58 (m, 4H), 1.15-1.12 (t, J=8, 3H). Exchangeable protons are not visible in MeOD.

Species shown in Table 8 were prepared using methods analogous to those used in the synthesis of L-9:

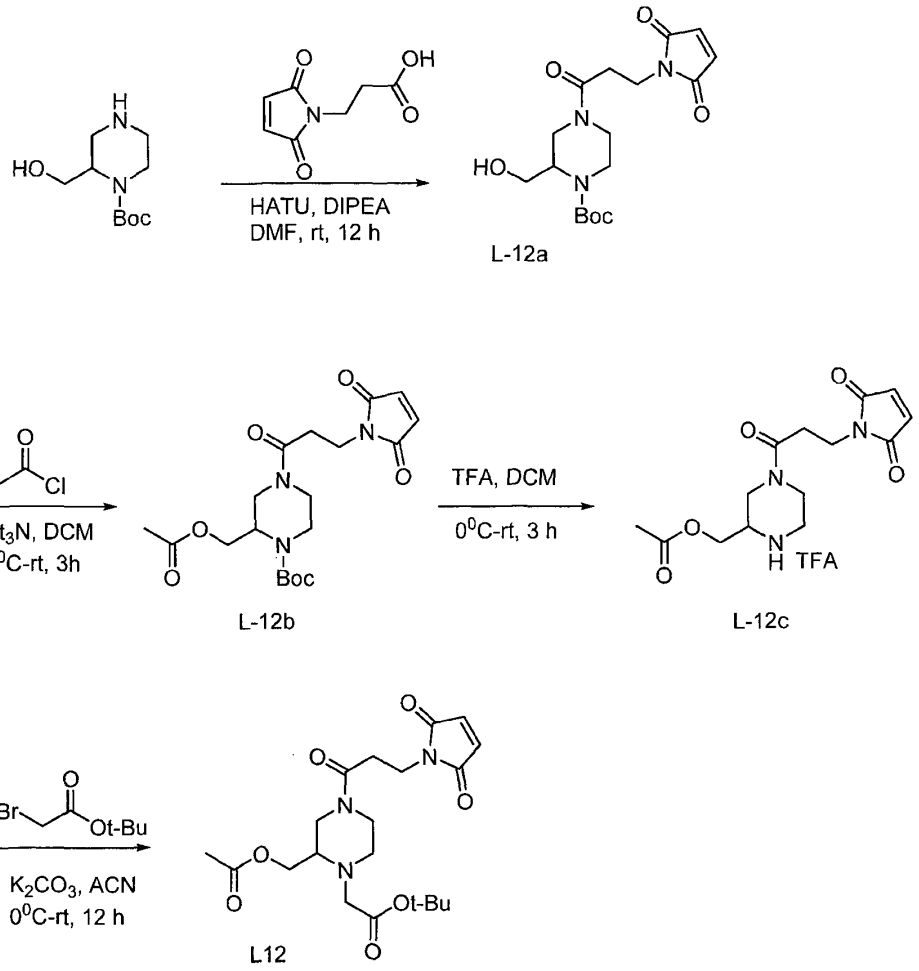


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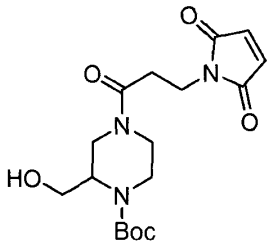
Table 8. Exemplary Traceless Linker Species			
R^4, Name	**Number (starting intermediate)**	**LCMS: [M+H]; retention time; method**	**^{1}H-NMR (300 MHz, CD_3OD)**
$R^4 = (CH_3)_2CH-$ 2-(isobutanoyloxymethyl)-4-(N-(5′-azidopentanoyl)-beta-alanyl)-1-piperazineacetic acid	**L10 (L-INT-4)**	441.1 0.16 min 5	4.21-4.15(m, 2H), 4.04-3.84(t, J=16, 1H), 3.81-3.73(m, 1H), 3.55(s, 2H), 3.49-3.45(t, J=8, 3H), 3.34(s, 1H), 3.27-3.22(m, 1H), 3.14-3.10(m, 2H), 2.99-2.80(m, 2H), 2.70-2.57(m, 3H), 1.72-1.58 (m,4H), 1.19-1.17(d, J=8, 6H).
$R^4 =$  2-(1′-methylcyclopropylcarbonyloxymethyl)-4-(N-(5″-azidopentanoyl)-beta-alanyl)-1-piperazineacetic acid	**L11 (L-INT-5)**	453.2 0.21 min 5	4.21-4.16 (dd, J=16,4, 2H), 4.05-3.99(t, J=12, 1H),3.83-3.72(m,1H), 3.56(s, 2H), 3.49-3.45(t, J=8, 3H), 3.28-3.20(m, 1H), 3.18-3.04(m,1H),3.02-2.76(m,2H),2.72-2.54(m, 2H), 2.24-2.21(t, J=8, 2H),1.72-1.56(m, 5H), 1.13(s,3H), 1.26-1.21(m, 2H),1.07-0.90(m, 1H), 0.76-0.74(t, J=4, 2H)

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Traceless linkers: L12 and L13



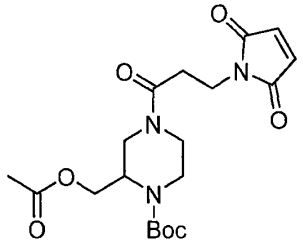
L-12a. 1-(Boc)-2-(hydroxymethyl)-4-(N-maleoyl-beta-alanyl)piperazine



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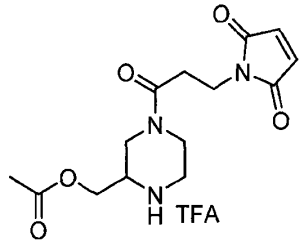
tert-Butyl 2-(hydroxymethyl)piperazine-1-carboxylate (200 mg, 0.93 mmol) was dissolved in dichloromethane (10 mL) at 0 °C. Diisopropylethylamine (359 mg, 2.78 mmol) and HATU (422 mg, 1.11 mmol) were added and the solution was stirred 15 min at 0 °C. 3-maleimidopropionic acid (187 mg, 1.11 mmol) was added, and then the reaction was stirred 12 h at room temperature under argon. The reaction was diluted with water and extracted with dichloromethane. The organic layer was washed with brine, dried over sodium sulfate and concentrated. Purification by flash chromatography (neutral alumina, 2% methanol : ethyl acetate provided the title material **L-12a**. LCMS (method 6): retention time = 2.87 min.

L-12b. 1-(Boc)-2-(acetoxymethyl)-4-(N-maleoyl-beta-alanyl)piperazine



To **L-12a** (300 mg, 0.82 mmol) and triethylamine (330 mg, 3.27 mmol) in dichloromethane (4 mL) at 0 °C under argon was dropwise added acetyl chloride (125 mg, 1.64 mmol). The reaction was stirred at room temperature for 3 h, then treated with water and extracted with dichloromethane. The organic layer was dried (sodium sulfate), concentrated and purified by flash chromatography (neutral alumina, 2% methanol : ethyl acetate) to provide the title material **L-12b**. LCMS (method 6): retention time = 3.2 min.

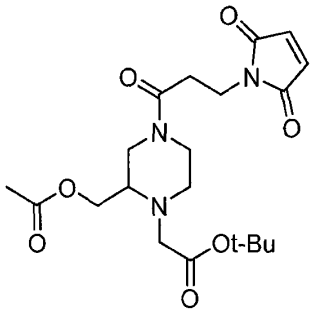
L-12c. 1-(N-maleoyl-beta-alanyl)-3-(acetoxymethyl)piperazine, trifluoroacetic acid salt



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L-12b (200 mg, 0.49 mmol) was dissolved in dichloromethane (5 mL) at 0 °C, then treated dropwise with trifluoroacetic acid (0.4 mL) and stirred at room temperature 3 h under argon. The reaction was evaporated, washed with pentane and dried to provide crude title material **L-12c.** LCMS (method 6): retention time = 1.709 min; MS (ESI+) *m/z* 310 (M+H).

L12. 2-(acetoxymethyl)-4-(N-maleoyl-beta-alanyl)-1-piperazineacetic acid t-butyl ester

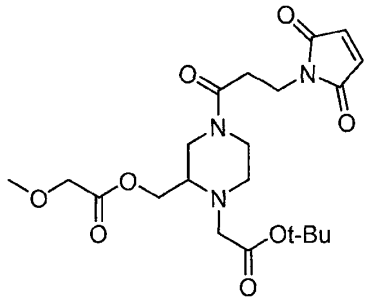


To **L-12c** (140 mg, 0.45 mmol) and potassium carbonate (156 mg, 1.13 mmol) in acetonitrile (5 mL) at 0 °C was dropwise added tert-butyl bromoacetate (100 mg, 0.54 mmol). The reaction was warmed to 10–15 °C, then allowed to stir for 12 h at room temperature under argon. The reaction was diluted with water and extracted with ethyl acetate. The organic layer was dried (sodium sulfate), concentrated and purified by preparative HPLC (column: zorbax eclipse XDB C18, 21.2 × 150 mm, 5 µm; Mobile phase A = water, mobile phase B=acetonitrile; time= 0 min: 30% B; 2 min: 40% B; 10 min: 60% B) to provide **L12**. LCMS (method 6): retention time = 3.08 min; MS (ESI+) *m/z* 424 (M+H). ^{1}H-NMR ($CDCl_3$, ppm) 6.69 (s, 2H), 4.25-4.14 (m, 2H), 4.09-3.96 (m, 2H), 3.88-3.83 (t, J=9, 2H), 3.65-3.54 (m, 1H), 3.46-3.33 (m, 2H), 3.31-3.17 (m, 2H), 3.05-2.98 (m ,2H), 2.84-2.75 (m, 2H), 2.68-2.63 (t, J=9,2H), 2.09-2.07 (d, J=6, 3H), 1.45 (s, 9H).

L13 was prepared using methods analogous to those used in the synthesis of **L12:**

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L13. 2-(2′-methoxyacetoxymethyl)-4-(N-maleoyl-beta-alanyl)-1-piperazineacetic acid t-butyl ester (1-(2-(tert-butoxy)-2-oxoethyl)-4-(3-(maleimidyl)propanoyl)piperazin-2-yl)methyl 2-methoxyacetate

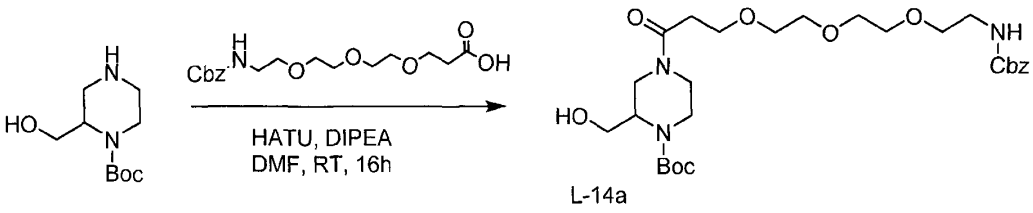


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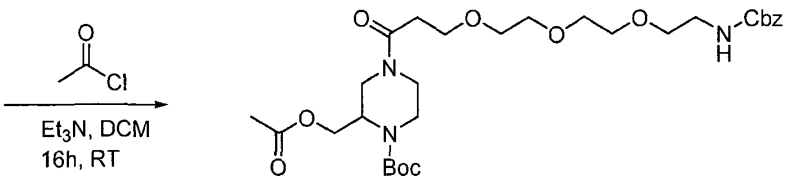
L13. LCMS (method 6): retention time = 3.04 min; MS (ESI+) *m/z* 454.5 (M+H). ^{1}H-NMR ($CDCl_3$, ppm) 6.70 (s, 2H), 4.40-4.24 (m, 1H), 4.20-4.10 (m, 1H), 4.07-4.60 (d, J=4, 2H), 4.02-3.94 (m, 1H), 3.87-3.83 (t, J=8, 2H), 3.70-3.60 (m, 1H), 3.68-3.60 (m, 1H),3.58-3.51 (m, 1H),
10 3.45 (s, 3H), 3.41-3.39 (d, J=8, 1H), 3.36-3.32 (s, 1H), 3.32-3.28 (s, 1H), 3.18-3.10 (m, 1H), 3.04-2.97 (m,1H), 2.83-2.81 (t, J= 4, 1H), 2.79-2.75 (m, 1H), 2.67-2.64 (t, J= 8, 2H), 1.46 (s,9H).

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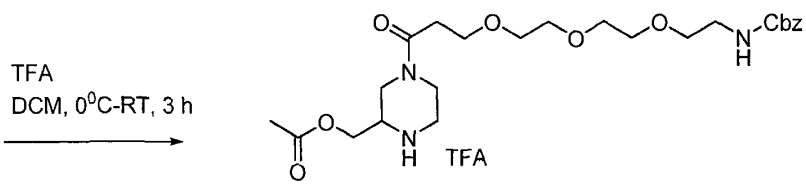
Traceless linkers; L14–L16



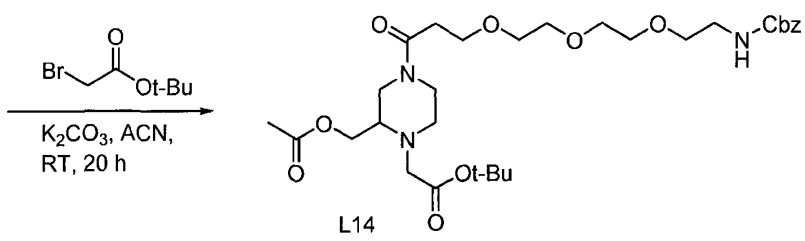
L-14a



L-14b

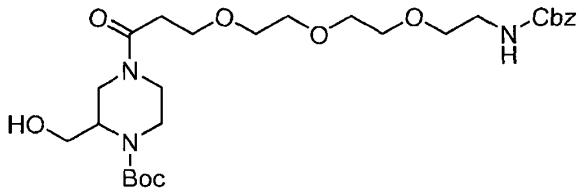


L-14c



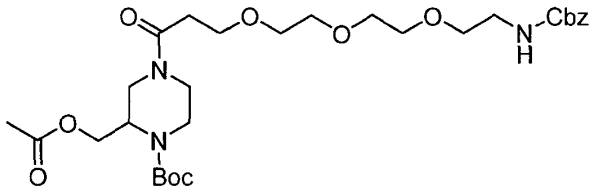
L14

L14a. 1-(Boc)-2-(hydroxymethyl)-4-(3′-(2″-(2‴-(2⁗-((N-Cbz)-amino)ethoxy)ethoxy)ethoxy)propanoyl)piperazine



To tert-butyl 2-(hydroxymethyl)piperazine-1-carboxylate (1.0 g, 4.63 mmol) dissolved in dichloromethane (20 mL) at 0 °C was added diisopropylethylamine (1.79 g, 13.9 mmol) and HATU (2.10 g, 5.55 mmol). The solution was stirred 15 min at 0 °C. 3-(2′-(2″-(2‴-((N-Cbz)-amino)ethoxy)ethoxy)ethoxy)propanoic acid (1.64 g, 4.63 mmol) was added and the reaction was stirred 16 h at room temperature under argon. The reaction was diluted with water and extracted with ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate and concentrated. Purification by flash chromatography (SiO_2, 1–4% methanol : dichloromethane) provided the title material **L-14a**. LCMS (method 5): retention time = 1.27 min; MS (ESI+) *m/z* 554.3 (M+H).

L-14b. 1-(Boc)-2-(acetoxymethyl)-4-(3′-(2″-(2‴-(2⁗-((N-Cbz)-amino)ethoxy)ethoxy)ethoxy)propanoyl)piperazine

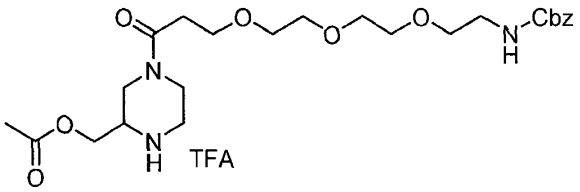


To **L-14a** (500 mg, 0.90 mmol) and triethylamine (228 mg, 2.26 mmol) dissolved in dichloromethane (20 mL) at 0 °C was dropwise added acetyl chloride (83 mg, 1.08 mmol). The reaction was stirred at room temperature under argon for 16 h. The reaction was diluted with water and extracted with dichloromethane. The combined organic layer was dried over sodium sulfate and concentrated, then purified by flash chromatography (SiO_2, 1–4% methanol :

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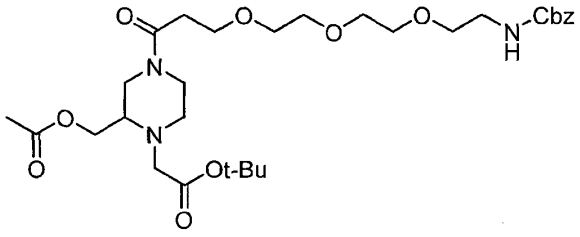
dichloromethane) to provide the title material **L-14b**. LCMS (method 7): retention time = 1.54 min; MS (ESI+) *m/z* 595.8 (M+H).

L-14c. 1-(3'-(2''-(2'''-(2''''-((N-Cbz)-amino)ethoxy)ethoxy)ethoxy)propanoyl)-3-(acetoxymethyl)piperazine, trifluoroacetic acid salt



L-14b (500 mg, 0.84 mmol) was dissolved in dichloromethane (10 mL) at 0 °C, then treated dropwise with trifluoroacetic acid (3 mL) and stirred at room temperature 3 h under argon. The reaction was evaporated, washed with pentane and dried to provide crude title material **L-14c**, which was carried into the next reaction without further purification or analysis.

L14. 2-(acetoxymethyl)-4-(3'-(2''-(2'''-(2''''-((N-Cbz)-amino)ethoxy)ethoxy)ethoxy)propanoyl)-1-piperazineacetic acid t-butyl ester



To **L-14c** (500 mg, 1.0 mmol) and potassium carbonate (418 mg, 3.03 mmol) in acetonitrile (10 mL) at 0 °C was dropwise added tert-butyl bromoacetate (295 mg, 1.51 mmol). The reaction was stirred for 20 h at room temperature under argon. The reaction was diluted with water and extracted with ethyl acetate. The organic layer was dried (sodium sulfate), concentrated and purified by an initial flash chromatography (SiO_2, 1–5% methanol : dichloromethane), followed by preparative HPLC (column: zorbax eclipse XDB C18, 4.6 × 150 mm, 5 μm; Mobile phase A = 0.01% TFA in water, mobile phase B=acetonitrile: methanol (1:1), 1 mL/min; time= 0 min: 30% B; 1 min: 70% B; 6 min: 100% B) to provide **L14**. LCMS (method 5): retention time = 3.43 min;

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MS (ESI+) *m/z* 610.35 (M+H). ^{1}H-NMR ($CDCl_3$, ppm) 7.36-7.31 (m, 5H), 5.49 (s, 1H), 5.09 (s, 2H), 4.26-4.16 (m, 1H), 4.07-3.98(m, 1H), 3.78-3.74(t, J=6, 2H), 3.60-3.53(m, 11H), 3.39-3.32(m, 4H), 2.84-2.72(m, 2H), 2.61-2.57(t, J=3, 2H), 2.08-2.06(d, J=6, 2H), 1.45(s, 9H).

Species shown in Table 9 were prepared using methods analogous to those used in the synthesis of **L14:**

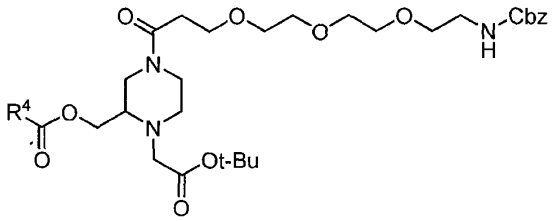
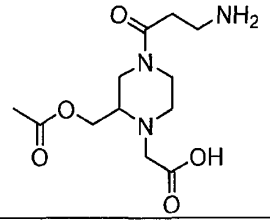
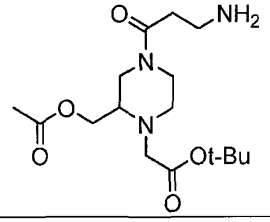
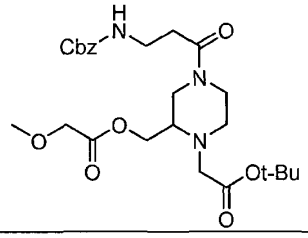
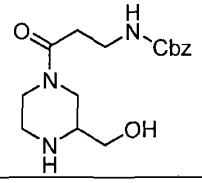
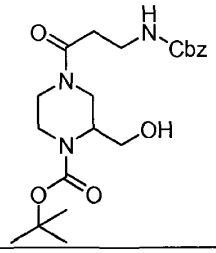
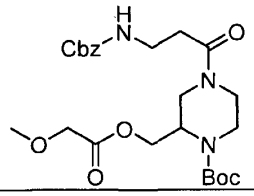
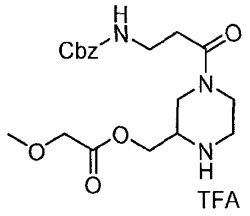
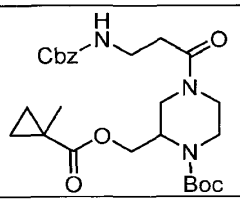
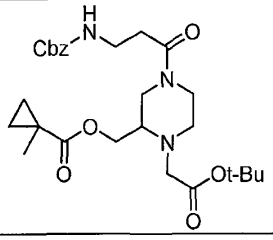
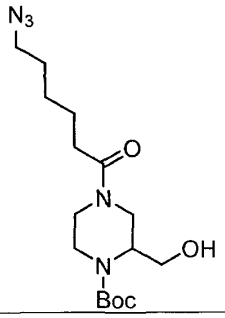
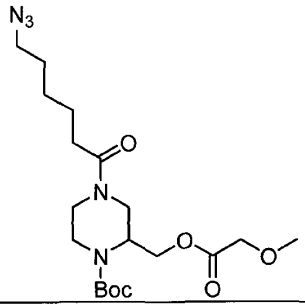


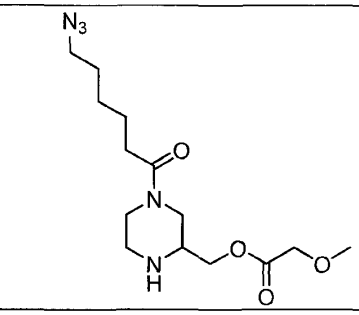
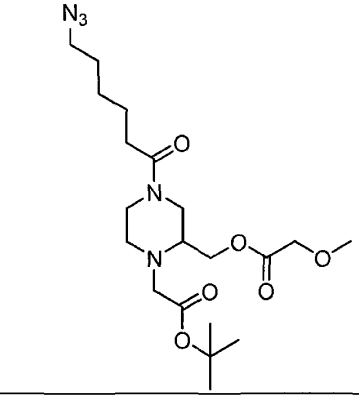
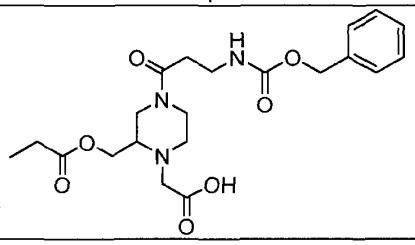
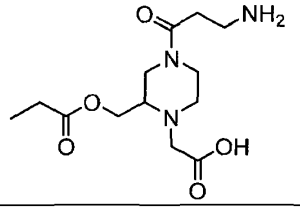
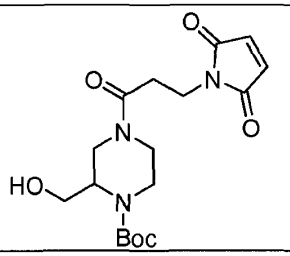
Table 9. Exemplary Traceless Linker Species

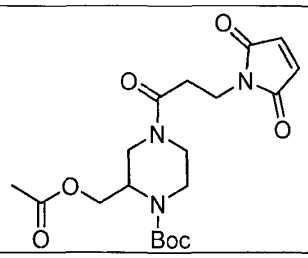
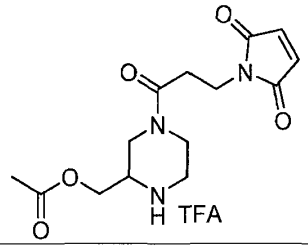
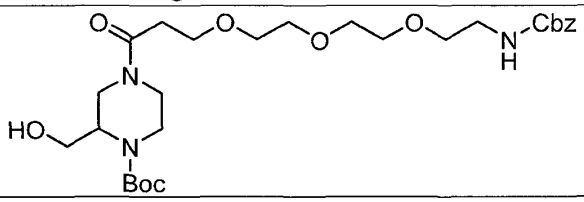
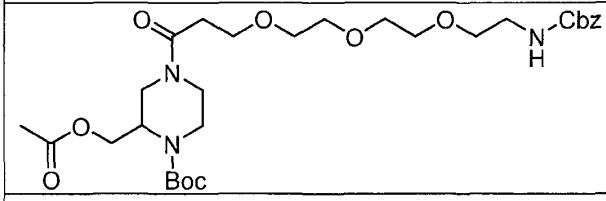
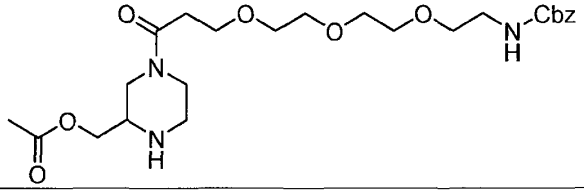
R^4, Name	**Number**	**LCMS: [M+H]; Retention Time; Method**	**^{1}H-NMR (300 MHz, $CDCl_3$)**
$R^4 = CH_3CH_2$- 2-(propanoyloxymethyl)-4-(3′-(2″-(2‴-(2⁗-((N-Cbz)-amino)ethoxy)ethoxy)ethoxy)propanoyl)-1-piperazineacetic acid t-butyl ester	**L15**	624.35; 3.60 min; 5	7.36-7.30 (m, 5H), 5.41 (s 1H), 5.09 (s, 2H), 4.28-3.96 (m, 3H), 3.78-3.74 (t, J=6, 3H), 3.60-3.53 (m, 12H), 3.43-3.22(m, 6H), 3.10-2.92 (m, 2H),2.81-2.74(m, 2H), 2.61-2.56(t, J=9, 2H), 2.39-20 (m, 2H), 1.63(s, 3H), 1.45(s, 9H), 1.16-1.10(t, J=6, 3H).
$R^4 = (CH_3)_2CH$- 2-(isobutanoyloxymethyl)-4-(3′-(2″-(2‴-(2⁗-((N-Cbz)-amino)ethoxy)ethoxy)ethoxy)propanoyl)-1-piperazineacetic acid t-butyl ester	**L16**	638.35; 3.60 min; 5	7.36-7.30 (m, 5H), 4.95 (s, 1H), 5.10(s, 2H), 4.26-4.12 (m, 2H), 4.08-3.96(m, 2H), 3.81-3.76(m, 3H), 3.61(s, 8H), 3.57-3.54 (t, J=4, 3H), 3.41-3.19(m, 6H),3.11-3.04(m, 1H), 3.02-2.88 (m, 1H), 2.84-2.72 (m, 2H), 2.60-2.57 (m, 3H), 1.45(s, 9H), 1.18-1.16(d, J=8, 6H).

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Table 10. Exemplary Traceless Linker Synthetic Intermediates		
Structure	**Number**	**Analogous compounds**
	**L-2b**	**L-1b, L-3b, L-4b, L-5b, L-6b, L-7b**
	**L-2a**	**L-1a, L-3a, L-4a, L-5a, L-6a, L-7a**
	**L-INT-1**	**L-INT-2, L-INT-3, L-INT-4**
	**L-INT-1b**	**L-INT-2b, L-INT-3b, L-INT-4b**
	**L-INT-1c**	**L-INT-2c, L-INT-3c, L-INT-4c**
	**L-INT-1d**	**L-INT-2c, L-INT-3c, L-INT-4c**

	**L-INT-1e**	**L-INT-2c, L-INT-3c, L-INT-4c**
	**L-INT-5a**	
	**L-INT-5b**	
	**L-8a**	
	**L-8b**	

	**L-8c**	
	**L-8d**	
	**L-9a**	**L-10a, L-11a**
	**L-9b**	**L-10b, L-11b**
	**L-12a**	**L-13a**

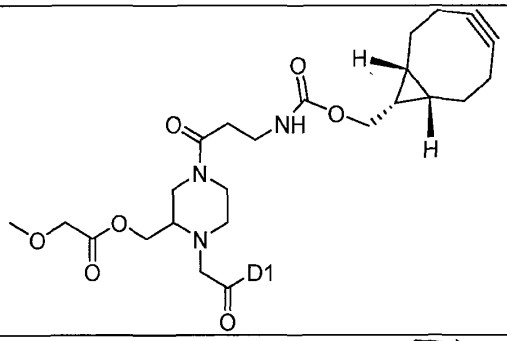
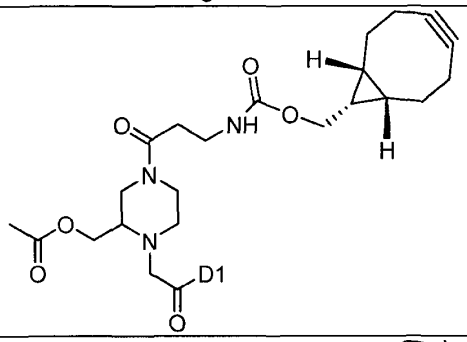
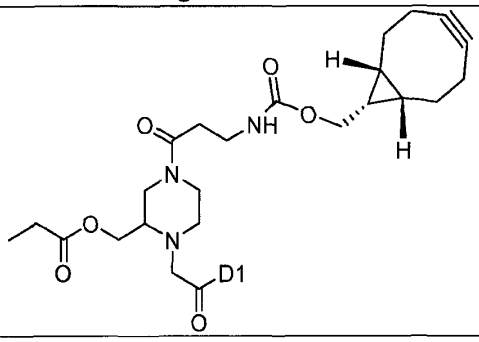
	**L-12b**	**L-13b**
	**L-12c**	**L-13c**
	**L-14a**	**L-15a, L-16a**
	**L-14b**	**L-15b, L-16b**
	**L-15c**	**L-15c, L-16c**

Example 2

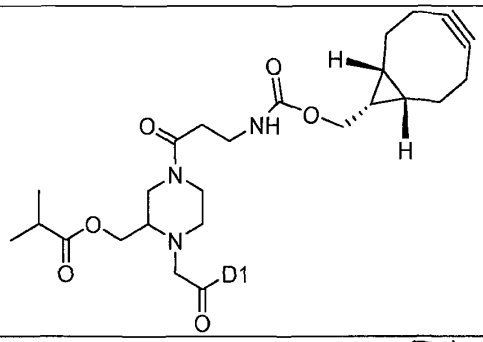
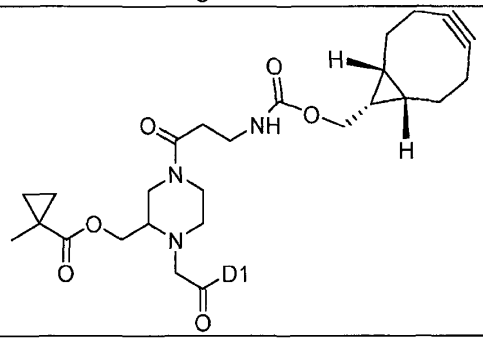
Adducts of Traceless Linkers with Biologically Active Moieties

This example describes the synthesis of a number of traceless linker-drug adducts, which are also capable of being conjugated to a carrier. D1 as depicted below comprises an ANGPTL3 polypeptide comprising amino acid residues 242–460 in reference to SEQ ID NO:1 and a K423Q substitution (SEQ ID NO:19).

Table 11. Exemplary Traceless Linker–Drug Adducts

Number	Structure	Traceless linker	Drug (D)
L1D1		L1	D1
L2D1		L2	D1
L3D1		L3	D1

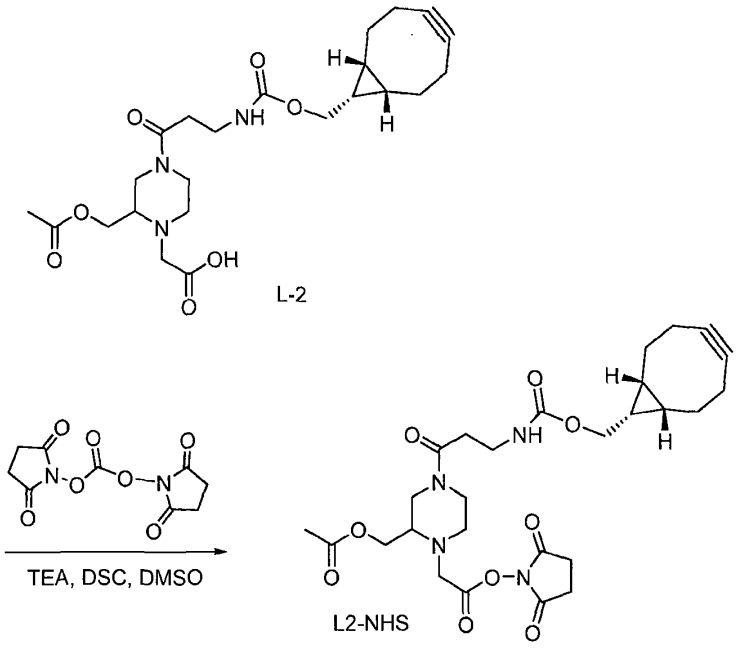
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L4D1		**L4**	**D1**
L5D1		**L5**	**D1**

Synthesis of Adducts

Acylation of biologically active moieties with a traceless linker:

L2-NHS. 2-(acetyloxymethyl)-4-(N-(((1*R*′,8′*S*,9′s)-bicyclo[6.1.0]non-4′-yn-9′-yl)methoxycarbonyl)-beta-alanyl)-1-piperazineacetic acid N-hydroxysuccinimidyl ester

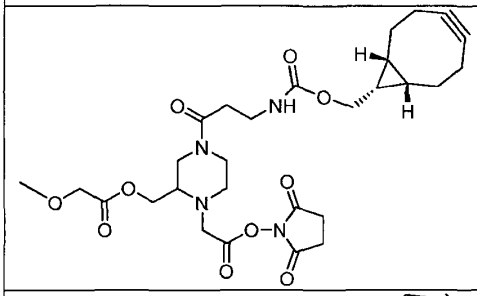
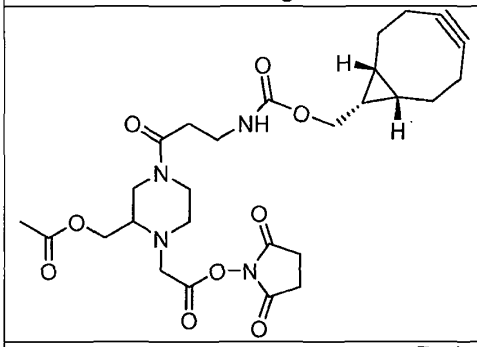
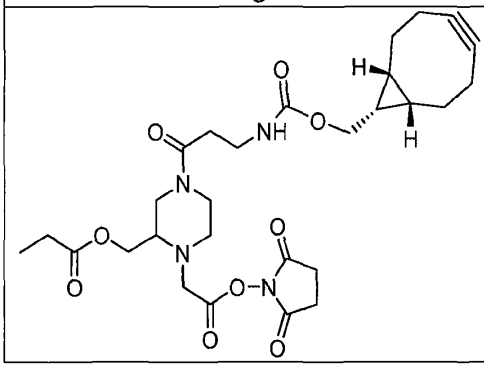


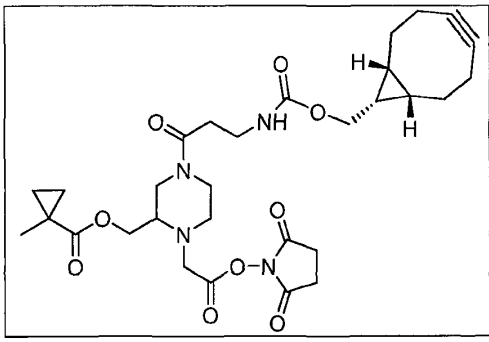
L2 (16 mg, 0.031 mmol) was dissolved in dimethylsulfoxide (0.818 mL) to reach a 38 mM concentration. Triethylamine (3.46 µL, 0.025 mmol) was added followed by N,N′-disuccinimidyl carbonate (11.94 mg, 0.047 mmol) and the resulting clear solution was stirred for 1 h at room temperature under argon. The solution was used in the next step as such without further purification. LCMS (method 10): retention time = 0.93 min; MS (ESI+) *m/z* 561.4 (M+H).

The species in Table 12 were prepared using methods analogous to those used in the synthesis of **L2-NHS**.

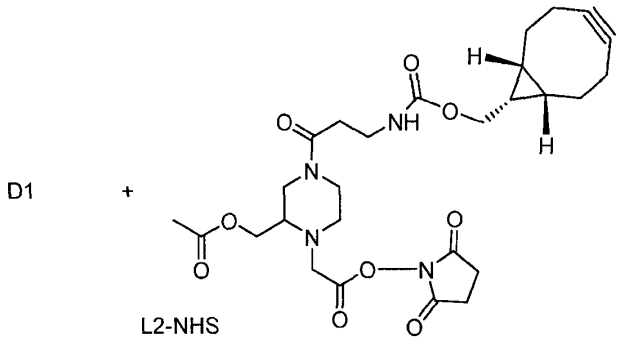
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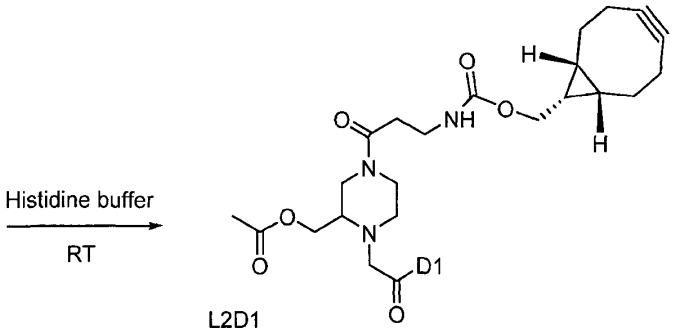
Table 12. Exemplary Activated Traceless Linker Species

Structure	Number (starting intermediate)	Name	MS (ESI+) *m/z* (M+H) retention time; method
	**L1-NHS (L1)**	2-(2′-methoxyacetoxymethyl)-4-(N-(((1″*R*,8″*S*,9″s)-bicyclo[6.1.0]non-4″-yn-9″-yl)methoxycarbonyl)-beta-alanyl)-1-piperazineacetic acid N-hydroxysuccinimidyl ester	591.4; 0.87 min; 2
	**L2-NHS (L2)**	2-(acetoxymethyl)-4-(N-(((1′*R*,8′*S*,9′s)-bicyclo[6.1.0]non-4′-yn-9′-yl)methoxycarbonyl)-beta-alanyl)-1-piperazineacetic acid N-hydroxysuccinimidyl ester	561.4; 0.93 min; 10
	**L3-NHS (L3)**	2-(propanoyloxymethyl)-4-(N-(((1′*R*,8′*S*,9′s)-bicyclo[6.1.0]non-4′-yn-9′-yl)methoxycarbonyl)-beta-alanyl)-1-piperazineacetic acid N-hydroxysuccinimidyl ester	575.5; 0.91 min; 4

	**L5-NHS (L5)**	2-(1′-methylcyclopropylcarbonyloxymethyl)-4-((N-(((1″*R*,8″*S*,9″s)-bicyclo[6.1.0]non-4″-yn-9″-yl)methoxycarbonyl)-beta-alanyl)-1-piperazineacetic acid N-hydroxysuccinimidyl ester	601.4; 0.99 min; 4

L2-D1 (SEQ ID NO:19) (L2D1)





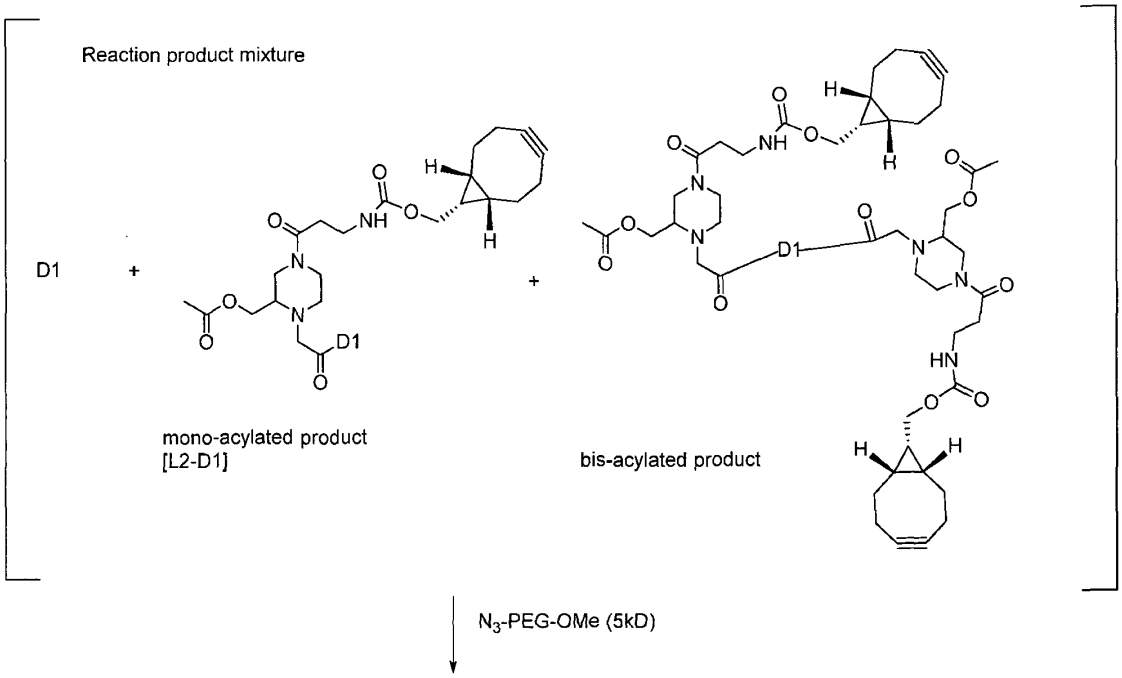
To a 10 mg/mL solution of D1 (SEQ ID NO:19) (30 mL, 9.66 mmol) in histidine buffer (20 mM, pH 5.8) or 1x PBS (pH 7.4) was slowly added a 38 mM solution of L2-NHS in DMSO (0.508 mL, 19.32 mmol). The reaction mixture was vigorously vortexed, and then shaken at room temperature for 2 h at 200 tr/min. Purification was performed by ultracentrifugation (Amicon filter, 3 kDa MWCO, 2000 rcf, 45 min, RT), washing with Histidine buffer or 1x PBS (3 times)

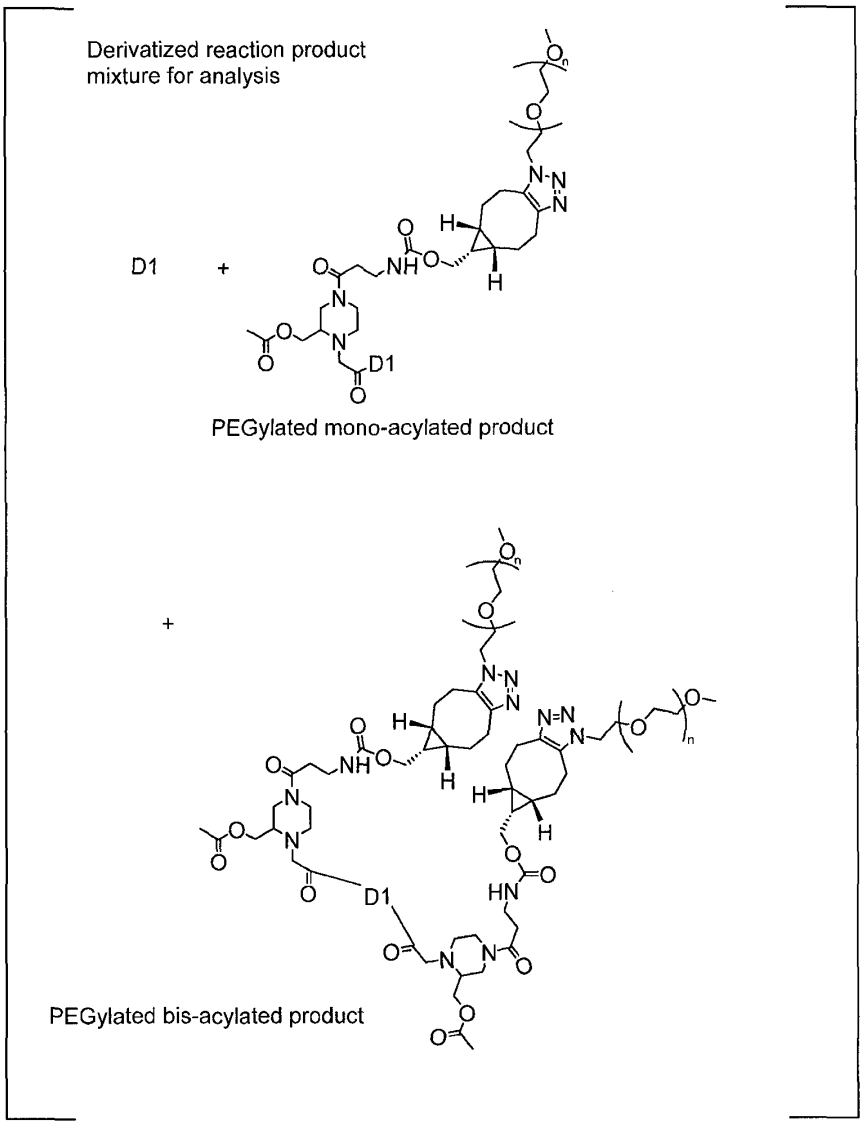
removed residual small molecule impurities, resulting in a mixture of **L2D1** and unmodified **D1**. MS (method 9) deconvoluted *m/z* 29969 (**D1**, M+H); deconvoluted *m/z* 30415 (**L2D1**, M+H, expected MW = MW (**D1**) + 446).

An important analytical parameter is determination of ratio of (unmodified **D1**, n = 0): (mono-acylated **D1**, n = 1):(poly-acylated **D1**, n > 1). Comparison of relative peak heights for the various species in a mass spectrum allows an estimate of the product ratio to be made.

An additional method for estimating the ratio of reaction products utilizes SEC (size exclusion chromatography) analysis of derivatized reaction products; derivitization is required in order to achieve separation of the reaction products.

L2D1 Derivatization Reaction





An aliquot from the reaction mixture (10 mg/mL **L2D1** solution, 0.020 mL, 0.060 μmol) was treated with a M_n ~ 5 kDa methoxy-PEG-azide (Sigma-Aldrich, 689475, 0.014 mL of a 100 mg/mL solution in 1× PBS, 0.28 μmol), vortexed, shaken at 37 °C for 30 min and diluted with PBS (0.066 mL).

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Analysis of L2D1-derivative

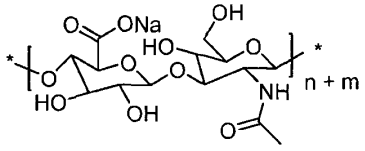
SEC: (Instrument: Agilent LC 1260 Infinity; column: Superdex 200 increase 10/300GL (28-9909-44); Column temperature: r.t., flow rate: 0.75 mL/min; injection volume: 10 µL; mobile phase: 1/9 PBS/water preliminary filtered through a 0.10 membrane filter (isocratic); run time: 40 min; detection wavelengths: 214 nm). **L2D1-derivative** determination of ratio of (unmodified D1, n=0, retention time = 20.90 min) : (mono-acylated D1, n=1, retention time = 18.23 min) : (bis-acylated D1, n=2, retention time = 16.64 min) = [(n=0) : (n=1) : (n=2)] = 87:12:1.

Example 3

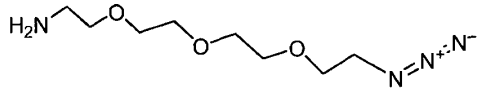
Functionalization of hyaluronic acid

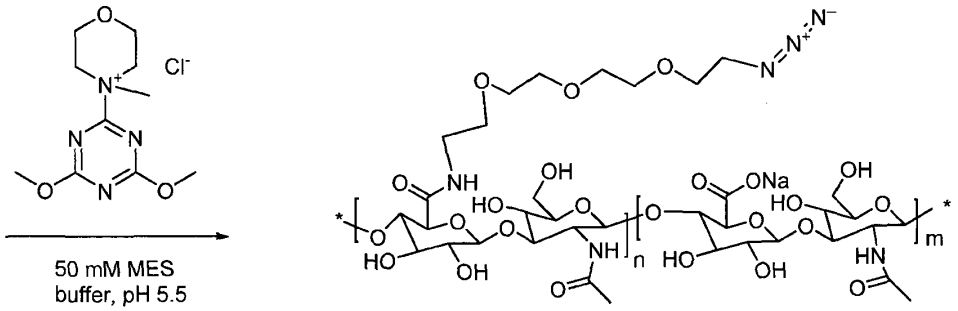
This example describes the synthesis of a functionalized hyaluronic acid, which may itself be a carrier or may also be reacted with a crosslinking moiety to form a hydrogel.

Synthesis of hyaluronic acid intermediate [HA-N3]:



Hyaluronic acid (HA), 200 kD





[HA-N_3]

Hyaluronic acid sodium salt is a linear polymer consisting of repeating dimeric units of glucuronic acid and N-acetylgalactosamine, with a repeating unit molecular weight of 401.3 Da. In this example the moles of hyaluronic acid reported refers to the moles of repeating unit and the equivalents of reagents used in the reaction with hyaluronic acid are reported relative to the moles of hyaluronic acid repeating unit. The average molecular weight of the polymer determines the average number of repeat units per polymer strand. Hyaluronic acid sodium salt labeled by the supplier, Lifecore Biomedical (HA200K, Chaska, MN) as having a nominal average molecular weight of 200kDa may vary from batch to batch in the range of 151–300 kDa, as determined by viscometry. In this example, such a molecule of hyaluronic acid sodium salt with an assumed nominal average molecular weight of 200 kDa would have an average length of approximately 500 monomer units.

Synthesis of [HA-N3]-23%

A solution of hyaluronic acid, sodium salt (nominal average molecular weight 200 kD; 250.1 mg, 0.623 mmol; Lifecore Biomedical, LLC; product number HA200K) was fully dissolved in 25 mL of MES buffer (50 mM, pH 5.5). To this solution was added 4-(4′,6′-dimethoxy-1′,3′,5′-triazin-2′-yl)-4-methylmorpholin-4-ium chloride (DMTMM, 295 mg, 1.07 mmol, 1.71 eq), followed after 5 min by addition of 11-azido-3,6,9-trioxaundecan-1-amine (N3-PEG3-NH2, 196 mg, 0.90 mmol). The reaction was stirred overnight, then diluted with 25 mL of 0.25 M NaCl solution and purified by tangential flow filtration.

Tangential flow filtration was carried out using a 30 kDa MWCO Vivaflow-50R hydrosart cartridge from Sartorius, eluting with 400 mL of 0.25 M NaCl solution, then 400 mL of water.

The product was flash frozen and lyophilized to provide the title material **[HA-N3]-23%**.

^{1}H NMR (400 MHz, D_2O) δ 4.45 (bs, 2H), 4.0–3.1 (m, 15.5H), 1.95 (s, 3H).

DOSY-NMR. One dimensional diffusion ordered NMR spectra (DOSY) were collected using the stimulated echo with one spoil gradient pulse sequence (stegp1s1d) on a Bruker AVANCE III 400 MHz (for ^{1}H) instrument with 5 mm DCH cryoprobe. The diffusion time and the diffusion gradient time were set to 50 ms and 4 ms, respectively. Two spectra were collected with gradient strength (gpz6) set to 2% and 95%. Comparison of the two spectra showed no differences apart from the solvent peak, indicating no small molecule impurities were present in the polymer.

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Elem. Anal: C: 40.05%: H: 5.67%; N: 5.70%.

The degree of substitution of the **[HA-N3]** is defined as the % of repeat units in which the carboxylate moiety has undergone reaction to give the depicted amide. Elemental analysis was used to determine the degree of substitution. The [%C/%N] ratio determined by elemental analysis of a purified sample is entered into the following formula to provide the degree of substitution. Where y = [(%C) / (%N)]) then:

Degree of substitution =

$$100 \times \frac{\frac{14 \times 12.01}{14.01 \times y} - 1}{4 - \frac{8 \times 12.01}{14.01 \times y}}$$

In this example, the degree of substitution (DS) of **[HA-N3]** was 23%.

This 200 kDa hyaluronic acid, functionalized with 23% of the azide linker is labeled **[HA-N3]-23%**.

In the rest of the examples, a 200 kDa hyaluronic acid, functionalized with X% of the azide linker is labeled **[HA-N3]-X%**.

The species in Table 13 were prepared and characterized using methods analogous to those used in the synthesis of **[HA-N3]-23%**. We find that the degree of substitution achieved depends on the given stock of DMTMM reagent used and can be idiosyncratic. In general, for a given stock of DMTMM, the degree of substitution increases as the number of equivalents of DMTMM used increases, and the degree of substitution decreases as the number of equivalents of DMTMM used decreases. Some of the [HA-N3] intermediates were purified by dialysis instead of tangential flow filtration (**[HA-N3]-23%b**, **[HA-N3]-37%**). In these cases, a crude reaction mixture was filled into a regenerated cellulose dialysis membrane (MWCO 1–25 kD), and dialyzed 1–3 days against 0.25 M–1 M NaCl, with several changes of the dialysis solution, followed by 1–3 day's dialysis against deionized water, also with multiple changes of the dialysis solution. Upon completion, the sample was removed from the dialysis tubing, flash-frozen, and lyophilized.

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Table 13. Exemplary Functionalized Hyaluronic Acid Species			
Hyaluronic acid derivative	**DS**	**Equivalents of DMTMM/N3-PEG3-NH2 used in reaction**	**Elemental analysis**
[HA-N3]-15%	15%	1.66/1.42	C: 39.80 H: 5.52 N: 4.93
[HA-N3]-19%	19%	1.63/1.08	C: 37.19 N: 4.88
[HA-N3]-22%	22%	1.65/1.42	C: 39.89 H: 5.76 N: 5.53
[HA-N3]-23%	23%	1.71/1.44	C: 40.05 H: 5.67 N: 5.70
[HA-N3]-23%b	23%	4.08/3.04	C:36.93 H: 5.97 N: 5.18
[HA-N3]-23%c	23%	1.60/1.02	C: 36.85 H: 6.44 N: 5.19
[HA-N3]-26%	26%	1.90/1.44	C: 39.78 H: 5.72 N: 5.92
[HA-N3]-33%	33%	1.75/1.43	C: 40.60 H: 5.97 N: 6.63
[HA-N3]-37%	37%	3.84/2.88	C: 39.87 H: 5.92 N: 6.82

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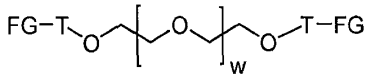
Example 4

Preparation of PEG polymers, XL-1–XL-10

This example describes the synthesis of crosslinkers that may be reacted with other functionalized polymers to form hydrogels.

PEG-based cross-linking polymers

General structure 1.



In some aspects, w can be 22, 45, 91, 136, 181, or 226 and correspond to a PEG having a nominal average molecular weight of about 1 kDa, about 2 kDa, about 4 kDa, about 6 kDa, about 8 kDa, or about 10 kDa, respectively.

General structure 2.

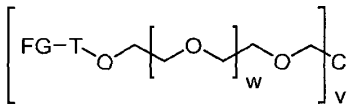
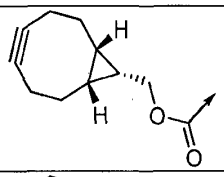
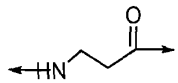
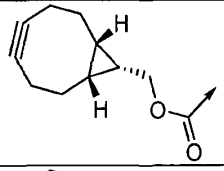
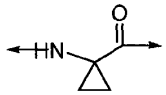
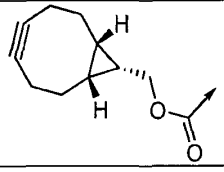
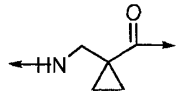
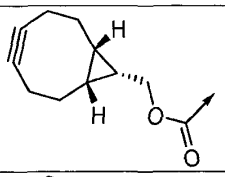
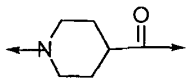
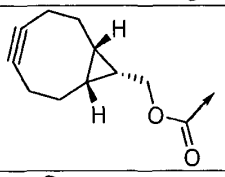
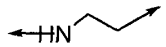
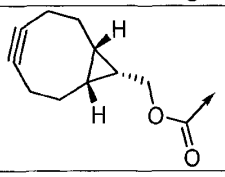
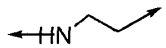
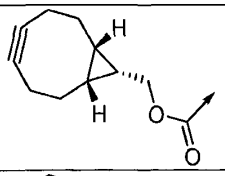
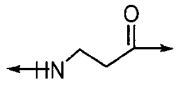
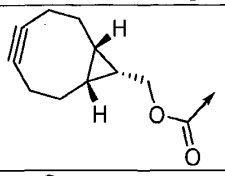
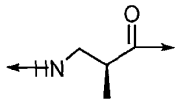
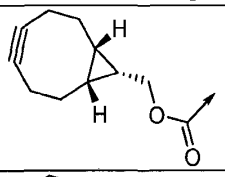
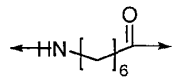
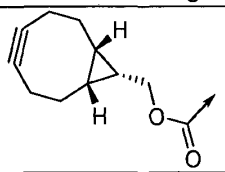
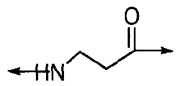
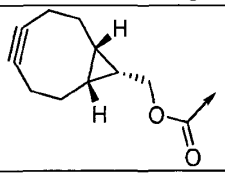
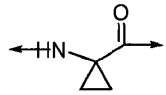


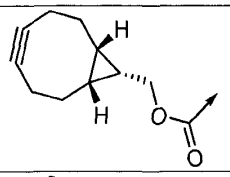
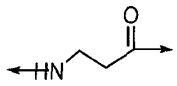
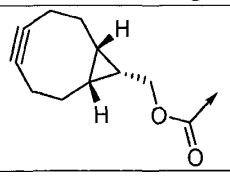
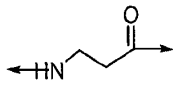
Table 14 Exemplary Crosslinking Species

Number	General structure	FG	T	v	M_n ~ starting PEG (Da)
XL-1	1			NA	2000 (w ~ 45)
XL-2	1			NA	2000 (w ~ 45)
XL-3	1			NA	2000 (w ~ 45)

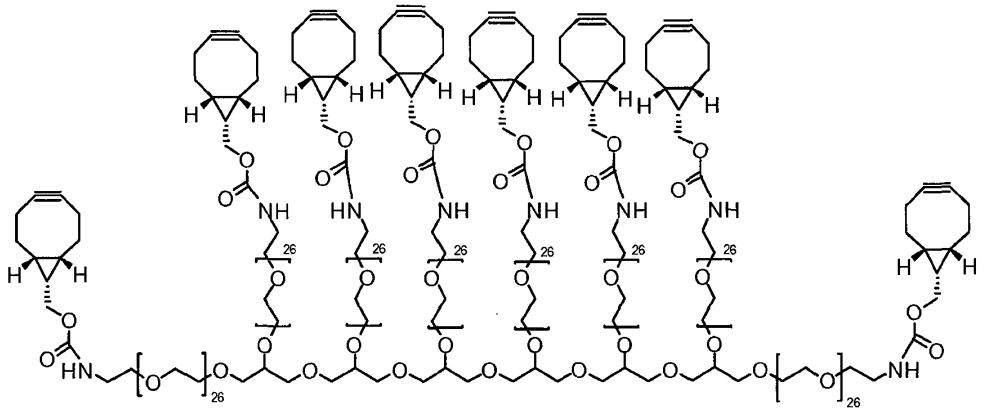
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XL-4	1			NA	2000 (w ~ 45)
XL-5	1			NA	2000 (w ~ 45)
XL-6	2			4	10000 (w ~ 226)
XL-7 (commercial product)	2	N_3	$-CH_2CH_2-$	4	10000 (w ~ 226)
XL-8 (shown below)	1			NA	10000 (w ~ 226)
XL-9	1			NA	2000 (w ~ 45)
XL-10	1			NA	2000 (w ~ 45)
XL-11	1			NA	1000 (w ~ 22)
XL-12	1			NA	4000 (w ~ 90)

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XL-13	1			NA	6000 (w ~136)
XL-14	1			NA	8000 (w ~ 181)

The structure of **XL-8**:



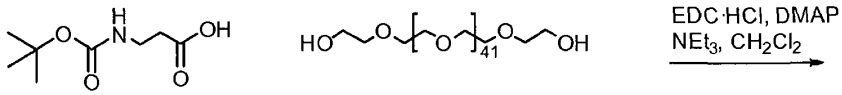
MW of starting PEG: M_n ~ 10,000 kDa.

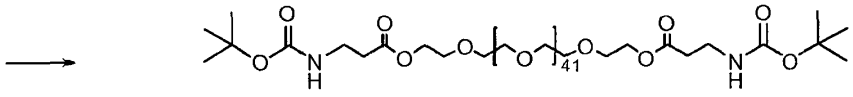
Synthesis of PEG-based Cross-Linking Polymers

Degree of substitution is an important parameter for PEG-derived cross-linkers and is defined as the percentage of PEG end groups substituted with the desired introduced functional group. Degree of substitution was determined using ^{1}H-NMR to compare the integration of a methylene group specific to the PEG end group to the integration of protons specific to the introduced functional group. For **XL-1–XL-6**, the methylene group specific to the PEG moiety was the ester or carbamate methylene [–(C=O)-O-CH_2-], which was compared to the integration of protons in the bicyclo[6.1.0]non-4-yne moiety. Degree of substitution = ((moles introduced functional group by ^{1}H-NMR) / (moles PEG end group by ^{1}H-NMR)) × 100.

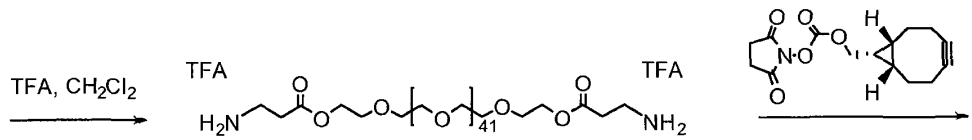
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XL-1. PEG(2000)-bis-[3-(((((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methoxy)carbonyl)amino)propanoate]

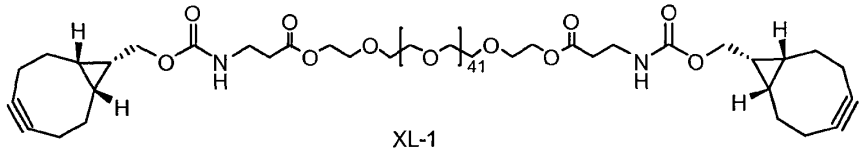




XL-1a

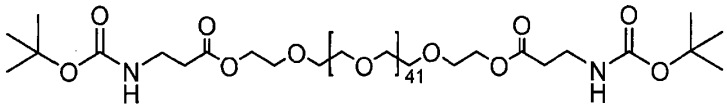


XL-1b



XL-1

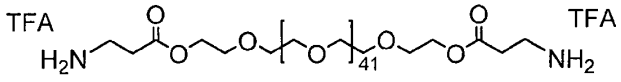
XL-1a. PEG(2000)-bis-[3-((tert-butoxycarbonyl)amino)propanoate]



3-((tert-butoxycarbonyl)amino)propanoic acid (1.15 g, 6.0 mmol) and M_n ~ 2 kDa PEG (3 g, 1.500 mmol) were dissolved in 30 mL dichloromethane. Dimethylaminopyridine (0.092 g,

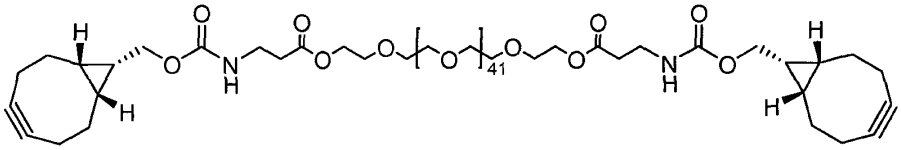
0.750 mmol) and EDC·HCl (1.211 g, 6.0 mmol) were added and the reaction mixture was stirred at room temperature overnight. The crude product was purified by flash column chromatography (with a ELSD detector) on silica with a 0–20% dichloromethane : dichloromethane / methanol (4: 2) gradient. The product containing fractions were pooled and concentrated to dryness to provide **XL-1a**. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 6.83 (t, *J* = 5.7 Hz, 2H), 4.16-4.09 (m, 4H), 3.61-3.56 (m, 5H), 3.51 (s, 184H), 3.15 (q, *J* = 6.7 Hz, 4H), 2.43 (t, *J* = 7.0 Hz, 4H), 1.37 (s, 18H).

XL-1b. PEG(2000)-bis-[3-(amino)propanoate], bis-trifluoroacetic acid



XL-1a (3.12 g, 1.357 mmol) was dissolved in dichloromethane (36 mL). Trifluoroacetic acid was added (36 mL) and the reaction mixture was stirred at room temperature for 2 hours. The solvent was removed under reduced pressure. The crude product was successively triturated with diethyl ether and filtered off. The operation was repeated three times and the resulting solid was dried under vacuum to provide **XL-1b**. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 7.80 (s, 4H), 4.24-4.11 (m, 4H), 3.72-3.32 (m, 182H), 3.09-2.95 (m, 4H), 2.67 (t, *J* = 6.9 Hz, 4H).

XL-1-1. PEG(2000)-bis-[3-(((((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methoxy)carbonyl)amino)propanoate]



XL-1b (600 mg, 0.286 mmol) was dissolved in acetonitrile (7 mL). Triethylamine (1.99 mL, 14.3 mmol) was added followed by ((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2,5-dioxopyrrolidin-1-yl) carbonate (333 mg, 1.14 mmol) and the reaction mixture was stirred at room temperature for 2 hours. The reaction mixture was directly purified by flash column chromatography (with a ELSD detector) on silica with a 0–40% dichloromethane : dichloromethane / methanol (4: 2) gradient. The product containing fractions were pooled and

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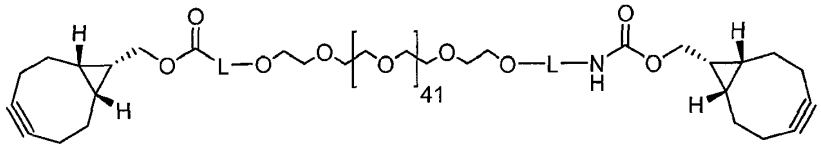
concentrated to dryness to afford **XL-1-1**. For storage purposes, **XL-1-1** was kept as a solution in acetonitrile (50 mg/mL) in placed in a freezer. Analytical UPLC (method B, see below): retention time = 1.19 min. ^{1}H NMR (500 MHz, MeOH-*d4*) δ 4.23 (m, 4H), 4.14 (m, 4H), 3.64 (br s, 214H), 2.55 (m, 4H), 2.22 (m, 12H), 1.61 (m, 4H), 1.35 (m, 2H), 0.92 (m, 4H).

Method A: analytical HPLC with CAD detector: Waters XBridge BEH300 C18; particle size: 3.5 μm; column size: 4.6 × 100 mm; eluent/gradient: 2% CH_3CN/H_2O/0.5 min, 2–98% CH_3CN/H_2O/17.5 min (CH_3CN containing 0.05% TFA and H_2O containing 0.1% TFA); flow rate: 1 mL/min; column temperature: 50 °C.

Method B: analytical UPLC with ELSD detector: Waters ACQUITY UPLC HSS T3; particle size: 1.8 μm; column size: 2.1 × 50 mm; eluent/gradient: 5–98% CH_3CN/H_2O in 1.4 min (CH_3CN containing 0.04% FA and H_2O containing 0.05% FA + 3.75 mM AA); flow rate: 1 mL/min; column temperature: 60 °C.

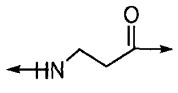
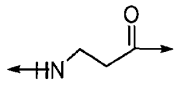
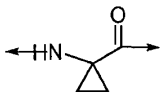
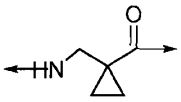
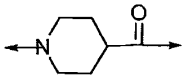
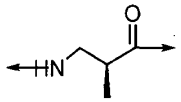
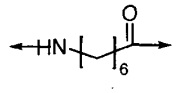
Method C: analytical UPLC with CAD: Waters ACQUITY BEH C18; particle size: 1.7 μm; pore size: 130Å; column size: 2.1 × 50 mm; eluent/gradient: 5% CH_3CN/H_2O/1.2 min, 5–95% CH_3CN/H_2O/1.8 min (CH_3CN containing 0.1% TFA and H_2O containing 0.1% TFA); flow rate: 1 mL/min; column temperature: 45 °C.

The species in Table 15 were prepared using methods analogous to those used in the synthesis of **XL-1** (except for **XL-1-2**):



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Table 15. Exemplary PEG-based Cross-Linking Polymers

Number	T	Number of PEG-end groups substituted with functional group	Anal Method / Retention time (min)	^{1}H NMR (400 MHz, Methanol-d4)
XL-1-1		2 of 2 100%	B 1.19 min	(500 MHz) δ 4.23 (m, 4H), 4.14 (m, 4H), 3.64 (br s, 214H), 2.55 (m, 4H), 2.22 (m, 12H), 1.61 (m, 4H), 1.35 (m, 2H), 0.92 (m, 4H).
XL-1-2		(1.5-1.8) of 2 75%	NA	δ 4.23 (m, 4H), 4.14 (m, 3.3H), 3.63 (br s, 156H), 2.55 (m, 4H), 2.21 (m, 9.6H), 1.60 (m, 3.6H), 0.94 (m, 3.9H).
XL-2		2 of 2 100%	B 1.20 min	δ 4.30-4.14 (m, 8H), 3.66 (s, 186H), 2.35-2.13 (m, 12H), 1.64 (d, *J* = 12.2 Hz, 4H), 1.51 (m, 4H), 1.41 (m, 2H), 1.17 (m, 4H), 1.02-0.93 (m, 4H).
XL-3		2 of 2 100%	A 12.93 min	δ 4.19 (m, 8H), 3.85-3.43 (m, 192H), 3.36 (br s, 4H), 2.22 (m, 12H), 1.60 (m, 4H), 1.38 (m, 2H), 1.19 (m, 4H), 0.95 (m, 8H).
XL-4		2 of 2 100%	A 13.25 min	δ 4.22 (m, 8H), 4.02 (m, 4H), 3.84-3.43 (m, 194H), 3.00 (br s, 4H), 2.60 (m, 2H), 2.23 (m, 12H), 1.91 (m, 4H), 1.61 (m, 8H), 1.42 (m, 2H), 0.97 (m, 4H).
XL-9		2 of 2 100%	C 1.85 min	δ 4.29-4.19 (m, 4H), 4.14 (d, J = 8.1 Hz, 4H), 3.87-3.41 (m, 179H), 3.28-3.18 (m, 3H), 2.78-2.62 (m, 2H), 2.37-2.06 (m, 12H), 1.72-1.51 (m, 4H), 1.47-1.31 (m, 2H), 1.21-1.09 (m, 6H), 1.04-0.87 (m, 4H).
XL-10		2 of 2 100%	C 1.96 min	δ 4.25-4.18 (m, 4H), 4.13 (d, *J* = 8.3 Hz, 4H), 3.85-3.40 (m, 178H), 3.09 (t, *J* = 7.0 Hz, 4H), 2.34 (t, *J* = 7.5 Hz, 4H), 2.31-2.09 (m, 11H), 1.72-1.54 (m,

				8H), 1.54-1.44 (m, 4H), 1.44-1.25 (m, 11H), 1.03-0.85 (m, 4H).

Intermediates used in the synthesis of PEG-based cross-linking polymers:

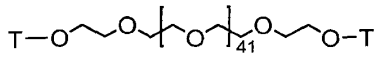
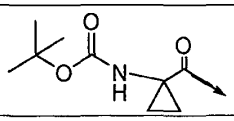
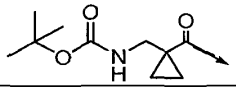
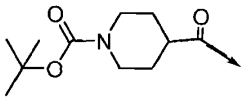
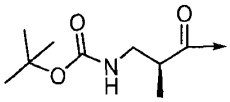
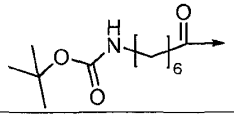
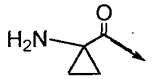
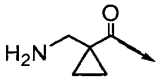
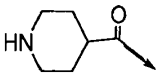
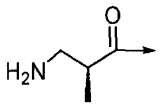
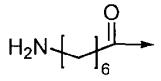
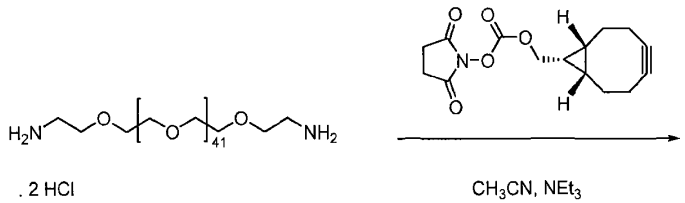


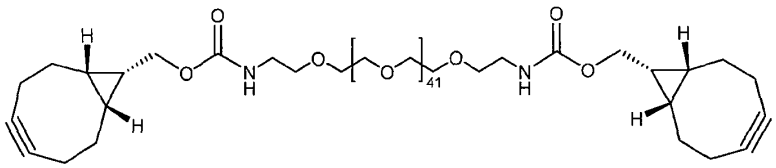
Table 16. Exemplary PEG-based Cross-Linking Polymers

Number	T	Number of PEG-end groups substituted with functional group	^{1}H NMR (400 MHz)
XL-2a		2 of 2 100%	(chloroform-d) δ 4.23 (m, 4H), 3.64 (br s, 165H), 1.52 (br m, 4H), 1.45 (br s, 18H), 1.16 (br m, 4H).
XL-3a		2 of 2 100%	(methanol-d4) δ 4.23 (m, 4H), 3.83-3.41 (m, 181H), 1.44 (br s, 18H) 1.17 (m, 4H), 0.94 (m, 4H).
XL-4a		2 of 2 100%	(methanol-d4) δ 4.23 (m, 4H), 3.95 (m, 4H), 3.85-3.41 (m, 180H), 2.92 (br s, 4H), 2.57 (m, 2H), 1.89 (m, 4H), 1.56 (m, 4H), 1.45 (m, 18H).
XL-9a		2 of 2 100%	(methanol-d_4) δ 4.31-4.17 (m, 4H), 3.83-3.44 (m, 179H), 3.27-3.15 (m, 4H), 2.72-2.61 (m, 2H), 1.50-1.39 (m, 18H), 1.14 (d, J = 7.2 Hz, 6H).
XL-10a		2 of 2 100%	(methanol-d_4) δ 4.27-4.15 (m, 4H), 3.83-3.44 (m, 180H), 3.04-2.99 (m, 4H), 2.34 (t, J = 7.3 Hz, 4H), 1.68-1.57 (m, 4H), 1.55-1.40 (m, 22H), 1.39-1.27 (m, 9H).
XL-2b	 Bis-TFA salt	2 of 2 100%	(chloroform-d) δ 4.23 (m, 4H), 3.64 (br s, 165H), 1.55 (br m, 4H), 1.19 (br m, 4H).
XL-3b	 Bis-TFA salt	2 of 2 100%	(methanol-d4) δ 4.33 (m, 4H), 3.88-3.42 (m, 184H), 2.91 (br m, 4H), 3.22 (br s, 2H), 1.41 (m, 4H), 1.15 (m, 4H),
XL-4b	 Bis-TFA salt	2 of 2 100%	(methanol-d4) δ 4.30 (m, 4H), 3.85-3.54 (m, 178H), 3.39 (m, 4H), 3.15, (m, 4H), 2.79 (m, 2H), 2.14 (m, 4H), 1.95 (m, 4H).

XL-9b	 Bis-TFA Salt	2 of 2 100%	(methanol-d_4) δ 4.56-4.43 (m, 2H), 4.32-4.20 (m, 2H), 3.92-3.41 (m, 180H), 3.25-3.12 (m, 4H), 2.99-2.84 (m, 2H), 1.28 (d, J = 7.0 Hz, 6H).
XL-10b	 Bis-TFA Salt	2 of 2 100%	(methanol-d_4) δ 4.25-4.19 (m, 4H), 4.10-3.41 (m, 180H), 3.04-2.90 (m, 4H), 2.44-2.30 (m, 4H), 1.77-1.55 (m, 8H), 1.54-1.33 (m, 8H).

XL-5. PEG(2000)-bis-[3-(((((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methylcarbamoyl]





XL-5

M_n ~ 2 kDa PEG diamine hydrochloride (JenKem Technology, 300 mg, 0.148 mmol) was dissolved in acetonitrile (3 mL). Triethylamine (0.413 mL, 2.96 mmol) was added, followed by ((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2,5-dioxopyrrolidin-1-yl) carbonate (345 mg, 1.184 mmol) and the reaction mixture was stirred at room temperature for 4 h. The reaction mixture was directly purified by preparative reverse phase HPLC with ELSD triggered fraction collection (method below). The product containing fractions were pooled, frozen, and lyophilized to provide **XL-5**. For storage purposes, **XL-5** was kept as an acetonitrile, DMSO, or methanol solution in a freezer. Analytical HPLC-CAD (method below): retention time = 11.85 min. NMR

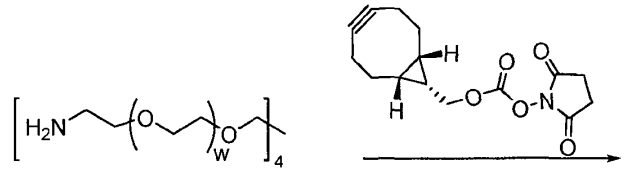
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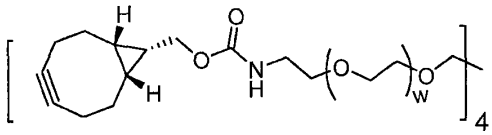
(400 MHz, MeOH-*d*4) δ 4.14 (m, 4H), 3.63 (br s, 186H), 3.54 (m, 4H), 2.22 (m, 12H), 1.61 (m, 4H), 1.38 (m, 2H), 0.94 (m, 4H). Exchangeable protons are not visible in MeOD.

Preparative HPLC conditions: Waters XBridge C18; particle size: 5 μm; column size: 19 × 250 mm; eluent/gradient: 5% CH_3CN/H_2O/0.5 min, 5–95% CH_3CN/H_2O/12.5 min, 95% CH_3CN/H_2O/3 min; flow rate: 30 mL/min; column temperature: room temperature.

Analytical HPLC-CAD conditions: Waters XBridge BEH300 C18; particle size: 3.5 μm; column size: 4.6 × 100 mm; eluent/gradient: 2% CH_3CN/H_2O/0.5 min, 2-98% CH_3CN/H_2O/17.5 min (CH_3CN containing 0.05% TFA and H_2O containing 0.1% TFA); flow rate: 1 mL/min; column temperature: 50 °C.

XL-6. PEG(10000)-tetra-[3-(((((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methylcarbamoyl]





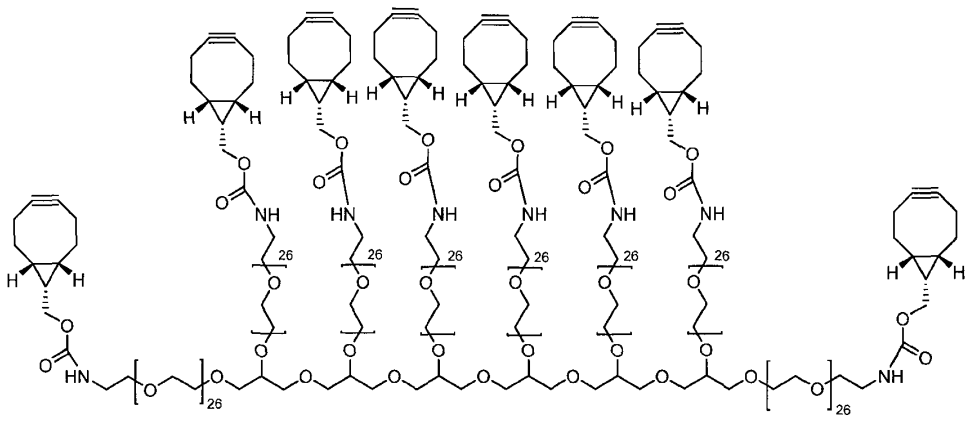
XL-6

M_n ~ 10 kDa 4-arm PEG amine hydrochloride (pentaerythritol core, JenKem Technology, 500 mg, 0.05 mmol) was dissolved in dichloromethane (15 mL). Triethylamine (0.554 mL, 4.00 mmol) was added, followed by ((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2,5-dioxopyrrolidin-1-yl) carbonate (175 mg, 0.600 mmol) and the reaction mixture was stirred at room temperature overnight. The reaction mixture was directly purified by dialysis against methanol using a 2 kDa MWCO Spectra/Por 6 regenerated cellulose dialysis membrane (Spectrum, Inc.). For analysis and utilization in further reactions, **XL-6** was isolated by

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concentration using a roto evaporator. For storage purposes the compound was kept as a methanol solution at room temperature. ^{1}H NMR (400 MHz, MeOH-*d*4) δ 4.14 (m, 8H), 3.63 (br s, 995H), 2.22 (m, 24H), 1.61 (m, 8H), 1.38 (m, 4H), 0.94 (m, 8H). Exchangeable protons are not visible in MeOD.

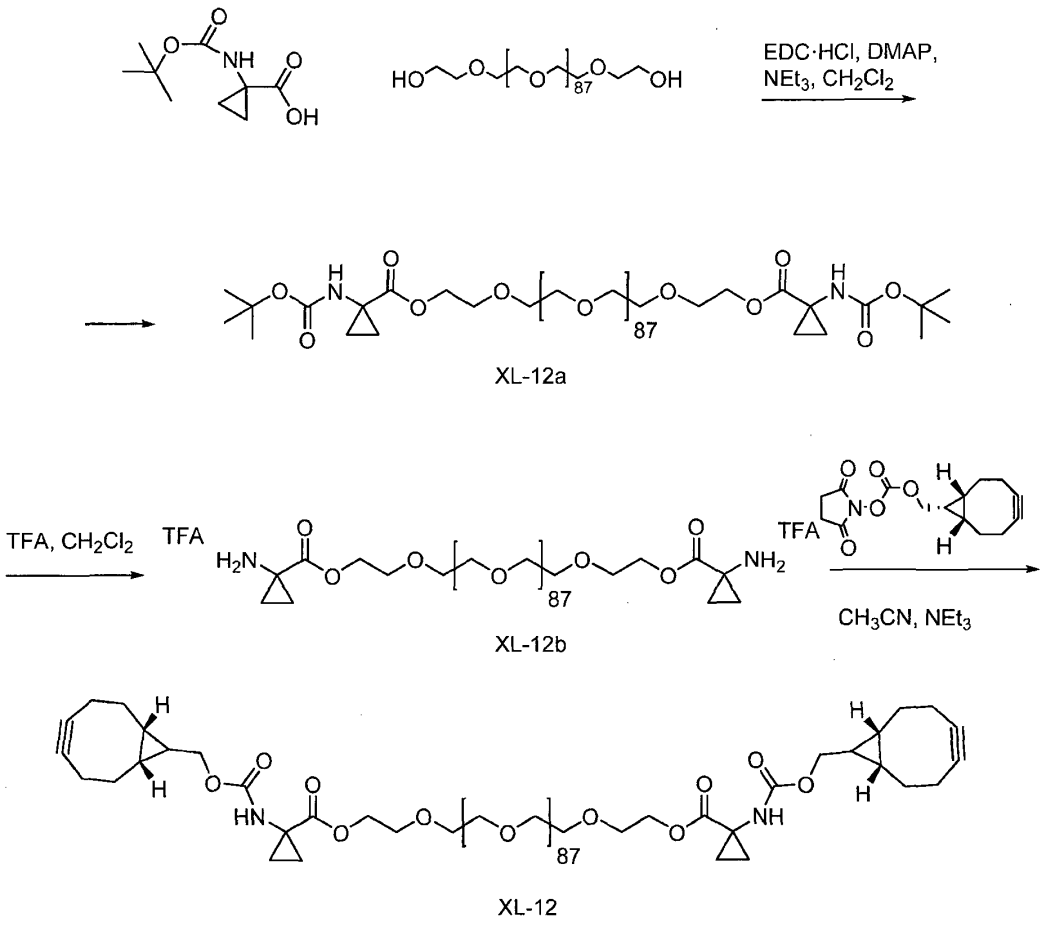
XL-8.



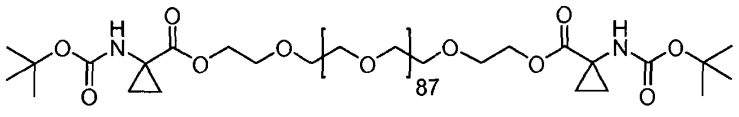
M_n ~ 10 kDa 8-arm PEG amine hydrochloride (hexaglycerol core, JenKem Technology, 800 mg, 0.08 mmol) was dissolved in dichloromethane (5 mL). Triethylamine (0.887 mL, 6.40 mmol) was added, followed by ((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2,5-dioxopyrrolidin-1-yl) carbonate (807 mg, 2.56 mmol) and the reaction mixture was stirred at room temperature for 5 h. The reaction mixture was concentrated using a rotoevaporator, then dissolved in methanol and purified by dialysis against methanol using a 2 kDa MWCO Spectra/Por 6 regenerated cellulose dialysis membrane (Spectrum, Inc.). For analysis and utilization in further reactions, **XL-8** was isolated by concentration using rotoevaporator, however for storage purposes **XL-8** was kept as a methanol solution at room temperature. ^{1}H NMR (400 MHz, MeOH-*d*4) δ 4.14 (m, 16H), 3.63 (br s, 935H), 2.23 (m, 48H), 1.61 (m, 16H), 1.38 (m, 8H), 0.95 (m, 16H). Exchangeable protons are not visible in MeOD.

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XL-12. PEG(4000)-((oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl) bis(1-(((((1*R*,8*S*)-bicyclo[6.1.0]non-4-yn-9-yl)methoxy)carbonyl)amino)cyclopropane-1-carboxylate)



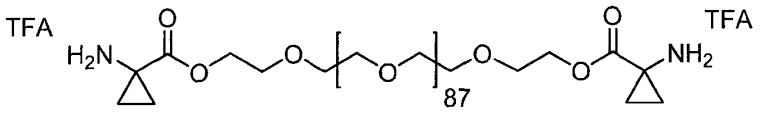
XL-12a. PEG(4000)-((oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl), bis(1-((tert-butoxycarbonyl)amino)cyclopropane-1-carboxylate)



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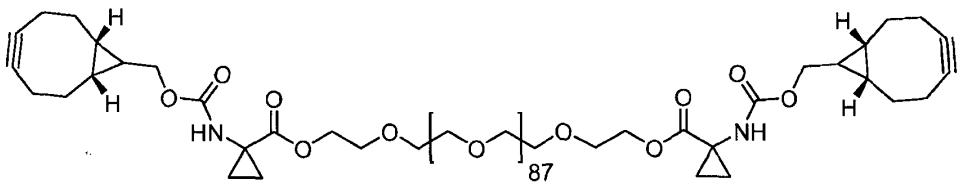
1-((tert-butoxycarbonyl)amino)cyclopropanecarboxylic acid (252 mg, 1.25 mmol) and M_n ~ 4 kDa PEG (1 g, 0.25 mmol) were dissolved in 10 mL dichloromethane. Dimethylaminopyridine (15 mg, 0.125 mmol) and EDC·HCl (192 mg, 1.0 mmol) were added and the reaction mixture was stirred at room temperature overnight. The crude product was purified by flash column chromatography (with a ELSD detector) on silica with a 0–100% Heptane : EtOAc, then 0-100% dichloromethane : dichloromethane / methanol (4: 2) gradient. The product containing fractions were pooled and concentrated to dryness to provide **XL-12a**. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.53 (s, 2H), 4.09 (t, 4H), 3.51 (s, 446H), 3.38-3.35 (m, 4H), 1.41-1.33 (m, 18H), 1.30 (d, 4H), 1.03-0.97 (m, 4H).

XL-12b. PEG(4000)-((oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl) bis(1-aminocyclopropane-1-carboxylate), bis-trifluoroacetic acid



XL-12a (1.03 g, 0.238 mmol) was dissolved in dichloromethane (4 mL). Trifluoroacetic acid was added (4 mL) and the reaction mixture was stirred at room temperature for 2 hours. The solvent was removed under reduced pressure. The crude product was successively triturated with diethyl ether and decanted off. The operation was repeated three times and the resulting solid was dried under vacuum to provide **XL-12b**. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.72 (s, 4H), 4.30-4.20 (m, 4H), 3.67-3.60 (m, 4H), 3.50 (s, 443H), 1.46-1.41 (m, 4H), 1.36-1.31 (m, 4H).

XL-12. PEG(4000)-((oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl) bis(1-(((((1*R*,8*S*)-bicyclo[6.1.0]non-4-yn-9-yl)methoxy)carbonyl)amino)cyclopropane-1-carboxylate)



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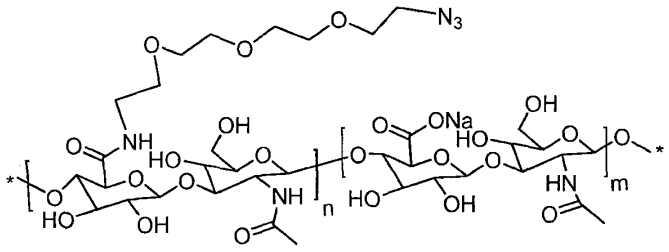
XL-12b (1.04 g, 0.238 mmol) was dissolved in acetonitrile (5 mL). Triethylamine (1.66 mL, 11.89 mmol) was added followed by ((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2,5-dioxopyrrolidin-1-yl) carbonate (277 mg, 0.952 mmol) and the reaction mixture was stirred at room temperature overnight. The reaction mixture was directly purified by flash column chromatography (with a ELSD detector) on silica with a 0–100% Heptane : EtOAc, then 0–100% dichloromethane : dichloromethane / methanol (4: 2) gradient. The product containing fractions were pooled and concentrated to dryness. Then, the solid was dissolved in dichloromethane and washed with deionized water. The organic layer was dried over phase separator to afford **XL-12**. For storage purposes, **XL-12** was kept as a solution in acetonitrile (50 mg/mL) and placed in a freezer. Analytical UPLC (method B, see above): retention time = 1.21 min. ^{1}H NMR (500 MHz, MeOH-d_4) δ 4.25-4.13 (m, 8H), 3.64 (s, 401H), 2.33-2.10 (m, 8H), 1.67-1.56 (m, 4H), 1.52-1.47 (m, 4H), 1.44-1.34 (m, 2H), 1.19-1.11 (m, 4H), 1.00-0.91 (m, 4H). Exchangeable protons are not visible in MeOD.

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Example 5

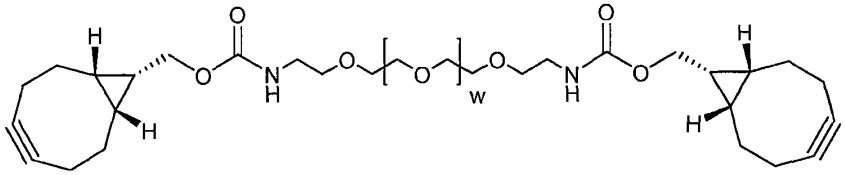
This example describes the synthesis of hydrogels prepared by reacting appropriately functionalized polymers with crosslinkers.

Synthesis of Hyaluronic Acid Hydrogel: H1g



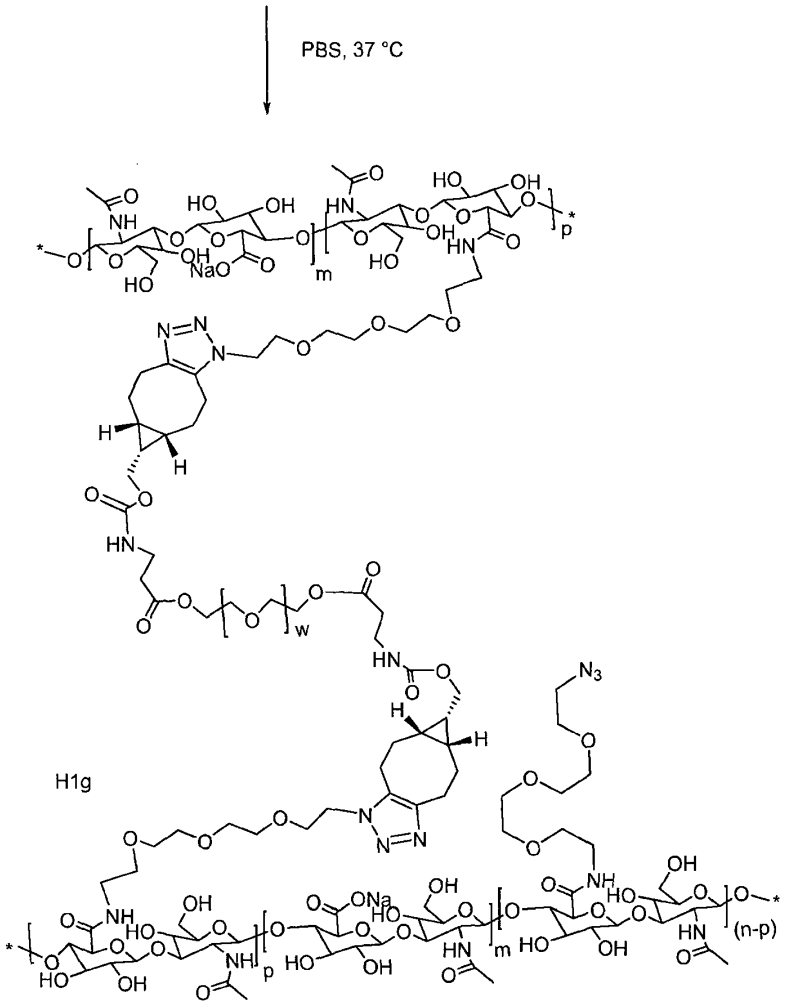
[HA-N_3]-19%

+



XL-1-1

PBS, 37 °C



[HA-N3]-19% (60 mg, 137 μmol, degree of substitution = 19%) was dissolved in 4.0mL sterile 1× PBS buffer, pH 7.4 at room temperature over 1 h in a 15 mL tube. The molecular weight of the unsubstituted carboxylate sodium salt repeat dimer unit is 401.3 Da. The MW of the azidylated repeat dimer unit is 579.6 Da. The average MW of a dimer unit for the sodium salt form of **[HA-N3]-19%** is 435.2 Da = ((401.3 × 0.81) + (579.6 × 0.19)). Using the average MW of a sodium salt dimer unit, the total moles of repeat dimer unit is 137 μmol and the number of moles of azidylated repeat dimer unit is 26 μmol.

To this solution was added a 50 mg/mL solution of **XL-1-1** (0.205 mL, 4.1 μmol of reagent assuming a MW of 2474 Da, 8.20 μmol of reactive functionality). This resulted in a solution

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which was 3% w/v with respect to **[HA-N3]-19%** and where 6% of the **[HA-N3]-19%** repeat units were predicted to be cross-linked by the **XL-1-1** ((8.20 μmol [XL-1-1-reactive functionality]/137 μmol [HA-units) × 100 = 6%).

The mixture was vortexed briefly to mix, then distributed into a 10 mL syringe, which was capped and held at 37 °C overnight to provide **H1g**. Visual inspection (inversion test) showed successful gelation.

Preparation of hyaluronic acid-based hydrogel particles

H1g, 2 mL, was forced through a 100 mesh stainless steel screen disc into a 5 mL syringe, yielding coarse gel particles. To this syringe 1 mL of 1× PBS was added, followed by vortexing to mix. The syringe was held at room temperature for 6 h to allow swelling of the hydrogel. This resulted in a mixture that was 1.5% with respect to **[HA-N3]-19%**. The swollen, coarse gel particles of **H1g** were forced 20 times through a 200-mesh stainless steel screen disc, yielding fine gel particles as the final product.

The hyaluronic acid-based hydrogels (and corresponding hydrogel particles) listed in Table 17 were prepared analogously to **H1g**. A hydrogel is defined by the **[HA-N3]** component used and its concentration in the crosslinking reaction, as well as by the PEG crosslinker used and the degree with which it is expected to form cross-links to the back bone polymer (% cross-link).

Table 17. Exemplary Hyaluronic Acid-based Hydrogels

Number	[HA-N_3]-X%	Conc of [HA-N3] in Hydrogel Reaction	XL-m	% cross-link in hydrogel
H1a	[HA-N_3]-23%	1.5%	**XL-1-1**	6.3
H1b	[HA-N_3]-23%	1.0%	**XL-1-1**	4.5
H1c	[HA-N_3]-37%	1.0%	**XL-1-1**	5.3
H1e	[HA-N_3]-22%	1.4%	**XL-1-1**	6.3
H1f	[HA-N_3]-23%	1.0%	**XL-1-1**	5.9
H1g	[HA-N_3]-19%	1.5%	**XL-1-1**	6.0
H2a	[HA-N_3]-19%	1.5%	**XL-2-1**	6.0
H12a	[HA-N_3]-19%	1.5%	**XL-12-1**	6.0

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Example 6

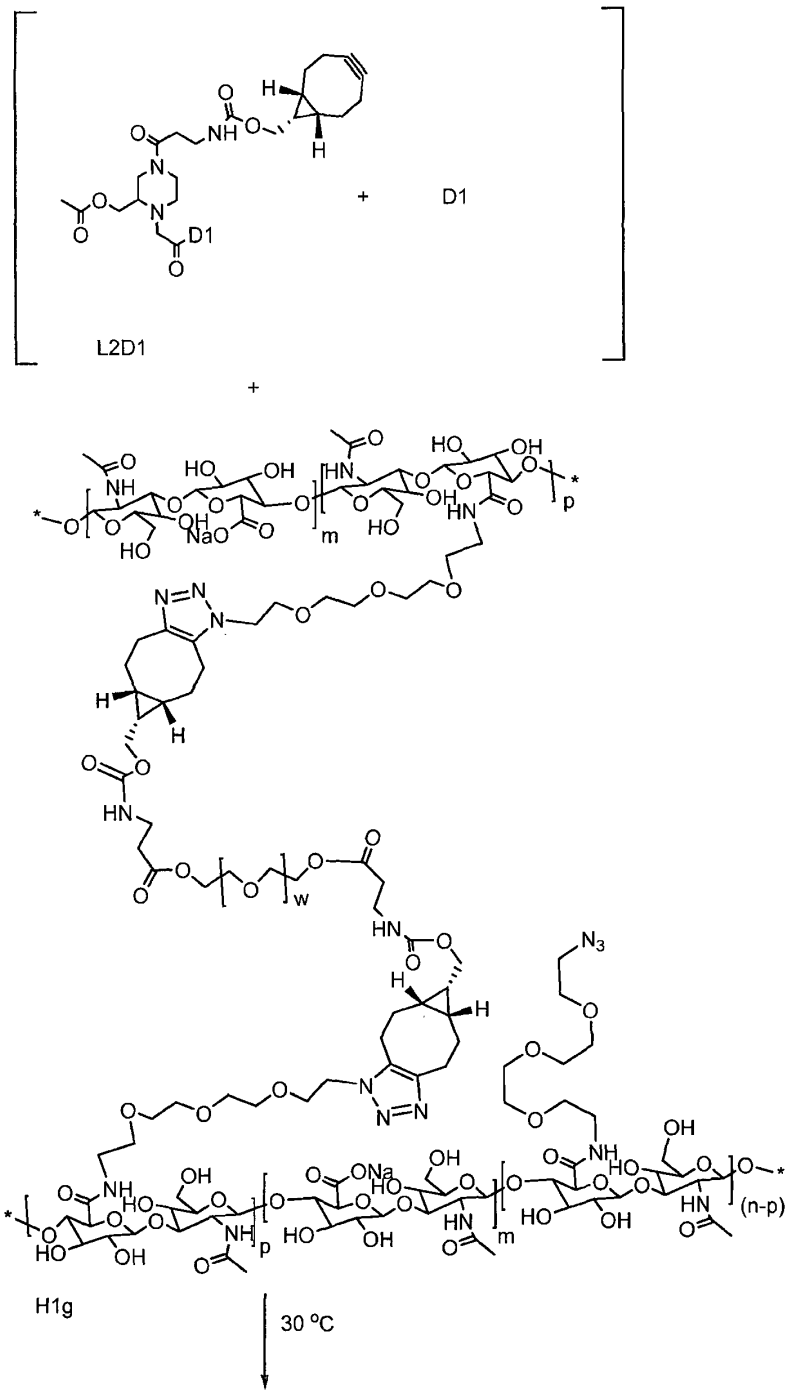
Synthesis of hydrogel-drug conjugates

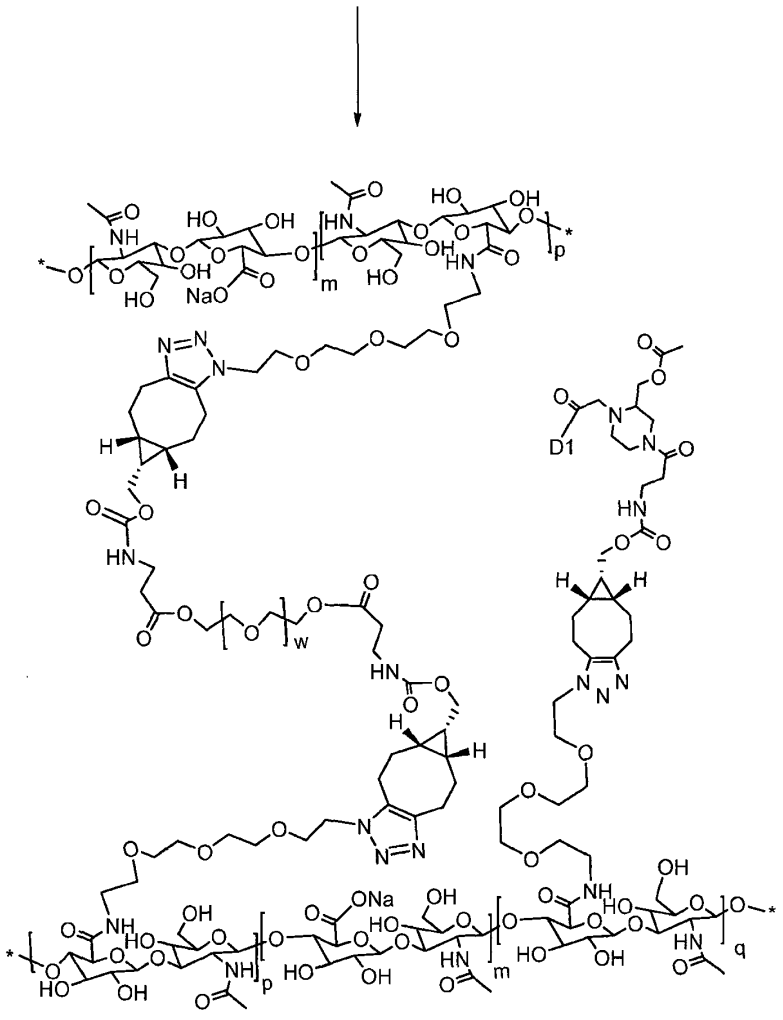
This example describes the synthesis of hydrogel-drug conjugates, in which the drug is conjugated to the carrier via a traceless linker.

When preparing samples for in vivo dosing, all manipulations of materials or solutions that were not capped took place in a laminar flow hood under aseptic conditions. All consumables used were previously unopened and were labeled "sterile."

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C1-2 (H1g-L2D1)





C1-2

Hydrogel particle suspension of **H1g** (1.08 g, 15 mg/mL, 19 mM total dimer repeat unit) was treated with a solution of **L2D1** (7 mL of 13.33 mg/mL solution in 20 mM histidine buffer (pH = 5.8)), vortexed and shaken overnight at 30 °C (600 rpm). The reaction tube was centrifuged (1500 rcf, 5 min) and the supernatant was removed from the tube using a needle and syringe. Fresh 1× PBS (10–15 mL) was added to the tube, which was shaken to resuspend the gel. Centrifugation, removal of supernatant, and dilution with fresh buffer was repeated. The washing continued until

no D1 was detected in the supernatant. A Nanodrop spectrophotometer used for detection of absorbance at 280 nm.

A key descriptor of hydrogel-drug conjugates is the amount of drug loaded per volume of hydrogel (= drug loading). When the drug is a protein, there are several methods to determine the loading.

Protein loading of the hydrogel was determined by forced release of the conjugated protein from a known volume of hydrogel, followed by quantification of the released protein. In this example: 20.0 mg of **[C1-2]** was weighed into a reaction tube. The sample was treated with 0.5M NaOH (4 mL, AVS Titrinorm, 31951.290), vortexed and shaken at 37 °C for 30 min. The concentration of released drug, D1 (SEQ ID NO:19) was measured, using a UV spectrophotometer, to be 11.68 mg/mL in this sample, corresponding to 11.68 mg D1/mL of hydrogel in **[C1-2]**. This analysis was carried out in duplicate. The average determined protein loading for **[C1-2]** prior to dilution was 12.14 mg of D1/mL of hydrogel.

In another method, 20.8 mg of **[C1-2]** was weighed into a reaction tube. The sample was treated with 1M Tris-HCl buffer pH = 9.5 (4 mL), vortexed and shaken at 37 °C for 24 hours (or until the measured concentration reached a steady state). The concentration of released drug D1 was measured, using a UV spectrophotometer, to be 13.3 mg/mL in this sample, corresponding to 13.3 mg D1/mL of hydrogel in **[C1-2]**. This analysis was carried out in triplicate. The average determined protein loading for **[C1-2]** prior to dilution was 12.72 mg of D1/mL of hydrogel.

In another method, 24.5 mg of **[C2-2]** was weight in a reaction tube and 2800 µL of water was added followed by 58.05 µL of a hyaluronidase solution (from Worthington, LS005475, 8140 U/mg). The reaction mixture was shaken for 3hr at RT and the absorption was measured, using a UV spectrophotometer, to be 19.68 mg/mL in this sample. This analysis was carried out in triplicate. The average determined protein loading for **[C2-2]** prior to dilution was 19.49 mg of D1/mL of hydrogel.

An alternate method of determining protein loading relies on difference calculations. In this method, the measured concentration of D1 (using a UV spectrophotometer) recovered in the hydrogel washes is subtracted from the known total added protein at the beginning of the experiment (for example: D1 + **L2D1**). The difference gives the D1 loading based on the volume of total hydrogel.

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The hydrogels in Table 18 were prepared using methods analogous to those used in the synthesis of **C1-2**.

Table 18. Exemplary Hydrogel-Drug Conjugates

Number	Study	Linker-drug adduct used	Hydrogel used	Drug loading (mg/mL)	Analytical method
C1-2	–	**L2D1**	**H1g**	12.1	Forced release (NaOH)
C1-2	–	**L2D1**	**H1g**	11.8	Difference
C2-1	–	**L1D1**	**H2a**	18.5	Forced release (NaOH)
C2-2	In vitro release Example 8.1 In vivo release Example 9	**L2D1**	**H2a**	19.5	Forced release (Hyaluronidase)
C2-2	In vitro release Example 8.1 In vivo release Example 9	**L2D1**	**H2a**	18.9	Forced release (NaOH)
C2-5	–	**L5D1**	**H2a**	18.2	Difference
C12-2	–	**L2D1**	**H12a**	18.8	Forced release (NaOH)

The hydrogel-drug conjugate may be diluted with 1× PBS to attain the desired final drug concentration for dosing. The amount of 1× PBS to be added can be calculated by the following equation: (PBS diluent (mL)) = [(total drug (mg) / desired drug loading (mg/mL)] – (initial volume of conjugate (mL)).

Conjugate **C2-2** was dispensed for dosing in 0.5 mL insulin syringes with attached 30 G needles, by removing the plunger and backfilling with the desired volume of **C2-2**.

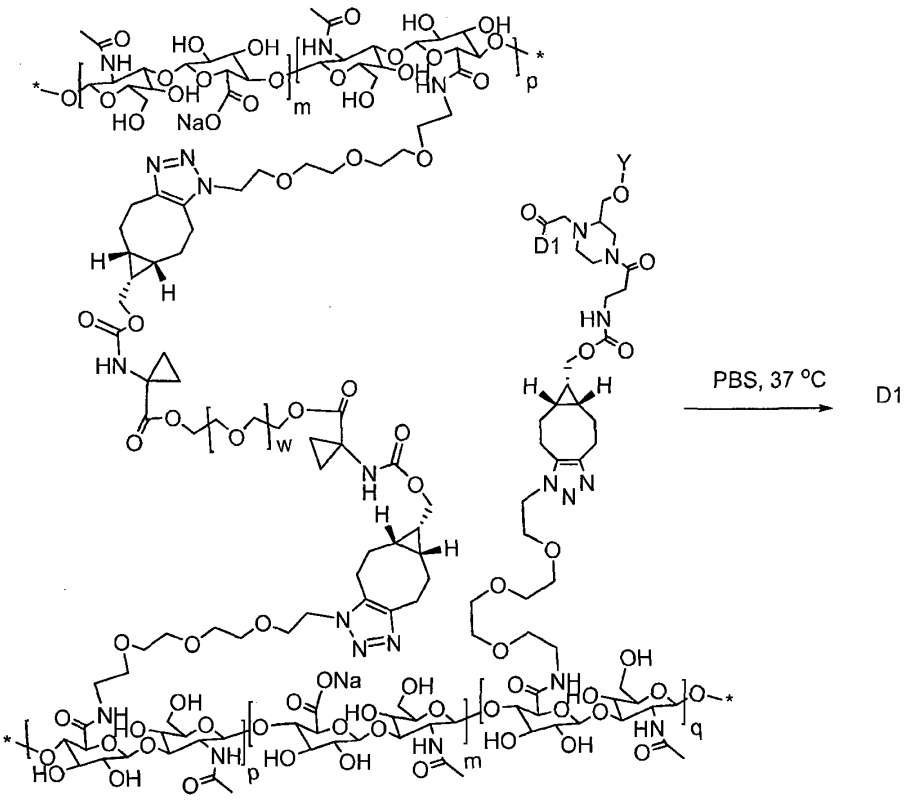
The hydrogel-drug conjugates were stored at RT.

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Example 7

In vitro drug release studies

7-1. Release of D1 (SEQ ID NO:19) from Hydrogel-Drug Conjugates



where Y is selected from Table 3, above.

Release of D1 (SEQ ID NO:19) from hydrogel conjugates **C1-2** and **C2-2** occurs as depicted in FIG. 4A. In this study a series of hydrogel-D1-conjugates was held in 1× PBS at 37 °C to assess the release of drug following the traceless linker cleavage reaction. These reactions were sampled at various timepoints and analyzed to determine the concentration of released D1 in the supernatant.

Each hydrogel (approximately 125 μL; exact amount determined by weighing, assumed density of hydrogel = 1 g/mL) was added into a Transwell® insert (Corning Inc; 6 well-plate, 24 mm diameter, polycarbonate membrane, 8.0 μm pore size). Wells of the plate were filled with 2.6 mL of PBS + (1%) Penicillin/Streptomycin. (Life Technologies, 10000 IU/μg/mL). Then, the

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hydrogel-containing inserts were placed into the wells and 1.375 mL of PBS + (1%) penicillin/streptomycin. was added on top of the hydrogel (in the insert). Three wells were prepared for each hydrogel sample. The plate was securely capped and held at 37 °C in a humid environment.

Samples of collection buffer were removed from the wells at various timepoints for up to 28 days. At each timepoint and for each well, the Transwell® insert was removed from the well, drip-dried and transferred to a new plate, in a well containing 3.6 mL of fresh 1x PBS + (1%) penicillin/streptomycin. The replenished plate was returned to the incubator. In the meantime, the entire volume of collection buffer was transferred to a clean tube and weighed, then a timepoint sample was removed from the collection buffer (0.10 mL to be used for analytical purposes) and the rest was stored at −80 °C until assaying.

Other buffers used in this assay are bovine synovial fluid (BSF), 1 M Tris·HCl solution (Teknova, pH 9.5) or Dulbecco's Modified Eagle Medium (Gibco, ref 41965) consisting in 1% fetal bovine serum (Gibco, ref 10270106), 12.5ug/ml insulin-transferrin-sodium selenite supplement (Roche, ref 11074547001), 50ug/ml L-ascorbic acid phosphate magnesium salt n-hydrate (Wako, ref 013-12061), 100 units/ml penicilin and 100 ug/ml streptomycin. The penicillin/streptomycin additive could be omitted if the experiment is run at 22 °C instead of 37 °C

For each timepoint samples, the protein concentration (which represents the protein concentration in each well at the timepoint) was determined by size-exclusion chromatography SEC preliminary calibrated with D1 (Instrument: Agilent LC 1260 Infinity; column: Superdex 200 increase 10/300GL (28-9909-44); Column temperature: r.t., flow rate: 0.75 mL/min; injection volume: 10 µL; mobile phase: 1/9 PBS/water preliminary filtered through a 0.10 µm membrane filter; run time: 40 min; detection wavelengths: 214, 280, and 680 nm). Each timepoint sample was analyzed in triplicate and the results were averaged.

The total amount of protein in a given well at a given timepoint was calculated by multiplying the protein concentration at the timepoint by the measured volume (assuming density of 1 g/mL). The cumulative D1 released from each hydrogel into each well up to that timepoint was calculated by adding the total amount of D1 in the well at each timepoint. The cumulative percent of protein released from conjugates **C2-2** up until a given timepoint is shown in FIG. 4B. The cumulative percent of D1 released is calculated by dividing the cumulative D1 released at a

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given timepoint by the total D1 present in the initial hydrogel sample and multiplying by 100. As evident from FIG. 4B, release of D1 from the carrier is slow but sustained over a period of one month.

Concentration of protein in a well at timepoint t:

$$[P](t)$$

Total volume in a well at timepoint t:

$$V(t)$$

Total mass of protein in a well at timepoint t:

$$m(P)_{well}(t) = [P](t) \times V(t)$$

Cumulative mass of protein released from hydrogel until timepoint t:

$$m(P)_{cum}(t) = \sum_{i=0}^{t} m(P)well$$

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Example 8

Comparative tissue tolerability of hydrogels

This study examined if hydrogels elicit a severe inflammatory response or any other adverse events over a period of 11-weeks after a single intra-articular injection in Lewis rats with knee osteoarthritis. The test articles were **H1a**, **H9a**, and Synvisc-One® hyaluronic acid-based injectable solution for treatment of osteoarthritis knee pain (Sanofi).

Preparation of H9a

Hyaluronic acid-based hydrogel particles of **H9a** were prepared as described for **H1a** (above, using cross-linker **XL-9-1**), where a half volume of 1× PBS was added to the particles following the first extrusion. Hydrogel particles of **H1a/H9a** were dispensed for dosing in 0.5 mL insulin syringes with attached 30 G needles by removing the plunger and backfilling with the desired volume of **H1a/H9a**. Air bubbles were removed by gentle manipulation of the syringe.

Synvisc-One®

Synvisc-One® was purchased and used as received.

Intra-articular injection

Male Lewis rats (LEW/OrlRj Janvier) underwent meniscal tear surgery of one knee. When knee osteoarthritis had developed due to joint stability about 7 days after the surgery, 25 µL of the test article or saline control were injected intra-articular in the operated knee joint under anesthesia (Table 19). One naive (not operated) rat served as control. In-life observations were recorded for 1–2 hours post dosing and at least once daily thereafter for one week. In the following weeks until necropsy, in-life observations were recorded once weekly. Body weights were taken weekly.

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Table 19. Study design, animal allocation and test article doses				
Group	***n***	**Treatment**	**Dose (μL)**	**Necropsy timepoint**
1.1	3	Synvisc-One®	25	4 weeks post injection
1.2	3	Synvisc-One®	25	6 weeks post injection
1.3	3	Synvisc-One®	25	8 weeks post injection
1.4	3	Synvisc-One®	25	11 weeks post injection
2.1	3	H1a	25	4 weeks post injection
2.2	3	H1a	25	6 weeks post injection
2.3	3	H1a	25	8 weeks post injection
2.4	3	H1a	25	11 weeks post injection
3.1	3	H9a	25	4 weeks post injection
3.2	3	H9a	25	6 weeks post injection
3.3	3	H9a	25	8 weeks post injection
3.4	3	H9a	25	11 weeks post injection
4	3	Saline	25	11 weeks post injection
5	1	—	—	12 weeks post study start

Rats were euthanized 4, 6, 8, or 11 weeks post intra-articular injection. Synovial fluid from the injected knee joint was collected by opening the knee joint and lavage with 25 μL saline. Macroscopic abnormalities were recorded during necropsy. The right operated/injected knees were collected from all animals, fixed in 10% neutral-buffered formalin, embedded in paraffin and sectioned. Hematoxylin and eosin and SafraninO/fast green stained knee joint sections were evaluated by a pathologist.

Results and Conclusions

The hydrogels did not cause any adverse events over 11 weeks when injected intra-articular in rat knee joints. The presence of hydrogel in the subsynovial space, blebbing of synovial cells into the joint space and proliferation of synovial and subsynovial cells was transient at early time points and less pronounced for HA hydrogel conjugates than for Synvisc-One®, which is marketed and frequently used for treatment of osteoarthritis knee pain (Sanofi).

Example 9

In vivo drug release study

This study examined the in vivo release profile of D1 from hydrogel drug conjugate C2-2 after intra-articular injection in rat knee joints.

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Preparation of C2-2

Hydrogel drug conjugate C2-2 was prepared as described in Example 6. D1 concentration in C2-2 was 19.0 mg/g, corresponding to a D1 dose of 0.475/joint.

Intra-articular injection

Both knee joints of 9 naïve male Lewis rats (LEW/OrlRj Janvier; 9 weeks old) were injected each with 25 µL of C2-2 under anesthesia. Blood/serum was collected at the time points indicated in Table 20. At time points 24, 72 and 168 hours after intraarticular injections, 3 rats each were euthanized. Synovial fluid and articular cartilage were collected from left knee joints, whole joint tissue from right knees.

Table 20

ER Formulation	Dose (mg/animal/joint)	Animal	Biological matrix collected	Time points post-dose
C2-2	0.25 / i.a.	01-03	Blood/Serum	0.5, 1, 3, 6, 24, 72,168 h
		01-03	SF; Cart; Knee	168 h
		04-06	B/S; SF; Cart; Knee	24 h
		07-09	B/S; SF; Cart; Knee	72 h

B/S: Blood /Serum; **SF**: Synovial fluid; **Cart:** Cartilage; **Knee:** Whole knee joints

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Synovial fluid from knee joints was collected by injecting 20 µL saline intra-articularly into the closed knee joint, moving the joint and collecting the fluid directly after opening the knee joint. Afterwards another 20 µL were injected into the open knee joint and collected. The lavage was repeated with another 20 µL saline resulting in a total lavage volume of 60 µL. The pre-weighed vial with the synovial fluid was centrifuged immediatelly after sampling to ensure that the hydrogel particles are located at the bottom of the tube. Afterwards 5 µL were removed from the pre-weighted vial before storing the main vial at -80°C. The separated 5 µL synovial fluid was kept as back-up sample to allow for analysis without hyaluronidase treatment and was also stored at -80°C. D1 concentrations were quantified in serum and synovial fluid by an ECLIA assay. The method is based on the capture of D1 using an anti-ANGPTL3 monoclonal antibody (Novartis, iProt109046) immobilized on a MSD plate. Captured D1 is detected using a biotinylated anti-ANGPTL3 polyclonal antibody (R&D Systems, BAF3829) followed by the incubation with Streptavidin-SulfoTag. Synovial fluid samples were either pre-treated with hyaluronidase (1500 U/mL) and incubated O/N at 4°C with shaking at 450 rpm before analysis or kept at -80°C without hyaluronidase pretreatment

Results and Conclusions

As shown in FIG. 5, C2-2 hydrogel provided prolonged serum exposure at very low levels for up to 1 week, as compared to the short and high systemic exposure observed with D1 immediate release formulation, which was no longer detectable at 1 week. C2-2 hydrogel intraarticular injection also resulted in high sustained D1 levels for at least one week in synovial fluid, which was hyaluronidase treated to release D1 from residual hydrogel in the synovial fluid. Free D1 levels detected in synovial fluid without hyaluronidase pretreatment were much lower, but also detectable for at least one week.

These results indicate that C2-2 hydrogel prolonged the release of D1 into synovial fluid for at least one week.

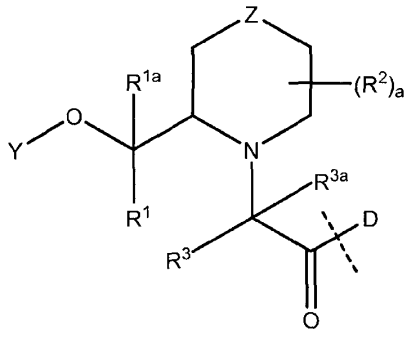
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CLAIMS

What is claimed is:

1. A drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (I), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and

R is a linker suitable for release of D:



(I),

where the dashed line indicates attachment to the primary amine;

R^1 is hydrogen or C_1-C_4alkyl;

R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination with the carbon atom(s) to which they are attached form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group;

a is 0, 1, 2, 3 or 4;

R^3 is hydrogen or C_1-C_4alkyl;

R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$;

R^{12} is hydrogen or C_1-C_4alkyl;

R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^{13} is C_1-C_4alkyl; or

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R^{12} and R^{13}, taken in combination with $C(R^{12a})$ and O form a 5, 6, or 7-member cyclic ether;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy;

R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl;

R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups;

b is an integer of from 1 to 10;

Z is CH-L-A, CH-A, N-L-A, or N-A;

L is an optionally substituted bivalent linker;

A is hydrogen, C_1-C_8alkyl, $C(O)C_1$-C_8alkyl, $C(O)N(H)C_1$-C_8alkyl, $C(O)OC_1$-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10};

R^{10} is a reactive functional group suitable for coupling Formula (I) to a carrier; and

R^{11} is a carrier.

2. The drug delivery system or pharmaceutically acceptable salt of claim 1, wherein D comprises an ANGPTL3 polypeptide having at least 95% identity to any one of SEQ ID NO: 1 or 3–45.

3. The drug delivery system or pharmaceutically acceptable salt of claim 1, wherein D comprises any one of SEQ ID NO:1 or 3–45.

4. The drug delivery system or pharmaceutically acceptable salt of claim 1, wherein D comprises an ANGPTL3 polypeptide having a K423Q substitution or a K423 deletion.

5. The drug delivery system or pharmaceutically acceptable salt of claim 1, wherein D comprises an ANGPTL3 polypeptide comprising amino acid residues 201–460; 207–460;

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225–455; 225–455; 225–460; 225–460; 226–455; 226–455; 226–460; 226–460; 228–455; 228–455; 228–460; 228–460; 233–455; 233–455; 233–460; 233–460; 241–455; 241–455; 241–460; 241–460; 242–455; 242–455; 242–460; or 242–460, each in reference to SEQ ID NO:1.

6. The drug delivery system or pharmaceutically acceptable salt of claim 1, wherein D comprises an ANGPTL3 polypeptide comprising at least 95% identity to amino acid residues 242–460 in reference to SEQ ID NO:1 and a K423Q substitution.

7. The drug delivery system or pharmaceutically acceptable salt of claim 1, wherein D comprises an ANGPTL3 polypeptide comprising amino acid residues 242–460 in reference to SEQ ID NO:1 and a K423Q substitution (SEQ ID NO:19).

8. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 7 wherein R^1 is hydrogen or methyl, R^{1a} is hydrogen or methyl, or CR^1R^{1a}, taken in combination form a cyclopropan-1,1-diyl group.

9. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 8, wherein the variable a is 0.

10. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 9, wherein R^3 and R^{3a} are each hydrogen.

11. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 10, wherein Y is $C(O)R^4$ and R^4 is C_1-C_6alkyl, C_3-C_6cycloalkyl or C_1-C_2alkoxyC_1-C_2alkyl.

12. The drug delivery system or pharmaceutically acceptable salt of claim 11, wherein R^4 is methyl, ethyl, propyl, isopropyl, 1-methyl-cyclopropyl, or methoxymethyl.

13. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 10, wherein Y is $SiR^5R^6R^7$;

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R^5 and R^6 are each methyl, ethyl, propyl or isopropyl; and

R^7 is C_1-C_4 alkyl, ethoxy, propoxy, isopropoxy, butoxy, iso-butoxy, sec-butoxy, 2-ethoxyethoxy, 2-isopropoxy-ethoxy, tetrahydropyranyloxy, or -$(OCHR^3CH_2)_bO$-C_1-C_4alkyl where b is 2, 3, or 4.

14. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 13, wherein Z is CH-L-A, CH-A, N-L-A, or N-A;

L is an optionally substituted bivalent linker Q-$[Sp\text{-}Q]_h$-Q;

Q is independently selected at each occurrence from a bond, O, C(O), N(H), N(C_1-C_4alkyl), C(O)NH, C(O)N(C_1-C_4alkyl), N(H)C(O), N(C_1-C_4alkyl)C(O), N(H)C(O)O, N(C_1-C_4alkyl)C(O)O, OC(O)N(H), OC(O)N(C1-C_4alkyl), N(H)C(O)N(H), N(C_1-C_4alkyl)C(O)N(H), N(H)C(O)N(C_1-C_4alkyl), N(C_1-C4alkyl)C(O)N(C_1-C_4alkyl), C(O)O, OC(O), OC(O)O, S, $S(O)_2$, N(H)$S(O)_2$, N(C_1-C_4alkyl)$S(O)_2$, $S(O)_2$N(H), $S(O)_2$N(C_1-C_4alkyl), C_1-C_2alkyl-C(O)N(H), N(H)C(O)C_1-C_2alkyl, C_1-C_2alkyl-C(O)O, OC(O)C_1-C_2alkyl, 1,2,3-triazole, $OP(O)_2$, $P(O)_2O$, C_1-C_4alkyl-$P(O)_2$-O, or O-$P(O)_2$-C_1-$_4$alkyl;

Sp is independently selected at each occurrence from an optionally substituted C_1-C_{20}alkyl, C_2-C_{20}alkenyl, C_2-C_{20}alkynyl, $[W\text{-}O]_g$, C_1-C_8alkyl-$[O\text{-}W]_g$, $[O\text{-}W]_g$-O-C_1-C_8alkyl, C_1-C_8Calkyl-$[O\text{-}W]_g$-O-C_1-C_8alkyl, or oligopeptide;

h is an integer of between 1 and 20;

g is a weighted average number of between about 2 and about 50;

W is C_2-C_4alkyl-1,2-diyl in which a hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom;

A is hydrogen, C_1-C_8alkyl, C(O)C_1-C_8alkyl, C(O)OC_1-C_8alkyl, C(O)N(H)C_1-C_8alkyl, R^{10}, or R^{11} wherein the alkyl group is optionally substituted with 0 or 1 R^{10};

R^{10} is a reactive functional group suitable for coupling Formula (I) to a carrier; and

R^{11} is a carrier.

15. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 13, wherein Z is CHR^8 or NR^9;

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R^8 and R^9 are each independently selected from hydrogen, C_1-C_8 alkyl, C(O)-$(CH_2)_n$-Q-A, C(O)C_1-C_8 alkyl, or $-C(O)(CH_2)_q[O-W]_g(NHC(O))_m(CH_2)_q[O-W]_p$-Q-A, wherein the alkyl group is optionally substituted with 0 or 1 Q-A;

q is independently at each occurrence 1, 2, or 3;

g and p each independently have a weighted average length of between about 2 and about 50;

m is 1 or 0;

W is C_2-C_4alkyl-1,2-diyl in which a hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom;

Q is a bond, O, N(H) or N(C_1-C_4alkyl);

A is hydrogen, C_1-C_8alkyl, C(O)C_1-C_8alkyl, C(O)N(H)C_1-C_8alkyl, C(O)OC_1-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10};

R^{10} is a reactive functional group suitable for coupling Formula (I) to a carrier; and

R^{11} is a carrier.

16. The drug delivery system or pharmaceutically acceptable salt of claim 15, wherein Z is NR^9;

R^9 is C(O)-$(CH_2)_n$-Q-A or -$C(O)(CH_2)_q[O-W]_g(NHC(O))_m(CH_2)_q[O-W]_p$-Q-A;

n is an integer of 1 to 8; and

A is R^{10} or R^{11}.

17. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 16, wherein R^{10} is azidyl, alkynyl, substituted or unsubstituted C_7-C_{12} cycloalkynyl, substituted or unsubstituted C_7-C_{12} heterocycloalkynyl, substituted or unsubstituted C_7-C_{12} cycloalkenyl, norbornyl, substituted or unsubstituted vinyl carboxyl, substituted or unsubstituted vinyl sulfonyl, substituted or unsubstituted C_2-C_8 alkenyl, amino, thiol, substituted or unsubstituted C_1-C_8 carboxyl, substituted or unsubstituted C_1-C_8 carbonyl, -O-NH_2, hydrazidyl, maleimide, alpha-halo carbonyl, furan, substituted or unsubstituted tetrazinyl, lysine, glutamine, cyclodextrin, or adamantanyl.

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18. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 17, wherein R^{10} comprises a reactive functional group suitable for coupling the drug delivery system or pharmaceutically acceptable salt of Formula (I) to a carrier.

19. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 16, wherein R^{11} is biodegradable.

20. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 16 or 19, wherein R^{11} comprises a polymer or cross-linked polymer.

21. The drug delivery system or pharmaceutically acceptable salt of claim 19 or 20, wherein R^{11} comprises a hydrogel comprising one or more cross-linked polymers.

22. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 16 and 19 to 21, wherein R^{11} comprises a polymer, cross-linked polymer, or hydrogel comprising one or more of hyaluronic acid, polyethylene glycol, polypropylene glycol, polyethylene oxide, polypropylene oxide, polyglutamate, polylysine, polysialic acid, polyvinyl alcohol, polyacrylate, polymethacrylate, polyacrylamide, polymethacrylamide, polyvinyl pyrrolidone, polyoxazoline, polyiminocarbonate, polyamino acid, hydrophilic polyester, polyamide, polyurethane, polyurea, dextran, agarose, xylan, mannan, carrageenan, alginate, gelatin, collagen, albumin, cellulose, methylcellulose, ethylcellulose, hydroxypropylmethylcellulose, hydroxyethyl starch, chitosan, nucleic acids, derivatives thereof, co-polymers thereof, or combinations thereof.

23. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 16 and 19 to 22, wherein R^{11} comprises hyaluronic acid, polyethylene glycol, a cross-linked hydrogel of hyaluronic acid, a cross-linked hydrogel of polyethylene glycol, or combinations thereof.

24. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 16 and 19 to 23, wherein R^{11} comprises hyaluronic acid or polyethylene glycol.

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25. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 16 and 19 to 23, wherein R^{11} comprises a hydrogel comprising cross-linked hyaluronic acid or cross-linked polyethylene glycol.

26. The drug delivery system or pharmaceutically acceptable salt of claim 24 or 25, wherein the hyaluronic acid or polyethylene glycol are functionalized with at least one functional group comprising azidyl, alkynyl, substituted or unsubstituted C_7-C_{12} cycloalkynyl, substituted or unsubstituted C_7-C_{12} heterocycloalkynyl, C_7-C_{12} cycloalkenyl, norbornyl, vinyl carboxyl, vinyl sulfonyl, C_2-C_8 alkenyl, amino, thiol, C_1-C_8 carboxyl, C_1-C_8 carbonyl, -O-NH2, carbohydrazide, maleimide, alpha-halo carbonyl, furan, substituted or unsubstituted tetrazinyl, lysine, glutamine, cyclodextrin, adamantanyl, or combinations thereof.

27. The drug delivery system or pharmaceutically acceptable salt of any one of claims 1 to 16 and 19 to 26, wherein R^{11} comprises a hydrogel comprising cross-linked hyaluronic acid, wherein the hyaluronic acid comprises at least one side chain selected from -NH(W1)$(O(W1))_d$-V, wherein W1 is C_2-C_4alkyl-1,2-diyl in which a hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom;
d is a number average of 0 to 500; and
V is a suitable functional group comprising azidyl, alkynyl, substituted or unsubstituted C_7-C_{12} cycloalkynyl, substituted or unsubstituted C_7-C_{12} heterocycloalkynyl, C_7-C_{12} cycloalkenyl, norbornyl, vinyl carboxyl, vinyl sulfonyl, C_2-C_8 alkenyl, amino, thiol, C_1-C_8 carboxyl, C_1-C_8 carbonyl, -O-NH2, carbohydrazide, maleimide, alpha-halo carbonyl, furan, substituted or unsubstituted tetrazinyl, lysine, glutamine, cyclodextrin, or adamantanyl.

28. The drug delivery system or pharmaceutically acceptable salt of claim 27, wherein V is azide.

29. A process for making a cross-linked carrier formulation, the process comprising:

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(a) functionalizing a carrier molecule, R^{11};

(b) preparing a reactive cross-linker; and

(c) reacting the functionalized carrier molecule with the reactive cross-linker to form a cross-linked carrier by incubation for about 0.5 hours to about 48 hours at a temperature of about 4 °C to about 60 °C.

30. The process of claim 29, wherein the carrier molecule comprises hyaluronic acid or polyethylene glycol.

31. The process of claim 30, wherein the carrier molecule is functionalized with azide, sulfhydryl, amine, aminoxy (O-NH_2) or aldehyde moieties to provide reactive functional groups for cross-linking.

32. The process of any one of claims 29 to 31, wherein the preparation of the reactive crosslinker comprises reacting a polyethylene glycol with 1-((tert-butoxycarbonyl)amino)cyclopropane-1-carboxylic acid, 3-((tert-butoxycarbonyl)amino)propanoic acid, 1-(tert-butoxycarbonyl)piperidine-4-carboxylic acid, 2-(1-(((tert-butoxycarbonyl)amino)methyl)cyclopropyl)acetic acid, 2-methyl-3-((tert-butoxycarbonyl)amino)propanoic acid, or 7-((tert-butoxycarbonyl)amino)heptanoic acid and deprotecting the functionalized polyethylene glycol ester.

33. The process of claim 32, wherein the preparation of the reactive crosslinker further comprises introduction at least two bicyclo[6.1.0]non-4-yn-9-yl)methyl groups after deprotection of the functionalzed polyethylene glycol ester.

34. A cross-linked hydrogel obtainable using the methods of any one of claims 29 to 33.

35. The cross-linked hydrogel of claim 34, wherein the carrier molecule is functionalized with azide.

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36. A process for preparing a drug adduct, comprising a traceless linker, R, coupled to an ANGPTL3 polypeptide comprising at least one primary amine, D, the process comprising:
    (a) providing an ANGPTL3 polypeptide comprising at least one primary amine, D;
    (b) reacting the biologically active agent with a traceless linker, R, that has an activated carbonyl functional group; and
    (c) purifying the drug adduct from the reagents.

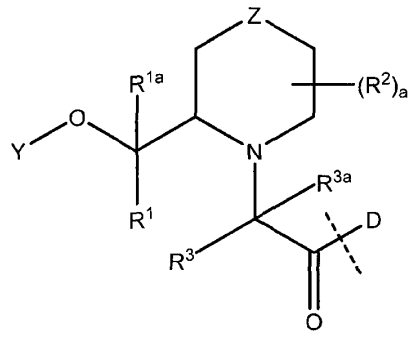
37. A drug adduct obtainable using the process of claim 36.

38. A process for making a drug delivery system, the process comprising:
    (a) preparing a carrier molecule, R^{11}, wherein R^{11} is a cross-linked hydrogel; optionally, step (a) may further comprise purifying the cross-linked hydrogel carrier molecule R^{11};
    (b) separately conjugating the traceless linker, R, to an ANGPTL3 polypeptide comprising at least one primary amine, D, thereby forming the traceless linker-D adduct; step (b) may optionally further comprise purification of the traceless linker-D adduct,
    (c) conjugating the carrier molecule, R^{11}, with the traceless linker-D adduct; and
    (d) purifying the drug delivery system from the reagents.

39. A drug delivery system made using the method of claim 38.

40. A method for treating macular degeneration comprising administering to a subject in need thereof a drug delivery system or pharmaceutically acceptable salt thereof comprising a conjugate D–R, that is represented by Formula (I), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine;
    R is a linker suitable for release of D:

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(I),

where the dashed line indicates attachment to the primary amine;

R^1 is hydrogen or C_1-C_4alkyl;

R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group;

a is 0, 1, 2, 3 or 4;

R^3 is hydrogen or C_1-C_4alkyl;

R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$;

R^{12} is hydrogen or C_1-C_4alkyl;

R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^{13} is C_1-C_4alkyl; or

$CHR^{12}OR^{13}$, taken in combination form a 5, 6, or 7-member cyclic ether;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by hydroxy, amino, C_1-C_4alkoxy or mono- and di- C_1-C_4alkylamino;

R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl;

R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring

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heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups;

b is an integer of from 1 to 10;

Z is CHR^8 or NR^9;

R^8 and R^9 are each independently selected from hydrogen, C_1-C_8 alkyl, $C(O)C_1$-C_8 alkyl, or $-C(O)(CH_2)_q[O-W]_g(NHC(O))_m(CH_2)_q[O-W]_p$-Q-A, wherein the alkyl group is optionally substituted with 0 or 1 Q-A;

q is independently at each occurrence 1, 2, or 3;

g and p each independently have a weighted average length of between about 2 and about 50;

m is 1 or 0;

W is C_2-C_4alkyl-1,2-diyl in which the hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom;

Q is a bond, O, N(H) or N(C_1-C_4alkyl);

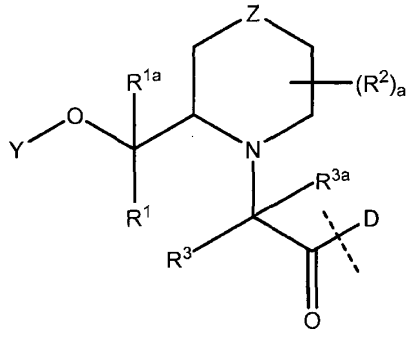
A is hydrogen, C_1-C_8alkyl, $C(O)C_1$-C_8alkyl, $C(O)N(H)C_1$-C_8alkyl, $C(O)OC_1$-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10};

R^{10} is a reactive functional group suitable for coupling Formula (I) to a carrier; and

R^{11} is a carrier.

41. A method for treating musculoskeletal disorders comprising administering to a subject in need thereof a drug delivery system or pharmaceutically acceptable salt thereof comprising a conjugate D–R, that is represented by Formula (I), where D an ANGPTL3 polypeptide comprising at least one primary amine;

R is a linker suitable for release of D:



(I),

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where the dashed line indicates attachment to the primary amine, secondary amine, or ring nitrogen atom of an azaheteroaryl ring;

R^1 is hydrogen or C_1-C_4alkyl;

R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group;

a is 0, 1, 2, 3 or 4;

R^3 is hydrogen or C_1-C_4alkyl;

R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$;

R^{12} is hydrogen or C_1-C_4alkyl;

R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^{13} is C_1-C_4alkyl; or

$CHR^{12}OR^{13}$, taken in combination form a 5, 6, or 7-member cyclic ether;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by hydroxy, amino, C_1-C_4alkoxy or mono- and di- C_1-C_4alkylamino;

R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl;

R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups;

b is an integer of from 1 to 10;

Z is CHR^8 or NR^9;

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R^8 and R^9 are each independently selected from hydrogen, C_1-C_8 alkyl, $C(O)C_1$-C_8 alkyl, or $-C(O)(CH_2)_q[O-W]_g(NHC(O))_m(CH_2)_q[O-W]_p$-Q-A, wherein the alkyl group is optionally substituted with 0 or 1 Q-A;

q is independently at each occurrence 1, 2, or 3;

g and p each independently have a weighted average length of between about 2 and about 50;

m is 1 or 0;

W is C_2-C_4alkyl-1,2-diyl in which the hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom;

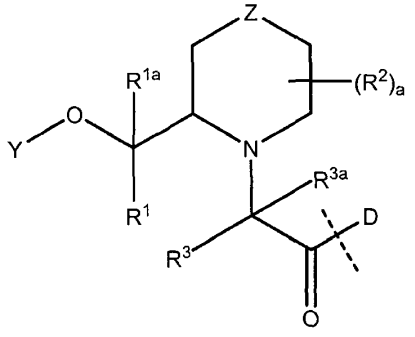
Q is a bond, O, N(H) or $N(C_1$-C_4alkyl);

A is hydrogen, C_1-C_8alkyl, $C(O)C_1$-C_8alkyl, $C(O)N(H)C_1$-C_8alkyl, $C(O)OC_1$-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10};

R^{10} is a reactive functional group suitable for coupling Formula (I) to a carrier; and

R^{11} is a carrier.

42. A method for treating a disease or disorder comprising administering to a subject in need thereof a drug delivery system or pharmaceutically acceptable salt thereof comprising a conjugate D–R, that is represented by Formula (I), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; R is a linker suitable for release of D:



(I),

where the dashed line indicates attachment to the primary amine;

R^1 is hydrogen or C_1-C_4alkyl;

R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

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R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group;

a is 0, 1, 2, 3 or 4;

R^3 is hydrogen or C_1-C_4alkyl;

R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$;

R^{12} is hydrogen or C_1-C_4alkyl;

R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^{13} is C_1-C_4alkyl; or

$CHR^{12}OR^{13}$, taken in combination form a 5, 6, or 7-member cyclic ether;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by hydroxy, amino, C_1-C_4alkoxy or mono- and di- C_1-C_4alkylamino;

R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl;

R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups;

b is an integer of from 1 to 10;

Z is CHR^8 or NR^9;

R^8 and R^9 are each independently selected from hydrogen, C_1-C_8 alkyl, $C(O)C_1$-C_8 alkyl, or $-C(O)(CH_2)_q[O-W]_g(NHC(O))_m(CH_2)_q[O-W]_p$-Q-A, wherein the alkyl group is optionally substituted with 0 or 1 Q-A;

q is independently at each occurrence 1, 2, or 3;

g and p each independently have a weighted average length of between about 2 and about 50;

m is 1 or 0;

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W is C_2-C_4alkyl-1,2-diyl in which the hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom;

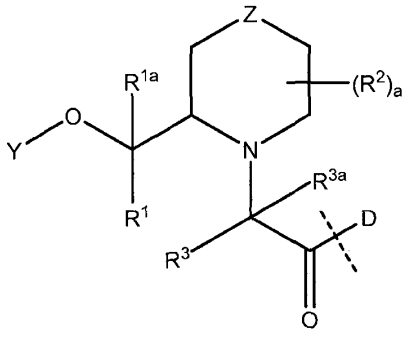
Q is a bond, O, N(H) or N(C_1-C_4alkyl);

A is hydrogen, C_1-C_8alkyl, C(O)C_1-C_8alkyl, C(O)N(H)C_1-C_8alkyl, C(O)OC_1-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10};

R^{10} is a reactive functional group suitable for coupling Formula (I) to a carrier; and

R^{11} is a carrier.

43. A means for extending half-life of an ANGPTL3 polypeptide comprising at least one primary amine, D, the means comprising attaching D to R, that is represented by Formula (I), where R is a linker suitable for release of D:



(I),

where the dashed line indicates attachment to the primary amine;

R^1 is hydrogen or C_1-C_4alkyl;

R^{1a} is hydrogen or C_1-C_4alkyl, or CR^1R^{1a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^2 is independently selected at each occurrence from C_1-C_4alkyl or oxo, or two R^2 groups taken in combination form a fused C_3-C_6 cycloalkyl or spiro C_3-C_6cycloalk-1,1-diyl group;

a is 0, 1, 2, 3 or 4;

R^3 is hydrogen or C_1-C_4alkyl;

R^{3a} is hydrogen, C_1-C_4alkyl, or CR^3R^{3a}, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

Y is $C(O)R^4$, $C(O)OR^4$, $C(O)NHR^4$, $C(O)NR^5R^6$, $SiR^5R^6R^7$, or $CR^{12}R^{12a}OR^{13}$;

R^{12} is hydrogen or C_1-C_4alkyl;

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R^{12a} is hydrogen or C_1-C_4alkyl, or $CR^{12}R^{12a}$, taken in combination form a C_3-C_6cycloalk-1,1-diyl;

R^{13} is C_1-C_4alkyl; or

$CHR^{12}OR^{13}$, taken in combination form a 5, 6, or 7-member cyclic ether;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by hydroxy, amino, C_1-C_4alkoxy or mono- and di- C_1-C_4alkylamino;

R^5 and R^6 are each independently selected from C_1-C_4alkyl and C_3-C_6cycloalkyl;

R^7 is C_1-C_8alkyl, C_3-C_7cycloalkyl, C_1-C_8alkoxy, C_3-C_7cycloalkyloxy, heterocycloalkyloxy, or $-(OCHR^3CH_2)_bO$-C_1-C_4alkyl, wherein the heterocycloalkyloxy is a 4 to 7 member saturated heterocyclic ring having one ring heteroatom selected from N, O, and S and optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups;

b is an integer of from 1 to 10;

Z is CHR^8 or NR^9;

R^8 and R^9 are each independently selected from hydrogen, C_1-C_8 alkyl, $C(O)C_1$-C_8 alkyl, or $-C(O)(CH_2)_q[O-W]_g(NHC(O))_m(CH_2)_q[O-W]_p$-Q-A, wherein the alkyl group is optionally substituted with 0 or 1 Q-A;

q is independently at each occurrence 1, 2, or 3;

g and p each independently have a weighted average length of between about 2 and about 50;

m is 1 or 0;

W is C_2-C_4alkyl-1,2-diyl in which the hydrogen, methyl, or ethyl side chain may be present on either backbone carbon atom;

Q is a bond, O, N(H) or N(C_1-C_4alkyl);

A is hydrogen, C_1-C_8alkyl, $C(O)C_1$-C_8alkyl, $C(O)N(H)C_1$-C_8alkyl, $C(O)OC_1$-C_8alkyl, R^{10}, or R^{11}, wherein the alkyl group is optionally substituted with 0 or 1 R^{10};

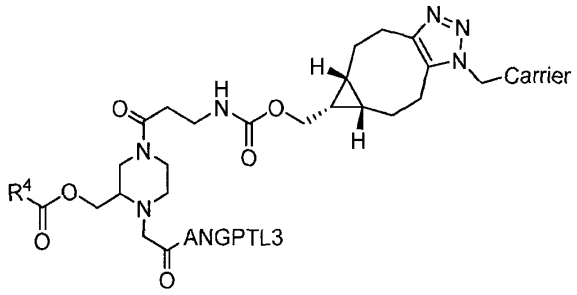
R^{10} is a reactive functional group suitable for coupling Formula (I) to a carrier; and

R^{11} is a carrier.

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44. The means of claim 43, wherein D comprises an ANGPTL3 polypeptide having at least 95% identity to any one of SEQ ID NO: 1 or 3–45.

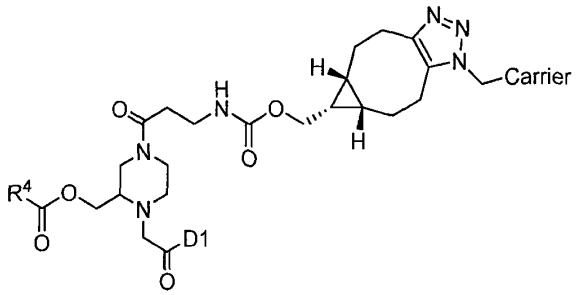
45. A drug delivery system comprising Formula (III):



(III),

wherein ANGPTL3 comprises an ANGPTL3 polypeptide having at least 95% identity to any one of SEQ ID NO: 1 or 3–45, or combinations thereof.

46. A drug delivery system comprising Formula (IV):



(IV),

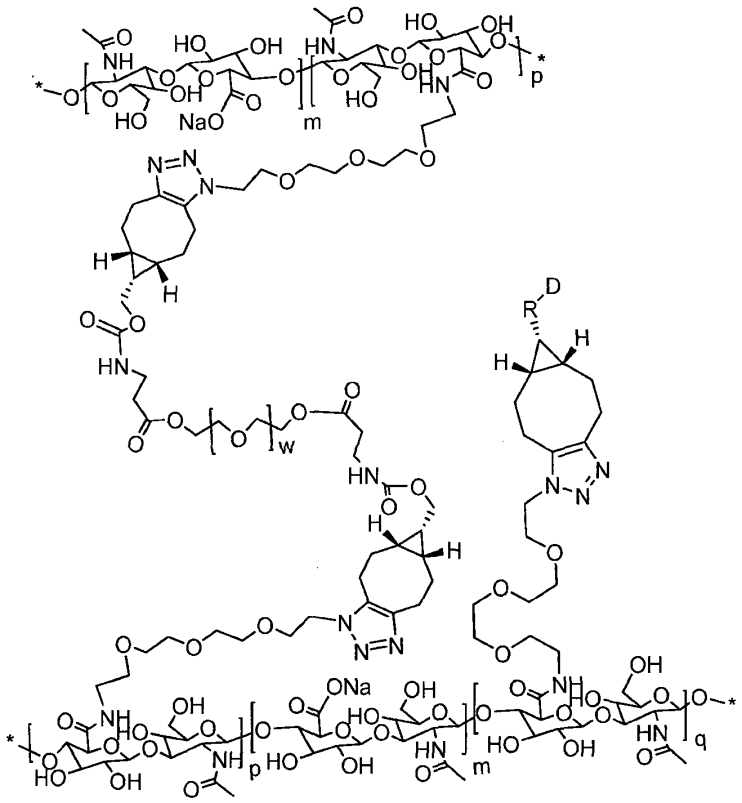
wherein SEQ ID NO:19 comprises an ANGPTL3 polypeptide comprising amino acid residues 242–460 in reference to SEQ ID NO:1 and a K423Q substitution.

47. A drug delivery system comprising D–R–R^{11}, wherein R comprises a traceless linker, coupled to a biologically-active agent, D; D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R^{11} comprises a plurality of hyaluronic acid polymers each of which comprises a random series of unfunctionalized D-glucuronic acid monomers, one or more D-glucuronic acid monomers functionalized with a cross-linker, or one or more D-glucuronic acid monomers functionalized with a drug adduct, D–R.

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48. The drug delivery system of claim 47, wherein D comprises an ANGPTL3 polypeptide having at least 95% identity to any one of SEQ ID NO: 1 or 3–45.

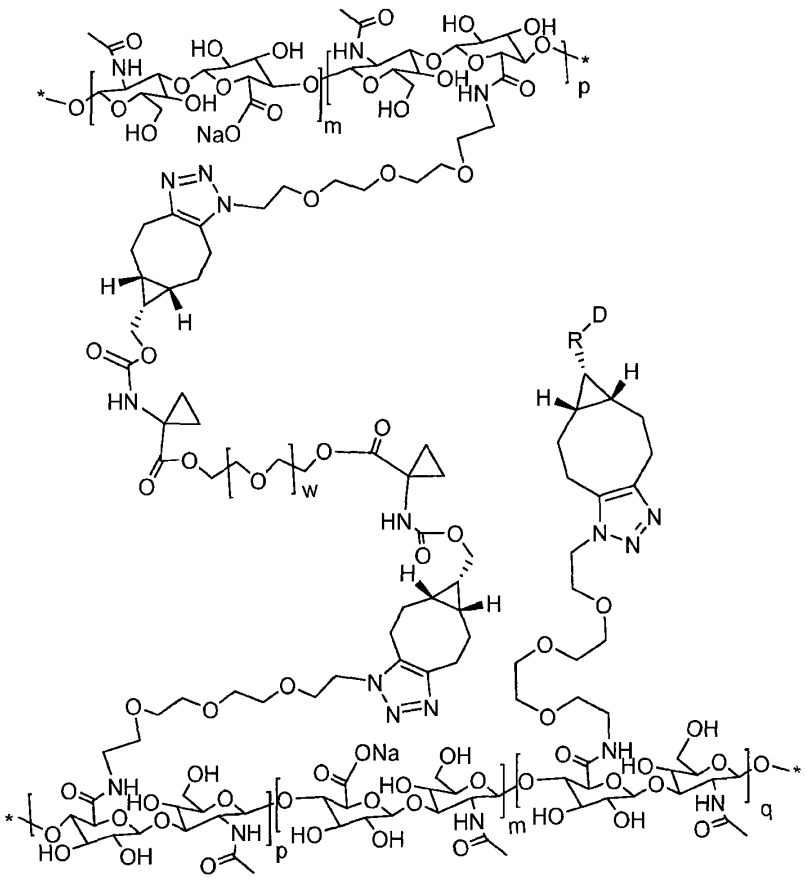
49. A drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXIV), where D is an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release D:



(XXIV).

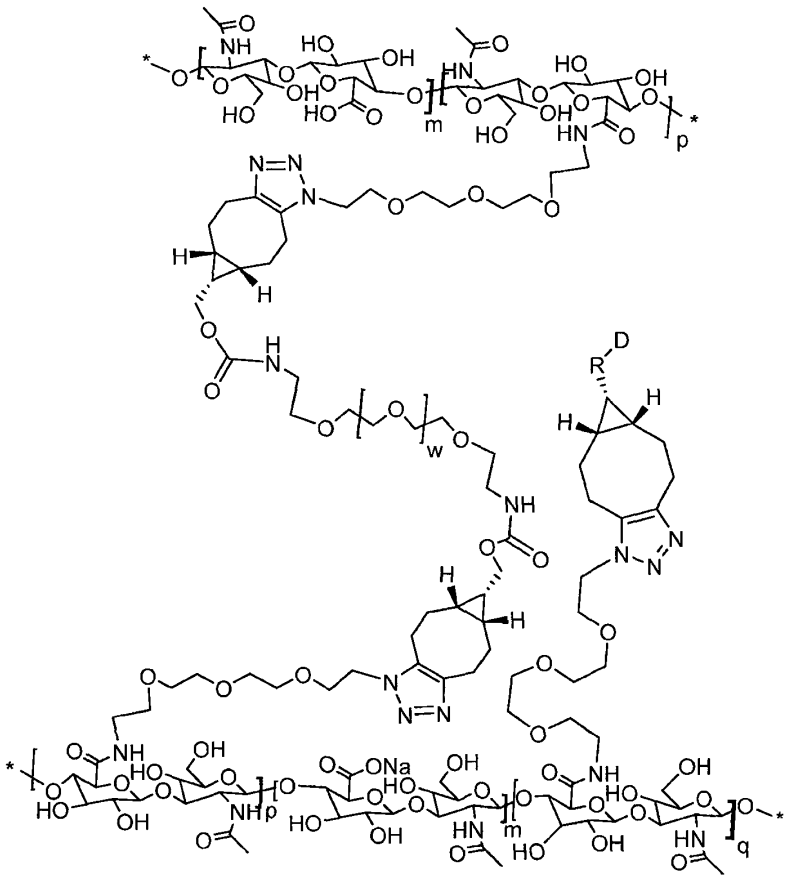
50. A drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXV), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:

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(XXV).

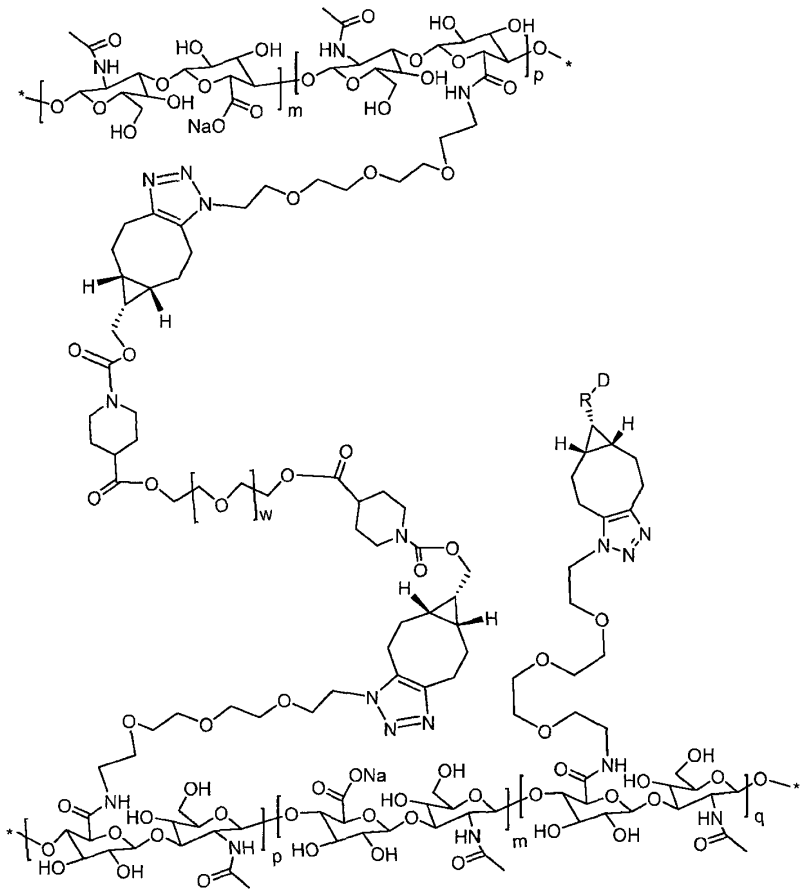
51. A drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXVI), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:



(XXVI).

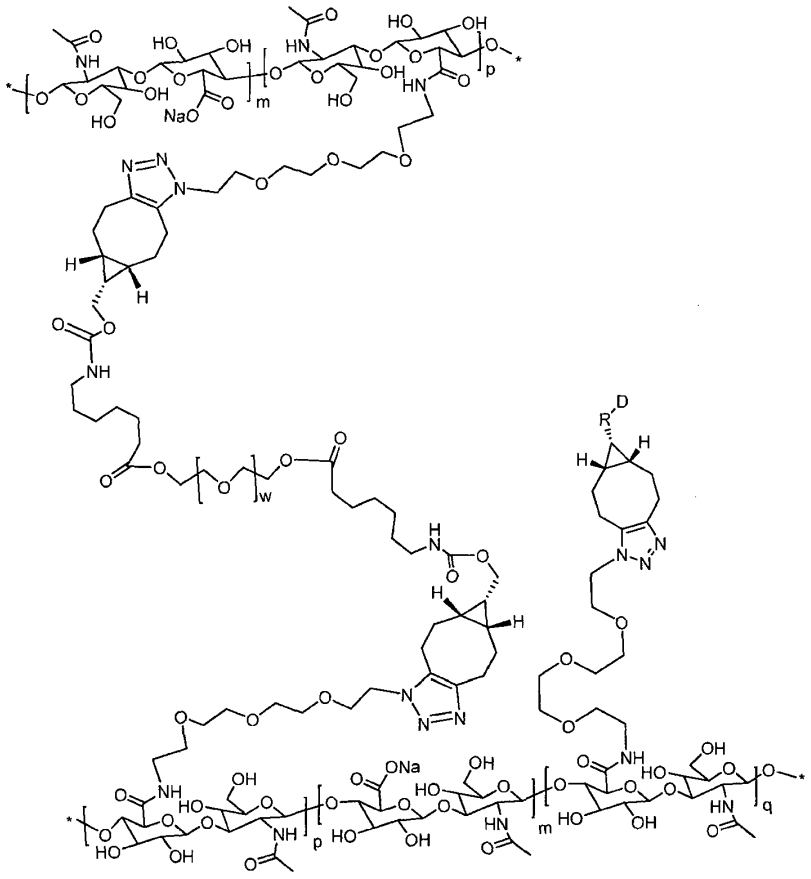
52. A drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXVII), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:

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(XXVII).

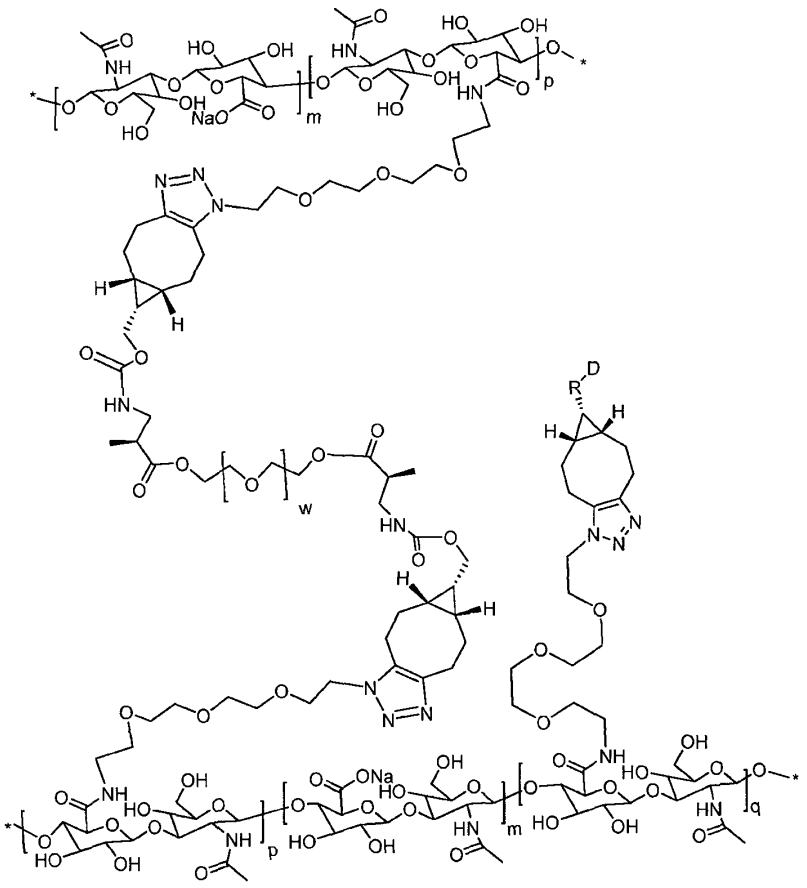
53. A drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXVIII), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:



(XXVIII).

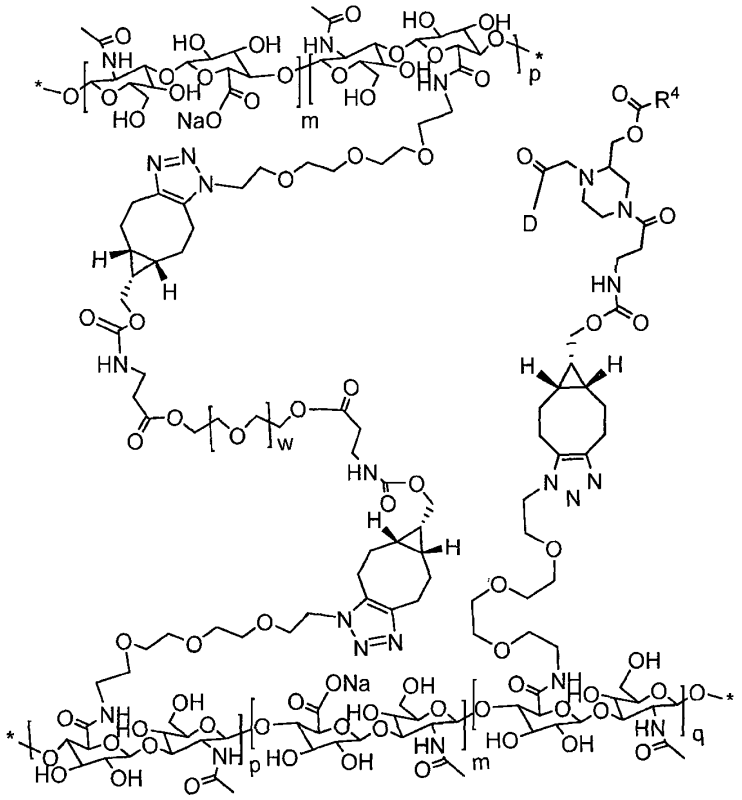
54. A drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXIX), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:

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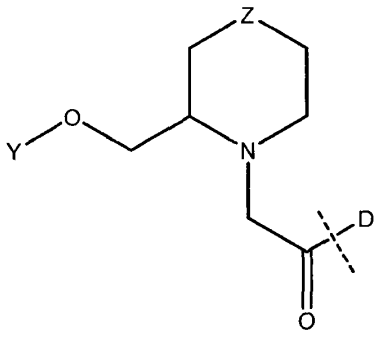
(XXIX).

55. A drug delivery system or pharmaceutically acceptable salt thereof that is represented by Formula (XXX), where Drug, D, comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R^4 is methyl, ethyl, propyl, isopropyl, 1-methyl-cyclopropyl, or methoxymethyl:



(XXX).

56. A drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXXI), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:



(XXXI),

where the dashed line indicates attachment to the primary amine;

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Y is $C(O)R^4$;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy;

Z is N-L-A;

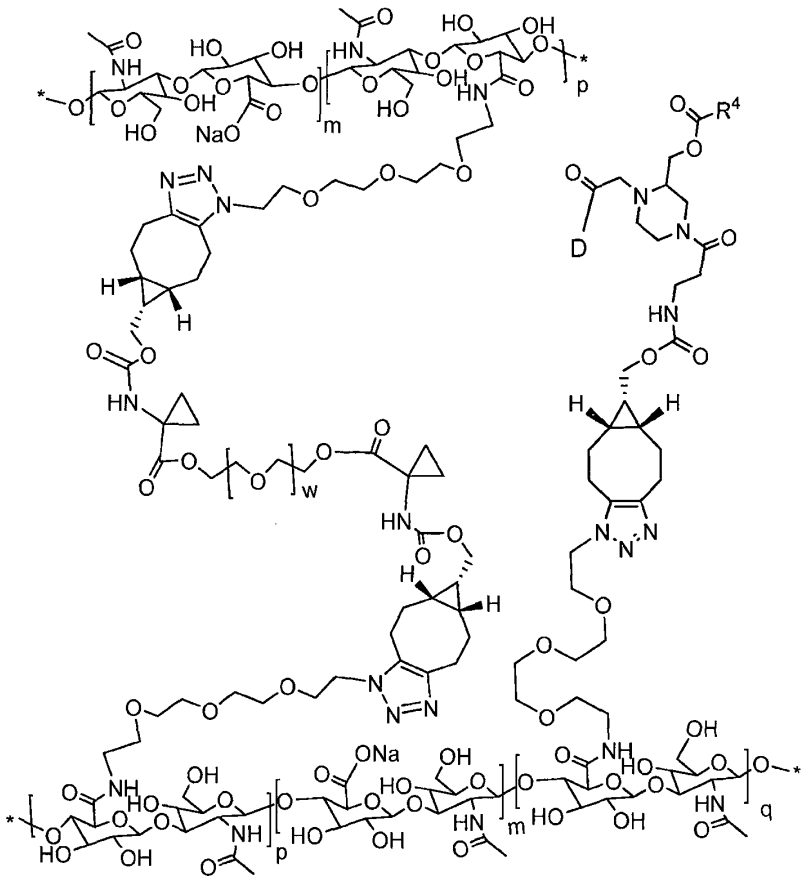
L is $C(O)CH_2CH_2NH$;

A is R^{11}; and

R^{11} is a hydrogel derived from a cross-linked hyaluronic acid, wherein the hyaluronic acid comprises at least one amide-linked side chain of $N(H)(CH_2CH_2O)_3$ $CH_2CH_2N_3$, and wherein the cross-linker used to form the hydrogel comprises PEG(2000)-bis-[3-(((((1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methoxy)carbonyl)amino)propanoate].

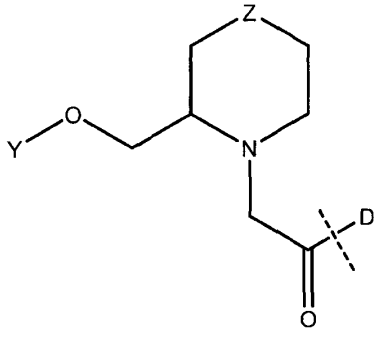
57. A drug delivery system or pharmaceutically acceptable salt thereof that is represented by Formula (XXXII), where Drug, D, comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R^4 is methyl, ethyl, propyl, isopropyl, 1-methyl-cyclopropyl, or methoxymethyl:

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(XXXII).

58. A drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula XXXIII, where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:



(XXXIII),

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where the dashed line indicates attachment to the primary amine;

Y is $C(O)R^4$;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy;

Z is N-L-A;

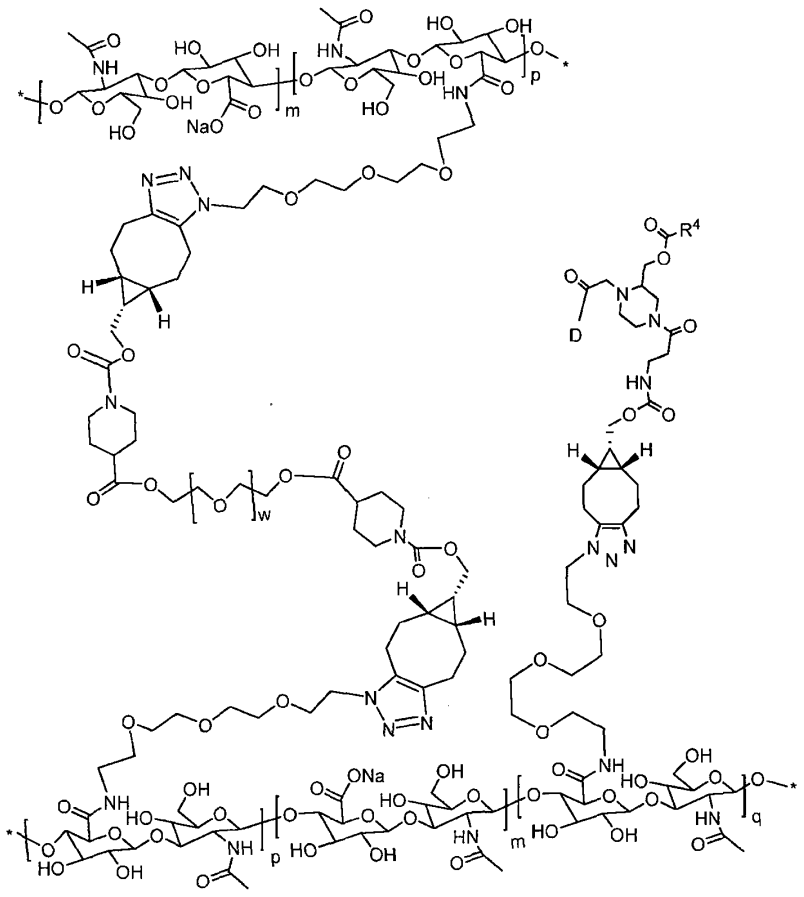
L is $C(O)CH_2CH_2NH$;

A is R^{11}; and

R^{11} is a hydrogel derived from a cross-linked hyaluronic acid, wherein the hyaluronic acid comprises at least one amide-linked side chain of $N(H)(CH_2CH_2O)_3$ $CH_2CH_2N_3$, and wherein the cross-linker used to form the hydrogel comprises PEG(2000)-bis-[1-((((1′*R*,8′*S*,9′s)-bicyclo[6.1.0]non-4′-yn-9′-yl)methoxy)carbonyl)amino-cyclopropane-1-carboxylic acid ester].

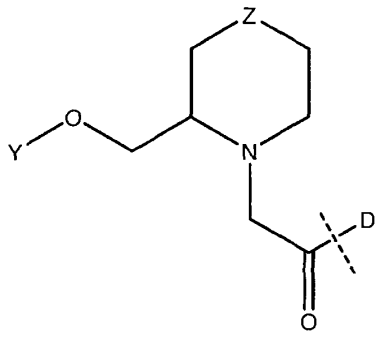
59. A drug delivery system or pharmaceutically acceptable salt thereof that is represented by Formula (XXXIV), where Drug, D, comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R^4 is methyl, ethyl, propyl, isopropyl, 1-methyl-cyclopropyl, or methoxymethyl:

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(XXXIV).

60. A drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula XXXV, where Drug, D, comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:



(XXXV),

where the dashed line indicates attachment to the primary amine;

Y is $C(O)R^4$;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy;

Z is N-L-A;

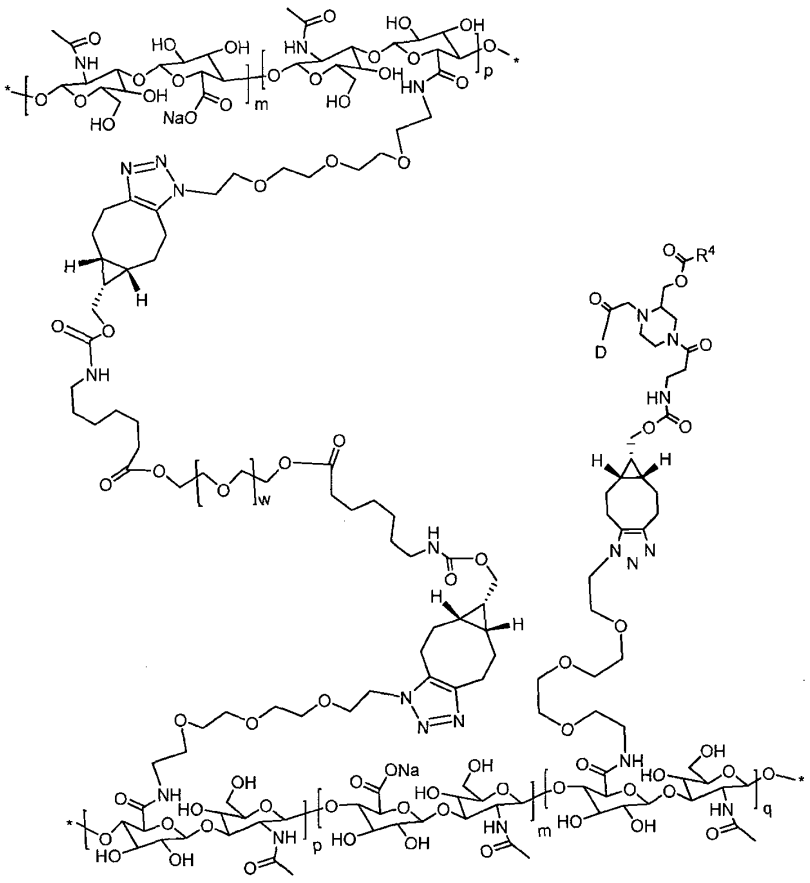
L is $C(O)CH_2CH_2NH$;

A is R^{11}; and

R^{11} is a hydrogel derived from a cross-linked hyaluronic acid, wherein the hyaluronic acid comprises at least one amide-linked side chain of $N(H)(CH_2CH_2O)_3$ $CH_2CH_2N_3$, and wherein the cross-linker used to form the hydrogel comprises PEG(2000)-bis-[1-((((1′*R*,8′*S*,9′s)-bicyclo[6.1.0]non-4′-yn-9′-yl)methoxy)carbonyl)piperidine-4-carboxylic acid ester].

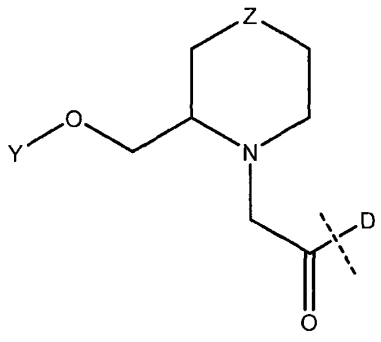
61. A drug delivery system or pharmaceutically acceptable salt thereof that is represented by Formula (XXXVI), where Drug, D, comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R^4 is methyl, ethyl, propyl, isopropyl, 1-methyl-cyclopropyl, or methoxymethyl:

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(XXXVI).

62. A drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula XXXVII, where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R is a linker suitable for release of D:



(XXXVII),

where the dashed line indicates attachment to the primary amine;

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Y is $C(O)R^4$;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy;

Z is N-L-A;

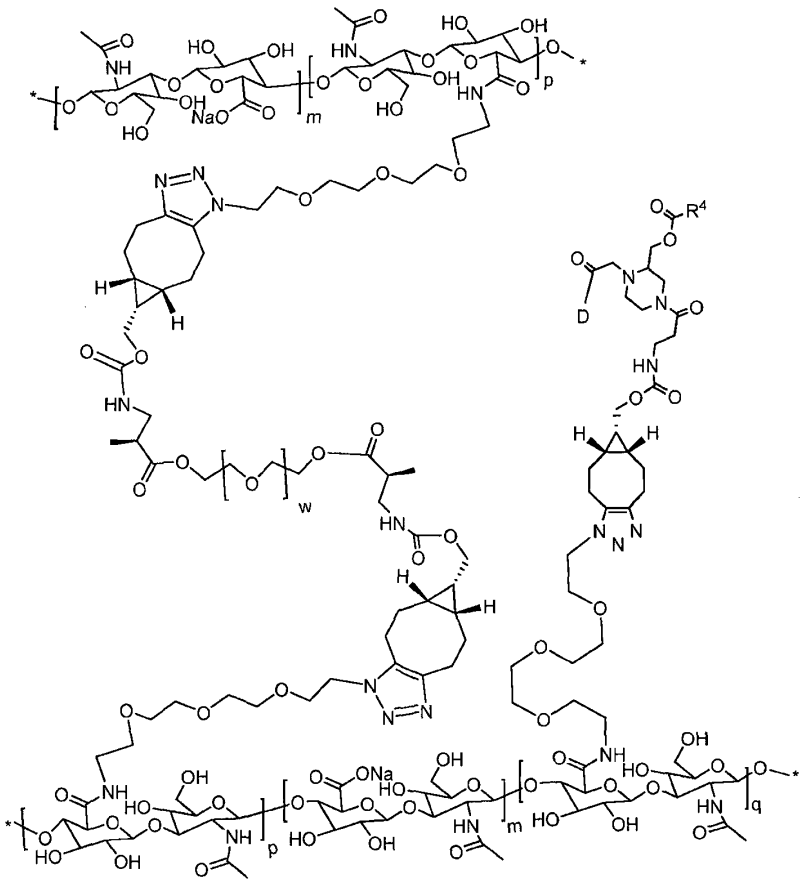
L is $C(O)CH_2CH_2NH$;

A is R^{11}; and

R^{11} is a hydrogel derived from a cross-linked hyaluronic acid, wherein the hyaluronic acid comprises at least one amide-linked side chain of $N(H)(CH_2CH_2O)_3$ $CH_2CH_2N_3$, and wherein the cross-linker used to form the hydrogel comprises PEG(2000)-bis-[7-(((((1′*R*,8′*S*,9′s)-bicyclo[6.1.0]non-4′-yn-9′-yl)methoxy)carbonyl)amino)heptanoate].

63. A drug delivery system or pharmaceutically acceptable salt thereof that is represented by Formula (XXXVIII), where Drug, D, comprises an ANGPTL3 polypeptide comprising at least one primary amine; and R^4 is methyl, ethyl, propyl, isopropyl, 1-methyl-cyclopropyl, or methoxymethyl:

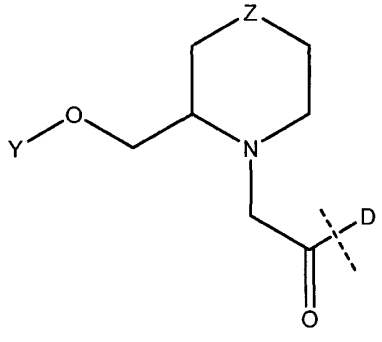
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(XXXVIII).

64. A drug delivery system or pharmaceutically acceptable salt thereof comprising D–R, that is represented by Formula (XXXIX), where D comprises an ANGPTL3 polypeptide comprising at least one primary amine; and

R is a linker suitable for release of D:



(XXXIX),

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where the dashed line indicates attachment to the primary amine, secondary amine, or ring nitrogen atom of an azaheteroaryl ring;

Y is $C(O)R^4$;

R^4 is C_1-C_8alkyl or C_3-C_7cycloalkyl, wherein cycloalkyl is optionally substituted with 0, 1, or 2 independently selected C_1-C_4alkyl groups and wherein alkyl is optionally substituted by C_1-C_4alkoxy;

Z is N-L-A;

L is $C(O)CH_2CH_2NH$;

A is R^{11}; and

R^{11} is a hydrogel derived from a cross-linked hyaluronic acid, wherein the hyaluronic acid comprises at least one amide-linked side chain of $N(H)(CH_2CH_2O)_3$ $CH_2CH_2N_3$, and wherein the cross-linker used to form the hydrogel comprises PEG(2000)-bis-[2-methyl-3-(((((1′*R*,8′*S*,9′s)-bicyclo[6.1.0]non-4′-yn-9′-yl)methoxy)carbonyl)amino)propanoate].

65. The drug delivery systems of any one of claims 55 to 64, wherein D is SEQ ID NO:19 and R^4 is -CH3, $-CH_2CH_3$, $-CH(CH_3)CH_3$, or methylcyclopropyl.

66. The drug delivery systems of any one of claims 55 to 65, wherein D is SEQ ID NO:19 and R^4 is $-CH_3$.

67. The drug delivery systems of any one of claims 55 to 66, wherein the D–R adduct comprises:

