

HYDROPHILIC LINKERS FOR MULTIVALENT PEPTIDE CONJUGATES

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Nos. 62/869,233, filed July 1, 2019, and 62/898,967, filed September 11, 2019, each of which is incorporated
5 herein in its entirety.

STATEMENT REGARDING SEQUENCE LISTING

[0002] This application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said
10 ASCII copy, created on June 30, 2020, is named
052566_505001WO_SequenceListing_ST25.txt and is 37,450 bytes in size.

BACKGROUND OF THE INVENTION

[0003] The use of biopolymers to modify the properties of biologically active agents is a
15 recurring theme across a wide range of medical and biological applications. A variety of chemical linkers can be used to attach bioactive peptides or proteins to biopolymers to modify the pharmacological properties of the resulting conjugate for use as a drug that can provide optimal treatment of specific diseases. Peptide-polymer conjugate comprising multiple copies of one or more species of peptide conjugated to a single biopolymer chain
20 have been employed to impart specific improvements to the pharmacological properties of the peptides, including: (1) higher binding affinity to the biological target, (2) slower diffusivity through a target tissue, and (3) inhibition of proteases that could deactivate the biological activity of the peptides or proteins.

[0004] These improved pharmacological properties of peptide-polymer conjugates are
25 particularly useful for the delivery of potent drugs that are be delivered directly into the diseased tissue. The dose delivered directly into the tissue can be lower than would be required to achieve the same therapeutic effect after systemic administration because the drug has been administered locally to the target tissue. It is also possible to administer to drugs to tissues that otherwise have poor transport properties from the blood. Specific examples of

tissues where direct drug administration is common include the posterior eye chamber via intravitreal injection and articular joints via intra-articular injection.

[0005] However, local tissue administration requires a professional to safely provide the required injection, which makes them more burdensome and costly to administer compared to systemic administration. When the peptide drug is administered as part of a peptide-polymer conjugate, it is possible to substantially reduce the frequency of drug administration, thereby reducing the burden on the patient to receive effective treatment. Furthermore, a reduction in the number of local injections reduces the risk of local tissue injury or adverse effects to the injection. Finally, the need for less frequent administrations can reduce the amount of time that the drug concentration in the target tissue is below the therapeutic concentration, thereby improving the overall efficacy of the drug. Based on these advantages, there is a strong motivation to develop protein-polymer drug products for a variety of diseases.

[0006] To appropriately formulate a peptide-polymer conjugate as a drug product, it is necessary to achieve sufficiently high drug concentrations to enable appropriate dosing in the patient. Achieving sufficient dosing requires achieving both an appropriate concentration of the peptide polymer conjugates in the drug product solution as well as the appropriate drug load of peptide conjugated to each polymer. It is also necessary to filter the peptide-polymer drug substance through a 0.22-micron filter to eliminate any bacteria or pathogens that may be contaminating the solution. Finally, the peptide-polymer drug product must exhibit shelf-stability by remaining in solution for up to two years from the date of manufacture to the date of clinical use. Interactions between the peptide-polymer conjugates can negatively impact the ability to complete any of these drug-enabling properties.

[0007] The linkers used to attach the polymer and the peptides can have a substantial impact on the pharmacological properties of the conjugates, intra-conjugate interactions, as well as conjugate-to-conjugate interactions. Therefore, there is a need to develop peptide-polymer conjugates with the specific linker chemistries that will enable them to achieve the preferred pharmacological properties for a given disease as well as to be successfully formulated into a drug product. The present invention meets this and other needs.

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BRIEF SUMMARY OF THE INVENTION

[0008] In one embodiment, the present invention provides a conjugate of Formula I:



wherein each X is independently a peptide having a molecular weight of from about 5 kDa to about 200 kDa; each Y is independently a hydrophilic linker; Z is a biocompatible polymer having a molecular weight of from about 0.1 MDa to about 3 MDa; and subscript n is an

5 integer from 10 to 1000.

[0009] In another embodiment, the present invention provides a pharmaceutical composition including a conjugate of the present invention and a pharmaceutically acceptable excipient.

[0010] In another embodiment, the present invention provides a method of treating an
10 ocular disease or disorder, the method comprising intravitreal administration to a subject in need thereof, a therapeutically effective amount of a conjugate of the present invention, thereby treating the ocular disease or disorder.

[0011] In another embodiment, the present invention provides a method of treating a disease or disorder in an articular joint, the method comprising injecting into the articular
15 joint an effective amount of a conjugate of the present invention, thereby treating the disease or disorder in the articular joint.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] FIG. 1 shows UV spectra of purified 830 kDa hyaluronic acid intermediates
20 synthesized using different thiol reactive linkers. See Table 2 for conjugates.

[0013] FIG. 2 shows reverse phase HPLC analysis of HyA-(Mal PEG2 Hydrazide) intermediate product before and after purification through a Zeba 7 kDa MWCO desalting column.

[0014] FIG. 3 shows SEC trace of conjugate 40 showing the conjugate peak, followed by
25 the smaller DARPin dimer and monomer peaks.

[0015] FIG. 4 shows percent loss after 0.22 μ m filtration for peptide protein conjugates 1 (EMCH), 3 (BMPH), 4 (MP2H), 16 (MP2A), 5 (MP3A), and 6 (n-AEM) linkers.

[0016] FIG. 5 shows improved log anti-VEGF potency for MVPs with a Mal-PEG2-hydrazide linker and a peptide of DARPin, E1-1 or VHH compared to MVPs with an EMCH
30 linker.

[0017] FIG. 6 shows a comparison of the amount of therapeutic protein loss after sterile (0.22.µm) filtering anti-TNFα polymer conjugate 19, 21, 22, and 16 made using anti-TNFα VHH antibodies and various crosslinkers. EMCH and BMPH resulted in substantial conjugate loss during filtration whereas the loss for the maleimide PEG2 hydrazide (MP2H) and maleimide PEG2 amine (MP2A) linkers was negligible. *p<0.001 compared to zero, ANOVA with Tukey and n=4.

[0018] FIG. 7 shows a comparison of the intra-articular (IA) retention of anti-TNFα MVP made using anti-TNFα VHH antibodies and either EMCH or MP2H. Both MVPs had substantial increases in IA half-life compared to the unconjugated anti-TNFα VHH. The MVPs made with MP2H had a significantly longer intra-articular half-life compared to those made with EMCH (ANOVA with Tukey and n=4).

[0019] FIG. 8A and FIG. 8B each show Reverse Phase HPLC 230 and 254 nm traces showing the purification of intermediates 40 (EMCH, top) and 41 (MP2H, bottom) intermediate reaction products, crude (left) or purified (right) with a Zeba 7 kDa MWCO desalting column.

[0020] FIG. 9 shows PAGE analysis of Avastin polymer conjugates made with 830 kDa HA intermediate with EMCH linker (labeled A) or MP2H linker (labeled B) done without heating PAGE samples. Band that did not migrate into the gel is indicative of conjugate formation.

[0021] FIG. 10 shows PAGE analysis of goat anti-human IgG polymer conjugates made with 830 kDa HA intermediate with MP2H linker (labeled). Band that did not migrate into the gel is indicative of conjugate formation.

[0022] FIG. 11 shows HPLC SEC 280 nm traces for anti-VEGF G5-1 polymer conjugates 24 and 25 synthesized with 830 kDa HA intermediate consisting of either MP2H or EMCH linker and filtration recovery for the conjugates using a 0.2 µm filter.

[0023] FIG. 12 shows HPLC SEC 280 nm traces for anti-VEGF DARPin conjugates 30 and 31 synthesized with 830 kDa HA intermediate consisting of either MP2H (30) or EMCH (31). EMCH linker shows lower reaction efficiency, size by retention time, and higher percent of unreacted DARPin compared to MP2H linker.

[0024] FIG. 13 shows BLI bioactivity data showing improved binding kinetics for MP2H Avastin IgG conjugate compared to EMCH Avastin conjugate or Avastin alone. Traut's

Avastin indicates binding data for the Avastin IgG after activation with 2-iminothiolane (Traut's reagent).

5 [0025] FIG. 14 shows Bioactivity data showing binding kinetics for MP2H G5-1 anti-VEGF conjugate and EMCH G5-1 conjugate compared to G5-1 alone. G5-1 MP2H= conjugate 24, G5-1 EMCH= conjugate 25.

[0026] FIG. 15A and FIG. 15B show filtration recovery and reaction efficiency for MP2H (conjugates 32-35) or EMCH (conjugates 36-39) anti-TNF α -polymer conjugates synthesized at different potencies.

10 [0027] FIG. 16 shows binding kinetics for protein conjugates 32-35 (MP2H) or 36-38 (EMCH) anti-TNF α -polymer conjugates synthesized at different potencies. While EMCH showed higher binding affinity at lower valency vs MPH2, the EMCH conjugates were limited in the maximum achievable valency (precipitated above peptide valency of 75) and displayed lower reaction efficiency as well as poor handleability at higher peptide valencies. K_d of 0.001 indicates a read that is < LOD.

15 [0028] FIG. 17 shows intravitreal half-life of peptide protein conjugate 41 or peptide alone (DARPin).

[0029] FIG. 18A and FIG. 18B shows PAGE analysis of purified peptide polymer conjugates using 2 kDa Natriuretic Peptide and polymer intermediates containing EMCH (labeled A or D) or MP2H (labeled B or C) ran with (FIG. 18B) and without (FIG. 18A) sample heat denaturation. Band that did not migrate into the gel is indicative of conjugate formation.

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DETAILED DESCRIPTION OF THE INVENTION

I. GENERAL

25 [0030] The present invention provides peptide-polymer conjugates using hydrophilic linkers to covalently link each peptide to the polymer. The hydrophilic polymers provide added stability to the peptide-polymer conjugates.

II. DEFINITIONS

[0031] Unless specifically indicated otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention belongs. In addition, any method or material similar or equivalent to a method or material described herein can be used in the practice of the present invention. For purposes of the present invention, the following terms are defined.

[0032] “Thiol reactive group” refers to a group capable of reacting with a thiol to form a covalent bond to the sulfur atom. Representative thiol reactive groups include, but are not limited to, thiol, TNB-thiol, haloacetyl, aziridine, acryloyl, vinylsulfone, APN (3-arylpropionitrile), maleimide and pyridyl disulfide. Reaction of the thiol reactive group with a thiol can form a disulfide or a thioether.

[0033] “Thiol” refers to the –SH functional group.

[0034] “Heteroalkyl” refers to an alkyl group of any suitable length and having from 1 to 6 heteroatoms such as N, O and S. Additional heteroatoms can also be useful, including, but not limited to, B, Al, Si and P. The heteroatoms can also be oxidized, such as, but not limited to, –S(O)– and –S(O)₂–. For example, heteroalkyl can include ethers, thioethers and alkyl-amines. The heteroatom portion of the heteroalkyl can replace a hydrogen of the alkyl group to form a hydroxy, thio or amino group. Alternatively, the heteroatom portion can be the connecting atom, or be inserted between two carbon atoms.

[0035] “Heteroalkylene” refers to a heteroalkyl group, as defined above, linking at least two other groups. The two moieties linked to the heteroalkylene can be linked to the same atom or different atoms of the heteroalkylene.

[0036] “Carboxy reactive group” refers to a group capable of reacting with a carboxy or carboxylic acid group, i.e., –COOH. Representative carboxy reactive groups include, but are not limited to, amine, hydrazide, alcohol and thiol. Reaction with a carboxy reactive group can form an amide, ester or thioester.

[0037] “HyA” as used herein refers to hyaluronic acid.

[0038] “CMC” refers to carboxymethyl cellulose.

[0039] “scFV” refers to small chain variable fragment antibody.

[0040] “VHH” as used herein refers to a single-domain heavy chain antibody.

[0041] “DARPin” refers to a designed ankyrin repeat protein, which is a genetically engineered antibody mimetic protein that can exhibit highly specific and high-affinity target protein binding.

5 [0042] “Articular joint” as used herein refers to the fibrous or cartilaginous joints, which is a fibrous or cartilaginous area wherein two or more bones connect to each other.

[0043] “Therapeutically effective amount” as used herein refers to a dose that produces therapeutic effects for which it is administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques (*see*,
10 *e.g.*, Lieberman, *Pharmaceutical Dosage Forms* (vols. 1-3, 1992); Lloyd, *The Art, Science and Technology of Pharmaceutical Compounding* (1999); Pickar, *Dosage Calculations* (1999); and *Remington: The Science and Practice of Pharmacy*, 20th Edition, 2003, Gennaro, Ed., Lippincott, Williams & Wilkins). In sensitized cells, the therapeutically effective dose can often be lower than the conventional therapeutically effective dose for non-sensitized
15 cells.

[0044] “Biocompatible polymer” as used herein refers to a polymer compatible with the joints at the injection site. Representative biocompatible polymers include, but are not limited to polysaccharides, glycosaminoglycans, and hyaluronic acid.

[0045] “Polymer molecular weight” as used herein refers to the molecular weight of the
20 polymer.

[0046] “Peptide,” “polypeptide,” and “protein” are used interchangeably herein, and refer to naturally occurring and synthetic amino acids of any length, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. The term “polypeptide” includes fusion proteins, including, but not limited to, fusion
25 proteins with a heterologous amino acid sequence, fusions with heterologous and homologous leader sequences, with or without N-terminal methionine residues; immunologically tagged proteins; and the like. The term “polypeptide” includes post-translationally modified polypeptides.

[0047] “Modulate” as used herein refers to the ability of a compound to increase or
30 decrease the function, or activity, of the associated activity (*e.g.*, immune cell function).

[0048] “Immune cell function” includes, for example, modulation of an immune response. The modulation can be immunosuppressive or immunostimulatory. Examples of immune responses can include, but are not limited to a humoral immune response, a cell-mediate immune response, or an inflammatory response.

- 5 [0049] “Inhibition”, “inhibits” and “inhibitor” as used herein refer to a compound that prohibits or a method of prohibiting, a specific action or function.

- [0050] “Antibody” as used herein refers to a polypeptide encoded by an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu
10 constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD, and IgE, respectively. Antibodies are representative of a wide variety of receptors including hormone receptors, drug targets such as peripheral benzodiazepine receptor, and
15 carrier proteins. Representative antibodies include, but are not limited to monoclonal IgG antibodies, IgG antibody fragments, single chain scFv antibodies, single-domain heavy-chain VHH antibodies, or engineered antibody-like scaffolds such as adnectins, affibodies, anticalins, DARPins, and engineered Kunitz-type inhibitors. Other examples also include receptor decoys of immunomodulatory cytokines such as Tumor Necrosis Factor- α and IL-
20 1 β , IL-6, or interferon- γ .

[0051] “Sulfide bond” as used herein refers to any moiety having a sulfur covalent bond.

[0052] “Diffusion half-life” as used herein refers to the time it takes for the initial concentration of the conjugate within a given volume or space to decrease by half, where the decrease in concentration is a function of the concentration gradient.

- 25 [0053] “Intra-articular half-life” as used herein refers to the time it takes for the initial concentration of the conjugate within a particular joint to decrease by half, where the transport out of the joint is via convection. Convective transport is the combination of transport via diffusion and advection, where advective transport is the transport of a substance by bulk motion.

- 30 [0054] “Pharmaceutical composition” as used herein refers to a product comprising the specified ingredients in the specified amounts, as well as any product, which results, directly

or indirectly, from combination of the specified ingredients in the specified amounts. The pharmaceutical composition is generally safe for biological use.

[0055] “Pharmaceutically acceptable carrier” and “pharmaceutically acceptable excipient” as used herein refers to a substance that aids the administration of an active agent to an absorption by a subject. Pharmaceutical carrier and/or excipient useful in the present invention include, but are not limited to, binders, fillers, disintegrants, lubricants, coatings, sweeteners, flavors and colors. One of skill in the art will recognize that other pharmaceutical carriers and/or excipients are useful in the present invention.

III. PEPTIDE-POLYMER CONJUGATES

[0056] The present invention provides conjugates of high molecular weight polymers and a plurality of peptides that possess a potency greater than a similar concentration of the unconjugated peptide, where the peptides are covalently linked to the polymer via a hydrophilic linker. In some embodiments, the present invention provides a conjugate of Formula I:

(X-Y)_n-Z Formula (I)

wherein each X is independently a peptide having a molecular weight of from about 5 kDa to about 200 kDa; each Y is independently a hydrophilic linker; Z is a biocompatible polymer having a molecular weight of from about 0.1 MDa to about 3 MDa; and subscript n is an integer from 10 to 1000.

anti-VEGF Peptides

[0057] Peptides that are suitable for inclusion in a conjugate, for use in a method of the present disclosure, include, but are not limited to, a neuroprotective polypeptide, an anti-angiogenic polypeptide, an anti-apoptotic factor, and a polypeptide that enhances function of a retinal cell.

[0058] Peptides that are suitable for inclusion in a conjugate, for use in a method of the present disclosure, include, but are not limited to, neuroprotective polypeptides (e.g., GDNF, CNTF, NT4, NGF, and NTN); anti-angiogenic polypeptides (e.g., a soluble vascular endothelial growth factor (VEGF) receptor; a VEGF-binding antibody; a VEGF-binding antibody fragment (e.g., a single chain anti-VEGF antibody); endostatin; tumstatin; angiostatin; a soluble Flt polypeptide (Lai et al. (2005) Mol. Ther. 12:659); an Fc fusion

protein comprising a soluble Flt polypeptide (see, e.g., Pechan et al. (2009) Gene Ther. 16:10); pigment epithelium-derived factor (PEDF); a soluble Tie-2 receptor; etc.); tissue inhibitor of metalloproteinases-3 (TIMP-3); a light-responsive opsin, e.g., a rhodopsin; anti-apoptotic polypeptides (e.g., Bcl-2, Bcl-Xl); and the like. Suitable polypeptides include, but are not limited to, glial derived neurotrophic factor (GDNF); fibroblast growth factor 2; neurturin (NTN); ciliary neurotrophic factor (CNTF); nerve growth factor (NGF); neurotrophin-4 (NT4); brain derived neurotrophic factor (BDNF); epidermal growth factor; rhodopsin; X-linked inhibitor of apoptosis; and Sonic hedgehog.

[0059] Peptides that are suitable for inclusion in a conjugate, for use in a method of the present disclosure, include, but are not limited to, a soluble vascular endothelial growth factor (VEGF) receptor; angiostatin, endostatin; vasostatin; retinal pigment epithelium-specific protein 65 kDa (RPE65); and compstatin. In some embodiments, the biologically active polypeptide is a soluble fms-like tyrosine kinase-1 (sFlt-1) polypeptide. In some embodiments, the biologically active polypeptide is a single-domain camelid (VHH) anti-VEGF antibody (VHH anti-VEGF antibody). In some embodiments, the biologically active polypeptide is a single chain Fv anti-VEGF antibody (scFv anti-VEGF antibody). In some embodiments, the peptide is an adnectin, an affibody, an anticalin, a DARPin, a Kunitz-type inhibitor, or a receptor decoy.

[0060] Peptides that are suitable for inclusion in a conjugate, for use in a method of the present disclosure, include, but are not limited to, glial derived neurotrophic factor, fibroblast growth factor 2, neurturin, ciliary neurotrophic factor, nerve growth factor, brain derived neurotrophic factor, epidermal growth factor, rhodopsin, X-linked inhibitor of apoptosis, retinoschisin, RPE65, retinitis pigmentosa GTPase-interacting protein-1, peripherin, peripherin-2, a rhodopsin, and Sonic hedgehog.

[0061] Suitable polypeptides also include retinoschisin. Suitable polypeptides include, e.g., retinitis pigmentosa GTPase regulator (RGPR)-interacting protein-1 (see, e.g., GenBank Accession Nos. Q96KN7, Q9EPQ2, and Q9GLM3); peripherin-2 (Prph2) (see, e.g., GenBank Accession No. NP_000313; and Travis et al. (1991) Genomics 10:733); peripherin; a retinal pigment epithelium-specific protein (RPE65) (see, e.g., GenBank AAC39660; and Morimura et al. (1998) Proc. Natl. Acad. Sci. USA 95:3088); and the like.

[0062] Suitable polypeptides also include: CHM (choroideremia (Rab escort protein 1)), a polypeptide that, when defective or missing, causes choroideremia (see, e.g., Donnelly et al.

(1994) Hum. Mol. Genet. 3:1017; and van Bokhoven et al. (1994) Hum. Mol. Genet. 3:1041); and Crumbs homolog 1 (CRB1), a polypeptide that, when defective or missing, causes Leber congenital amaurosis and retinitis pigmentosa (see, e.g., den Hollander et al. (1999) Nat. Genet. 23:217; and GenBank Accession No. CAM23328).

- 5 **[0063]** Suitable peptides also include peptides that, when defective or missing, lead to achromotopsia, where such polypeptides include, e.g., cone photoreceptor cGMP-gated channel subunit alpha (CNGA3) (see, e.g., GenBank Accession No. NP_001289; and Booij et al. (2011) Ophthalmology 118:160-167); cone photoreceptor cGMP-gated cation channel beta-subunit (CNGB3) (see, e.g., Kohl et al. (2005) Eur J Hum Genet. 13(3):302); guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2 (GNAT2) 10 (ACHM4); and ACHM5; and polypeptides that, when defective or lacking, lead to various forms of color blindness (e.g., L-opsin, M-opsin, and S-opsin). See Mancuso et al. (2009) Nature 461(7265):784-787.

- 15 **[0064]** Peptides that are suitable for inclusion in a conjugate, for use in a method of the present disclosure, include an antibody. Suitable antibodies include, e.g., an antibody specific for VEGF; an antibody specific for tumor necrosis factor-alpha (TNF- α); and the like.

- [0065]** Suitable antibodies include, but are not limited to, adalimumab, alemtuzumab, basiliximab, belimumab, bevacizumab, briakinumab, brodalumab, canakinumab, certolizumab, claakizumab, daclizumab, denosumab, efalizumab, epratuzumab, 20 etaracizumab, fezakinumab, figitumumab, fontolizumab, gevokizumab, gotimumab, infliximab, namilumab, namilumab, natalizumab, neutrazumab, nextomab, ocaratuzumab, ofatumumab, olokizumab, pateclizumab, priliximab, ranibizumab, rituximab, secukinumab, sirukumab, sonpcepizumab, tabalumab, tocilizumab, toralizumab, ustekinumab, vapaliximab, vedolizumab, veltuzumab, visilizumab, vorsetuzumab, and ziralimumab.

- 25 **[0066]** In some embodiments, the peptide is a soluble fms-like tyrosine kinase-1 (sFlt-1) polypeptide. In some embodiments, the peptide comprises an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to a contiguous stretch of from 100 amino acids (aa) to 200 aa, from 200 aa to 300 aa, from 300 aa to 400 aa, from 400 aa to 500 aa, from 500 aa to 600 aa, 30 from 600 aa to 700 aa, or from 700 aa to 755 aa, of the amino acid sequence depicted in SEQ ID NO:12. In some embodiments, the peptide comprises an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%,

amino acid sequence identity to the amino acid sequence depicted in SEQ ID NO:13. In some embodiments, the peptide comprises an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence depicted in SEQ ID NO:14. In some embodiments, the peptide comprises an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence depicted in SEQ ID NO:15. In some embodiments, the peptide comprises the amino acid sequence depicted in SEQ ID NO:15.

[0067] In some embodiments, the peptide is an sFlt-1 polypeptide having a length of from 150 amino acids to 200 amino acids, from 200 to amino acids to 250 amino acids, from 250 amino acids to 300 amino acids, from 300 amino acids to 350 amino acids, or from 350 amino acids to 400 amino acids.

[0068] In some embodiments, the peptide is a scFv anti-VEGF antibody. Any suitable scFv anti-VEGF antibody can be used. A non-limiting example of an amino acid sequence of a scFv anti-VEGF antibody is provided in SEQ ID NO:16. An enterokinase cleavage site (DDDDK) and a poly(His) tract (HHHHHH) are present at the carboxyl terminus of the scFv anti-VEGF antibody depicted in F SEQ ID NO:16. In some embodiments, a scFv anti-VEGF antibody does not include an enterokinase cleavage site or a poly(His) tract.

[0069] In some embodiments, the peptide is a single domain camelid (VHH) anti-VEGF antibody. Any suitable VHH anti-VEGF antibody can be used. A non-limiting example of an amino acid sequence of a VHH anti-VEGF antibody is provided in SEQ ID NO:17. An enterokinase cleavage site (DDDDK) and a poly(His) tract (HHHHHH) are present at the carboxyl terminus of the VHH anti-VEGF antibody depicted in SEQ ID NO:17. In some embodiments, a VHH anti-VEGF antibody does not include an enterokinase cleavage site or a poly(His) tract.

[0070] In some embodiments, the peptide is an inhibitor of angiogenesis. In some embodiments, the peptide is a soluble vascular endothelial growth factor (VEGF) receptor, angiostatin, endostatin, vasostatin, an antibody specific for VEGF, or a DARPin specific for VEGF. In some embodiments, the peptide inhibits VEGF-A, VEGF-B, VEGF-C, VEGF-D, Ang-1, Ang-2, PDGF, or PlGF. In some embodiments, the peptide is a monoclonal IgG antibody, an IgG antibody fragment, a single-chain variable region antibody, a single-domain

heavy chain antibody, an adnectin, an affibody, an anticalin, a DARPin, a Kunitz-type inhibitor, or a receptor decoy.

anti-TNF alpha Peptides

[0071] Peptides suitable in the present invention are those having a molecular weight of at least about 2 kDa, and exhibit tertiary structure. Representative peptides include, but are not limited to, polypeptides, one or more aptamers, avimer scaffolds based on human A domain scaffolds, diabodies, camelids, shark IgNAR antibodies, fibronectin type III scaffolds with modified specificities, antibodies, antibody fragments, proteins, peptides, polypeptides.

[0072] In some embodiments, the peptide is a therapeutic protein. Numerous therapeutic proteins are disclosed throughout the application such as, and without limitation, erythropoietin, granulocyte colony stimulating factor (G-CSF), GM-CSF, interferon alpha, interferon beta, human growth hormone, and imiglucerase.

[0073] In some embodiments, the peptide can be selected from specifically identified protein or peptide agents, including, but not limited to: A β , agalsidase, alefacept, alkaline phosphatase, asparaginase, amdoxovir (DAPD), antide, becaplermin, botulinum toxin including types A and B and lower molecular weight compounds with botulinum toxin activity, calcitonins, cyanovirin, denileukin diftitox, erythropoietin (EPO), EPO agonists, dornase alpha, erythropoiesis stimulating protein (NESP), coagulation factors such as Factor V, Factor VII, Factor VIIa, Factor VIII, Factor IX, Factor X, Factor XII, Factor XIII, von Willebrand factor; ceredase, cerezyme, alpha-glucosidase, N-Acetylgalactosamine-6-sulfate sulfatase, collagen, cyclosporin, alpha defensins, beta defensins, desmopressin, exendin-4, cytokines, cytokine receptors, granulocyte colony stimulating factor (G-CSF), thrombopoietin (TPO), alpha-1 proteinase inhibitor, elcatonin, granulocyte macrophage colony stimulating factor (GM-CSF), fibrinogen, filgrastim, growth hormones human growth hormone (hGH), somatropin, growth hormone releasing hormone (GHRH), GRO-beta, GRO-beta antibody, bone morphogenic proteins such as bone morphogenic protein-2, bone morphogenic protein-6, parathyroid hormone, parathyroid hormone related peptide, OP-1; acidic fibroblast growth factor, basic fibroblast growth factor, Fibroblast Growth Factor 21, CD-40 ligand, heparin, human serum albumin, low molecular weight heparin (LMWH), interferon alpha, interferon beta, interferon gamma, interferon omega, interferon tau, consensus interferon, human lysyl oxidase-like-2 (LOXL2); interleukins and interleukin receptors such as interleukin-1 receptor, interleukin-2, interleukin-2 fusion proteins,

interleukin-1 receptor antagonist, interleukin-3, interleukin-4, interleukin-4 receptor, interleukin-6, interleukin-8, interleukin-12, interleukin-15, interleukin-17, interleukin-21, interleukin-23, p40, interleukin-13 receptor, interleukin-17 receptor; lactoferrin and lactoferrin fragments, luteinizing hormone releasing hormone (LHRH), insulin, pro-insulin, 5 insulin analogues, leptin, ghrelin, amylin, C-peptide, somatostatin, somatostatin analogs including octreotide, vasopressin, follicle stimulating hormone (FSH), imiglucerase, influenza vaccine, insulin-like growth factor (IGF), insulintropin, macrophage colony stimulating factor (M-CSF), plasminogen activators such as alteplase, urokinase, reteplase, streptokinase, pamiteplase, lanoteplase, and tenecteplase; nerve growth factor (NGF), 10 osteoprotegerin, platelet-derived growth factor, tissue growth factors, transforming growth factor-1, vascular endothelial growth factor, leukemia inhibiting factor, keratinocyte growth factor (KGF), glial growth factor (GGF), T Cell receptors, CD molecules/antigens, tumor necrosis factor (TNF) (e.g., TNF- α and TNF- β), TNF receptors (e.g., TNF- α receptor and TNF- β receptor), CTLA4, CTLA4 receptor, monocyte chemoattractant protein-1, endothelial 15 growth factors, parathyroid hormone (PTH), glucagon-like peptide, somatotropin, thymosin alpha 1, rasburicase, thymosin alpha 1 IIb/IIIa inhibitor, thymosin beta 10, thymosin beta 9, thymosin beta 4, alpha-1 antitrypsin, phosphodiesterase (PDE) compounds, VLA-4 (very late antigen-4), VLA-4 inhibitors, bisphosphonates, respiratory syncytial virus antibody, cystic fibrosis transmembrane regulator (CFTR) gene, deoxyribonuclease (Dnase), 20 bactericidal/permeability increasing protein (BPI), and anti-CMV antibody. Exemplary monoclonal antibodies include etanercept (a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human 75 kD TNF receptor linked to the Fc portion of IgG1), abciximab, adalimumab, afelimomab, alemtuzumab, antibody to B-lymphocyte, atlizumab, basiliximab, bevacizumab, biciromab, bertilimumab, CDP-484, CDP- 25 571, CDP-791, CDP-860, CDP-870, cetuximab, clenoliximab, daclizumab, eculizumab, edrecolomab, efalizumab, epratuzumab, fontolizumab, gavilimumab, gentuzumab, ozogamicin, ibritumomab tiuxetan, infliximab, inolimumab, keliximab, labetuzumab, lerdelimomab, olizumab, radiolabeled lym-1, metelimomab, mepolizumab, mitumomab, muromonad-CD3, nebacumab, natalizumab, odulimumab, omalizumab, oregovomab, 30 palivizumab, pentumomab, pexelizumab, rhuMAb-VEGF, rituximab, satumomab pendetide, sevirumab, sipilizumab, tositumomab, I¹³¹tositumomab, trastuzumab, tuvirumab, visilizumab, and fragments and mimetics thereof.

[0074] In some embodiments, the peptide is a fusion protein. For example, and without limitation, the peptide can be an immunoglobulin or portion of an immunoglobulin fused to one or more certain useful peptide sequences. For example, the peptide may contain an antibody Fc fragment. In one embodiment, the peptide is a CTLA4 fusion protein. For example, the peptide can be an Fc-CTLA4 fusion protein. In another embodiment, the peptide is a Factor VIII fusion protein. For example, the peptide can be an Fc-Factor VIII fusion protein.

[0075] In some embodiments, the peptide is a human protein or human polypeptide, for example, a heterologously produced human protein or human polypeptide. Numerous proteins and polypeptides are disclosed herein for which there is a corresponding human form (i.e., the protein or peptide is normally produced in human cells in the human body). Therefore, in one embodiment, the peptide is the human form of each of the proteins and polypeptides disclosed herein for which there is a human form. Examples of such human proteins include, without limitation, human antibodies, human enzymes, human hormones and human cytokines such as granulocyte colony stimulation factor, granulocyte macrophage colony stimulation factor, interferons (e.g., alpha interferons and beta interferons), human growth hormone and erythropoietin.

[0076] Other examples of therapeutic proteins include, without limitation, factor VIII, b-domain deleted factor VIII, factor VIIa, factor IX, anticoagulants; hirudin, alteplase, tpa, reteplase, tpa, tpa--3 of 5 domains deleted, insulin, insulin lispro, insulin aspart, insulin glargine, long-acting insulin analogs, hgh, glucagons, tsh, follitropin-beta, fsh, gm-csf, pdgh, ifn alpha2, ifn alpha2a, ifn alpha2b, inf-apha1, consensus ifn, ifn-beta, ifn-beta 1b, ifn-beta 1a, ifn-gamma (e.g., 1 and 2), ifn-lambda, ifn-delta, il-2, il-11, hbsag, ospa, murine mab directed against t-lymphocyte antigen, murine mab directed against tag-72, tumor-associated glycoprotein, fab fragments derived from chimeric mab directed against platelet surface receptor gpII(b)/III(a), murine mab fragment directed against tumor-associated antigen cal 25, murine mab fragment directed against human carcinoembryonic antigen, cea, murine mab fragment directed against human cardiac myosin, murine mab fragment directed against tumor surface antigen psma, murine mab fragments (fab/fab2 mix) directed against hmw-maa, murine mab fragment (fab) directed against carcinoma-associated antigen, mab fragments (fab) directed against nca 90, a surface granulocyte nonspecific cross reacting antigen, chimeric mab directed against cd20 antigen found on surface of b lymphocytes, humanized mab directed against the alpha chain of the il2 receptor, chimeric mab directed

against the alpha chain of the il2 receptor, chimeric mab directed against tnfr-alpha, humanized mab directed against an epitope on the surface of respiratory syncytial virus, humanized mab directed against her 2, human epidermal growth factor receptor 2, human mab directed against cytokeratin tumor-associated antigen anti-ctla4, chimeric mab directed against cd 20 surface antigen of b lymphocytes dornase-alpha dnase, beta glucocerebrosidase, 5 tnfr-igg fragment fusion protein laronidase, dnaases, alefacept, darbepoetin alpha (colony stimulating factor), tositumomab, murine mab, alemtuzumab, rasburicase, agalsidase beta, teriparatide, parathyroid hormone derivatives, adalimumab (lgg1), anakinra, biological modifier, nesiritide, human b-type natriuretic peptide 10 (hbnp), colony stimulating factors, pegvisomant, human growth hormone receptor antagonist, recombinant activated protein c, omalizumab, immunoglobulin e (ige) blocker, lbratumab tiuxetan, ACTH, glucagon, somatostatin, somatotropin, thymosin, parathyroid hormone, pigmentary hormones, somatomedin, erythropoietin, luteinizing hormone, chorionic gonadotropin, hypothalamic releasing factors, etanercept, antidiuretic hormones, prolactin and 15 thyroid stimulating hormone. And any of these can be modified to have a site-specific conjugation point (a N-terminus, or C-terminus, or other location) using natural (for example, a serine to cysteine substitution) (for example, formylaldehyde per method of Redwood Biosciences) or non-natural amino acid.

[0077] Examples of therapeutic antibodies (or their respective scFv or Fab fragments) 20 useful in the present invention include, but are not limited to, Anti-TNF inhibitors such as the TNF receptor decoy etanercept and the monoclonal antibodies adalimumab, infliximab, golimumab, and certolizumab, the IL-6 monoclonal antibody inhibitor siltuximab, the IL-17 monoclonal antibody inhibitors secukinumab and ixekizumab, the IL-12/23 monoclonal antibody inhibitor ustekinumab, integrin receptor antagonists such as the monoclonal 25 antibody inhibitors natalizumab and etrolizumab, the CLTA receptor antagonist abatacept, the IL-13 monoclonal antibody inhibitor tralokinumab, chemokine inhibitors such as the monoclonal antibodies eldelumab and bertilumab, and IL-1 inhibitors such as the receptor decoy rilonacept and the such as the monoclonal antibody canakinumab.

[0078] Other examples of therapeutic antibodies (or their respective scFv or Fab fragments) 30 useful in the present invention include, but are not limited, to HERCEPTINTM (Trastuzumab) (Genentech, CA) which is a humanized anti-HER2 monoclonal antibody for the treatment of patients with metastatic breast cancer; REOPROTM (abciximab) (Centocor) which is an anti-glycoprotein IIb/IIIc receptor on the platelets for the prevention of clot formation;

ZENAPAX™ (daclizumab) (Roche Pharmaceuticals, Switzerland) which is an immunosuppressive, humanized anti-CD25 monoclonal antibody for the prevention of acute renal allograft rejection; PANOREX™ which is a murine anti-17-1A cell surface antigen IgG2a antibody (Glaxo Wellcome/Centocor); BEC2 which is a murine anti-idiotypic (GD3 epitope) IgG antibody (ImClone System); IMC-C225 which is a chimeric anti-EGFR IgG antibody (ImClone System); VITAXIN™ which is a humanized anti- α V β 3 integrin antibody (Applied Molecular Evolution/MedImmune); Campath; Campath 1H/LDP-03 which is a humanized anti CD52 IgG1 antibody (Leukosite); Smart M195 which is a humanized anti-CD33 IgG antibody (Protein Design Lab/Kanebo); RITUXAN™ which is a chimeric anti-CD20 IgG1 antibody (IDEC Pharm/Genentech, Roche/Zetenyaku); LYMPHOCIDE™ which is a humanized anti-CD22 IgG antibody (Immunomedics); ICM3 is a humanized anti-ICAM3 antibody (ICOS Pharm); IDEC-114 is a primate anti-CD80 antibody (IDEC Pharm/Mitsubishi); ZEVALIN™ is a radiolabelled murine anti-CD20 antibody (IDEC/Schering AG); IDEC-131 is a humanized anti-CD40L antibody (IDEC/Eisai); IDEC-151 is a primatized anti-CD4 antibody (IDEC); IDEC-152 is a primatized anti-CD23 antibody (IDEC/Seikagaku); SMART anti-CD3 is a humanized anti-CD3 IgG (Protein Design Lab); 5G1.1 is a humanized anti-complement factor 5 (C5) antibody (Alexion Pharm); D2E7 is a humanized anti-TNF- α antibody (CATIBASF); CDP870 is a humanized anti-TNF- α Fab fragment (Celltech); IDEC-151 is a primatized anti-CD4 IgG1 antibody (IDEC Pharm/SmithKline Beecham); MDX-CD4 is a human anti-CD4 IgG antibody (Medarex/Eisai/Genmab); CDP571 is a humanized anti-TNF- α IgG4 antibody (Celltech); LDP-02 is a humanized anti- α 4 β 7 antibody (LeukoSite/Genentech); OrthoClone OKT4A is a humanized anti-CD4 IgG antibody (Ortho Biotech); ANTOVA™ is a humanized anti-CD40L IgG antibody (Biogen); ANTEGREN™ is a humanized anti-VLA-4 IgG antibody (Elan); CAT-152, a human anti-TGF- β .sub.2 antibody (Cambridge Ab Tech); Cetuximab (BMS) is a monoclonal anti-EGF receptor (EGFR) antibody; Bevacizumab (Genentech) is an anti-VEGF human monoclonal antibody; Infliximab (Centocor, JJ) is a chimeric (mouse and human) monoclonal antibody used to treat autoimmune disorders; Gemtuzumab ozogamicin (Wyeth) is a monoclonal antibody used for chemotherapy; and Ranibizumab (Genentech) is a chimeric (mouse and human) monoclonal antibody used to treat macular degeneration.

[0079] Proteins and peptides disclosed herein can be produced by any useful method including production by in vitro synthesis and by production in biological systems. Typical examples of in vitro synthesis methods which are well known in the art include solid-phase

synthesis ("SPPS") and solid-phase fragment condensation ("SPFC"). Biological systems used for the production of proteins are also well known in the art. Bacteria (e.g., *E. coli* and *Bacillus* sp.) , yeast (e.g., *Saccharomyces cerevisiae* and *Pichia pastoris*) tobacco leaves (via tobacco mosaic virus) are widely used for the production of heterologous proteins. In addition, heterologous gene expression for the production of peptides for use as disclosed herein can be accomplished using animal cell lines such as mammalian cell lines (e.g., CHO cells). In one particularly useful embodiment, the peptides are produced in transgenic or cloned animals such as cows, sheep, goats and birds (e.g., chicken, quail, ducks and turkey), each as is understood in the art. See, for example, U.S. Pat. No. 6,781,030, issued Aug. 24, 2004, the disclosure of which is incorporated in its entirety herein by reference.

[0080] Protein or polypeptides useful in the present invention may also comprise non-naturally occurring amino acids in addition to the common naturally occurring amino acids found in proteins and polypeptides. In addition to being present for the purpose of altering the properties of a polypeptide or protein, non-naturally occurring amino acids can be introduced to provide a functional group that can be used to link the protein or polypeptide directly to the random copolymer. Furthermore, naturally occurring amino acids, e.g., cysteine, tyrosine, tryptophan can be used in this way.

[0081] Non-naturally occurring amino acids can be introduced into proteins and peptides by a variety of means. Some of the techniques for the introduction of non-natural amino acids are discussed in U.S. Pat. No. 5,162,218, the disclosure of which is incorporated in its entirety herein by reference. First, non-naturally occurring amino acids can be introduced by chemical modification of a polypeptide or protein on the amino acid side chain or at either the amino terminus or the carboxyl terminus. Non-limiting examples of chemical modification of a protein or peptide might be methylation by agents such as diazomethane, or the introduction of acetylation at an amino group present in lysine's side chain or at the amino terminus of a peptide or protein. Another example of the protein/polypeptide amino group modification to prepare a non-natural amino acid is the use of methyl 3-mercaptopropionimidate ester or 2-iminothiolane to introduce a thiol (sulfhydryl, --SH) bearing functionality linked to positions in a protein or polypeptide bearing a primary amine. Once introduced, such groups can be employed to form a covalent linkage to the protein or polypeptide.

[0082] Second, non-naturally occurring amino acids can be introduced into proteins and polypeptides during chemical synthesis. Synthetic methods are typically utilized for

preparing polypeptides having fewer than about 200 amino acids, usually having fewer than about 150 amino acids, and more usually having 100 or fewer amino acids. Shorter proteins or polypeptides having less than about 75 or less than about 50 amino acids can be prepared by chemical synthesis.

- 5 **[0083]** The synthetic preparation methods that are particularly convenient for allowing the insertion of non-natural amino acids at a desired location are known in the art. Suitable synthetic polypeptide preparation methods can be based on Merrifield solid-phase synthesis methods where amino acids are sequentially added to a growing chain (Merrifield (1963) J. Am. Chem. Soc. 85:2149-2156). Automated systems for synthesizing polypeptides by such
- 10 techniques are now commercially available from suppliers such as Applied Biosystems, Inc., Foster City, Calif. 94404; New Brunswick Scientific, Edison, N.J. 08818; and Pharmacia, Inc., Biotechnology Group, Piscataway, N.J. 08854.

- [0084]** Examples of non-naturally occurring amino acids that can be introduced during chemical synthesis of polypeptides include, but are not limited to: D-amino acids and
- 15 mixtures of D and L-forms of the 20 naturally occurring amino acids, N-formyl glycine, ornithine, norleucine, hydroxyproline, beta-alanine, hydroxyvaline, norvaline, phenylglycine, cyclohexylalanine, t-butylglycine (t-leucine, 2-amino-3,3-dimethylbutanoic acid), hydroxy-t-butylglycine, amino butyric acid, cycloleucine, 4-hydroxyproline, pyroglutamic acid (5-oxoproline), azetidine carboxylic acid, pipecolic acid, indoline-2-carboxylic acid,
- 20 tetrahydro-3-isoquinoline carboxylic acid, 2,4-diaminobutyric acid, 2,6-diaminopimelic acid, 2,4-diaminobutyric acid, 2,6-diaminopimelic acid, 2,3-diaminopropionic acid, 5-hydroxylysine, neuraminic acid, and 3,5-diiodotyrosine.

- [0085]** Third, non-naturally occurring amino acids can be introduced through biological synthesis in vivo or in vitro by insertion of a non-sense codon (e.g., an amber or ochre codon)
- 25 in a DNA sequence (e.g., the gene) encoding the polypeptide at the codon corresponding to the position where the non-natural amino acid is to be inserted. Such techniques are discussed for example in U.S. Pat. Nos. 5,162,218 and 6,964,859, the disclosures of which are incorporated in their entirety herein by reference. A variety of methods can be used to insert the mutant codon including oligonucleotide-directed mutagenesis. The altered sequence is
- 30 subsequently transcribed and translated, in vivo or in vitro in a system which provides a suppressor tRNA, directed against the nonsense codon that has been chemically or enzymatically acylated with the desired non-naturally occurring amino acid. The synthetic

amino acid will be inserted at the location corresponding to the nonsense codon. For the preparation of larger and/or glycosylated polypeptides, recombinant preparation techniques of this type are usually preferred. Among the amino acids that can be introduced in this fashion are: formyl glycine, fluoroalanine, 2-Amino-3-mercapto-3-methylbutanoic acid,

- 5 homocysteine, homoarginine and the like. Other similar approaches to obtain non-natural amino acids in a protein include methionine substitution methods.

[0086] Where non-naturally occurring amino acids have a functionality that is susceptible to selective modification, they are particularly useful for forming a covalent linkage to the protein or polypeptide. Circumstances where a functionality is susceptible to selective
10 modification include those where the functionality is unique or where other functionalities that might react under the conditions of interest are hindered either stereochemically or otherwise.

[0087] Other antibodies, such as single domain antibodies are useful in the present invention. A single domain antibody (sdAb, called Nanobody by Ablynx) is an antibody
15 fragment consisting of a single monomeric variable antibody domain. Like a whole antibody, the sdAb is able to bind selectively to a specific antigen. With a molecular weight of only 12-15 kDa, single domain antibodies are much smaller than common whole antibodies (150-160 kDa). A single domain antibody is a peptide chain of about 110 amino acids in length, comprising one variable domain (VH) of a heavy chain antibody, or of a common IgG.

20 [0088] Unlike whole antibodies, single domain antibody (sdAbs) such as VHH do not show complement system triggered cytotoxicity because they lack an Fc region. Camelid and fish derived sdAbs are able to bind to hidden antigens that are not accessible to whole antibodies, for example to the active sites of enzymes.

[0089] A sdAb can be obtained by immunization of dromedaries, camels, llamas, alpacas
25 or sharks with the desired antigen and subsequent isolation of the mRNA coding for heavy chain antibodies. Alternatively they can be made by screening synthetic libraries. Camelids are members of the biological family Camelidae, the only living family in the suborder Tylopoda. Camels, dromedaries, Bactrian Camels, llamas, alpacas, vicunas, and guanacos are in this group.

30 [0090] Peptides useful in the present invention also include, but are not limited to, a macrocyclic peptide, a cyclotide, an LDL receptor A-domain, a soluble receptor, an enzyme, a peptide multimer, a domain multimer, an antibody fragment multimer, and a fusion protein.

[0091] In some embodiments, the peptide modulates the activity of immune cell function. In some embodiments, the peptide inhibits tumor necrosis factor- α , interleukin-1 β , interleukin-6, or interferon- γ . In some embodiments, the peptide inhibits tumor necrosis factor- α . In some embodiments, the peptide is a monoclonal IgG antibody, an IgG antibody
5 fragment, a single-chain variable region antibody, a single-domain heavy chain antibody, an adnectin, an affibody, an anticalin, a DARPIn, a Kunitz-type inhibitor, or a receptor decoy.

[0092] In some embodiments, the peptide can be anti-TNF α single-domain heavy-chain (VHH) antibody. In some embodiments, the peptide can be anti-TNF α affibody. In some
10 embodiments, the peptide can be anti-TNF α designed ankyrin repeat protein (DARPIn). In some embodiments, the peptide can be anti-IL-1B single-chain (scFv) antibody. In some embodiments, the peptide can be soluble interleukin receptor 2 (sILR2). In some
embodiments, the peptide has an amino acid sequence that is SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10 or SEQ ID
NO:11. In some embodiments, the peptide has an amino acid sequence that is SEQ ID NO:1,
15 SEQ ID NO:2, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID
NO:10. In some embodiments, the peptide has an amino acid sequence that is SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10 or SEQ ID
NO:11. In some embodiments, the peptide has an amino acid sequence that is SEQ ID NO:6,
SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11. In some embodiments, the peptide has an
20 amino acid sequence that is SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:9. In some
embodiments, the peptide has an amino acid sequence that is SEQ ID NO:6. In some
embodiments, the peptide has an amino acid sequence that is SEQ ID NO:7. In some
embodiments, the peptide has an amino acid sequence that is SEQ ID NO:9.

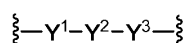
[0093] Peptides useful in the present invention can have a molecular weight of at least 2
25 kDa. Peptides useful in the present invention can have a molecular weight of at least 2 kDa
and exhibit a tertiary structure. For example, the molecular weight of the peptide can be from
about 2 kDa to about 150 kDa, from about 5 kDa to about 150 kDa, from about 5 kDa to
about 100 kDa, from about 2 kDa to about 50 kDa, from about 5 kDa to about 50 kDa, from
about 5 kDa to about 30 kDa, from about 10 kDa to about 30 kDa, or from about 10 kDa to
30 about 20 kDa. Representative molecular weights for the peptide includes about 2 kDa, or 3,
4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30,
35, 40, 45, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or about 150 kDa. In some
embodiments, the peptide has a molecular weight of from about 5kDa to about 30 kDa. In

some embodiments, the peptide has a molecular weight of from about 10 kDa to about 20 kDa.

Linkers

[0094] The linkers of the present invention are hydrophilic linkers. The hydrophilic linkers can include naturally occurring molecules like nucleic acid bases, dimers, and oligomers, carbohydrate monomers or oligosaccharides of a variety of compositions, dextrans, dipeptides or oligopeptides. Other hydrophilic linkers can include, but are not limited to, ethylene glycol dimers, trimers, oligomers and polymers, as well as polyvinyl alcohol, polyvinyl acetate, polyacrylate, peptoids, D- or artificial amino acid containing peptides, polymer brushes, polyelectrolyte brushes, synthetic carbohydrate mono and oligomers, cleavable linkers. Any combination of the above like nucleic acid-amino acid-synthetic polymer, etc.

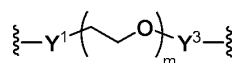
[0095] In some embodiments, each hydrophilic linker independently has the formula:



wherein Y¹ is a thiol reactive group; Y² is a C₃₋₂₀ heteroalkylene having from 1 to 6 heteroatoms each independently N, O or S, or -(CH₂CH₂O)_m-, wherein subscript m is an integer of from 1 to 100; and Y³ is a carboxy reactive group.

[0096] In some embodiments, Y² is a C₃₋₂₀ heteroalkylene having from 1 to 6 heteroatoms each independently N, O or S. In some embodiments, Y² is -(CH₂CH₂O)_m-, wherein subscript m is an integer of from 1 to 100.

[0097] In some embodiments, each hydrophilic linker independently has the formula:

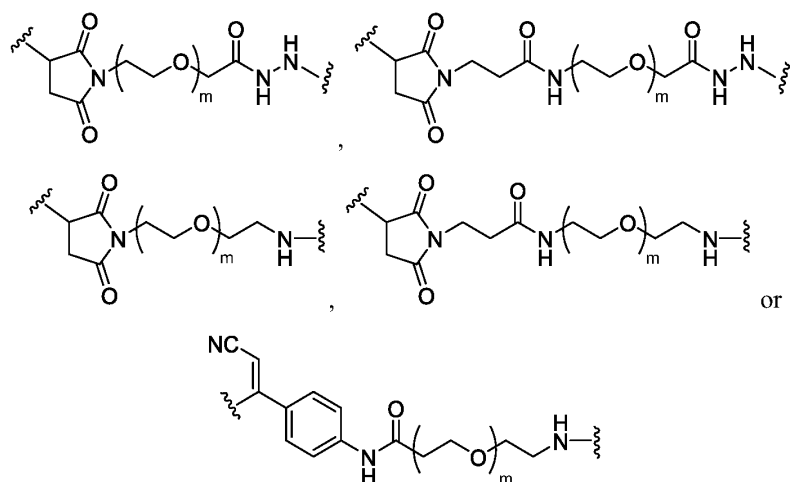


wherein Y¹ is a thiol reactive group; Y³ is a carboxy reactive group; and subscript m is an integer of from 1 to 100. In some embodiments, subscript m is an integer of from 1 to 10. In some embodiments, subscript m is an integer of from 1 to 5. In some embodiments, subscript m is an integer of from 2 to 5. In some embodiments, subscript m is 2. In some embodiments, subscript m is 3. In some embodiments, subscript m is 4.

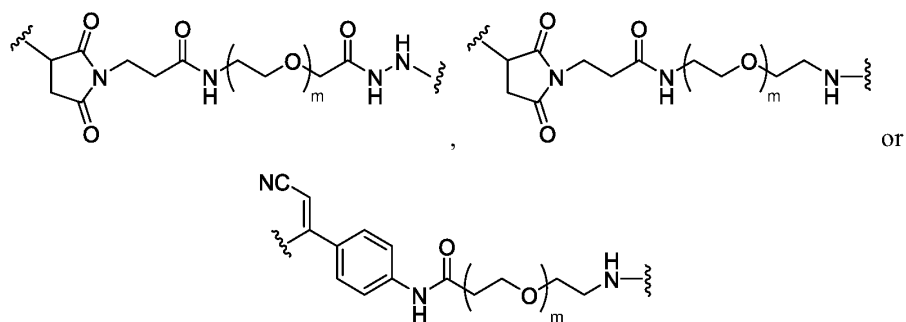
[0098] In some embodiments, Y¹ is a thiol, arylpropionitrile or maleimide; and Y³ is an amine or a N-acylhydrazide. In some embodiments, Y¹ is maleimide; and Y³ is an amine or a

N-acylhydrazide. In some embodiments, Y¹ is maleimide; and Y³ is an amine. In some embodiments, Y¹ is maleimide; and Y³ is a N-acylhydrazide. In some embodiments, Y¹ is arylpropiolonitrile; and Y³ is an amine or a N-acylhydrazide. In some embodiments, Y¹ is arylpropiolonitrile; and Y³ is an amine. In some embodiments, Y¹ is arylpropiolonitrile; and Y³ is a N-acylhydrazide.

[0099] In some embodiments, the hydrophilic linker has the formula:

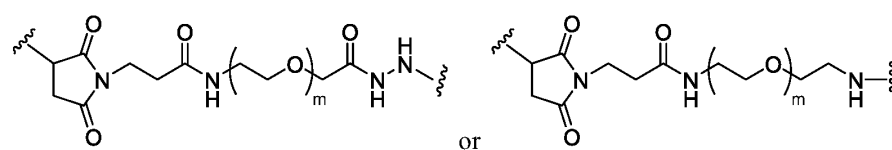


10 wherein subscript m is an integer of from 1 to 100. In some embodiments, the hydrophilic linker has the formula:



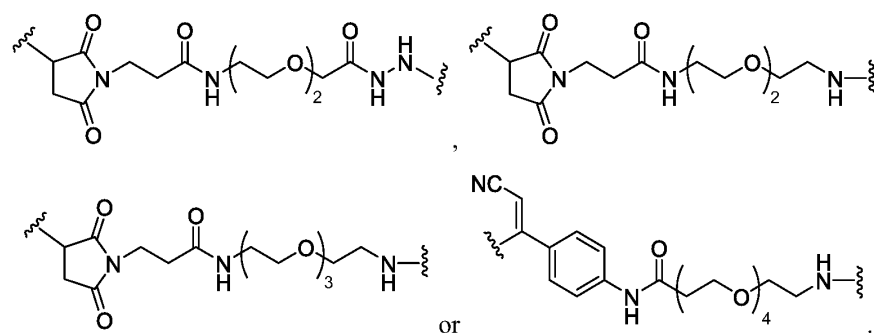
wherein subscript m is an integer of from 1 to 100. In some embodiments, subscript m is an integer of from 1 to 10. In some embodiments, subscript m is an integer of from 1 to 5. In some embodiments, subscript m is an integer of from 2 to 5. In some embodiments, subscript m is 2. In some embodiments, subscript m is 3. In some embodiments, subscript m is 4.

[0100] In some embodiments, the hydrophilic linker has the formula:

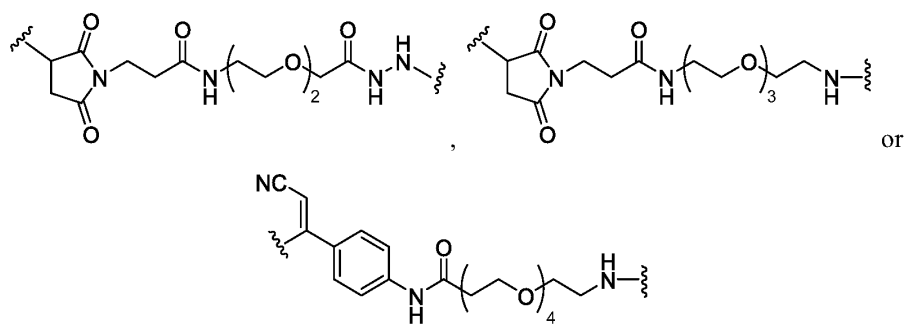


wherein subscript m is an integer of from 1 to 100. In some embodiments, subscript m is an integer of from 1 to 10. In some embodiments, subscript m is an integer of from 1 to 5. In some embodiments, subscript m is an integer of from 2 to 5. In some embodiments, subscript m is 3.

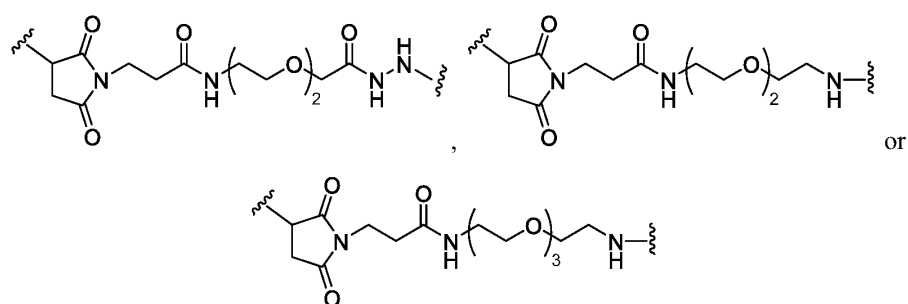
[0101] In some embodiments, the hydrophilic linker has the formula:



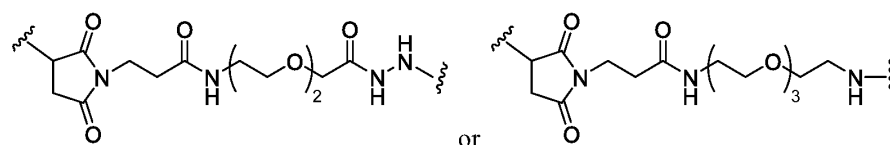
[0102] In some embodiments, the hydrophilic linker has the formula:



[0103] In some embodiments, the hydrophilic linker has the formula:



[0104] In some embodiments, the hydrophilic linker has the formula:



5 Biocompatible polymers

[0105] Polymers useful in the conjugates of the present invention include any suitable biocompatible polymer. Biocompatible polymers are hydrophilic polymers that generally do not trigger an immune response. Suitable biocompatible polymers include, but are not limited to, polysaccharides, glycosaminoglycan, hyaluronic acid and derivatives thereof, cellulose, carboxymethylcellulose and derivatives thereof, heparin and derivatives thereof, dermatin, starch and modified starches, chondroitin, chitosan, carboxymethyl chitosan and others. The biocompatible polymer can also include polyvinylchloride, polytetrafluoroethylene, polyethersulfone, polyethylene, polyetheretherketone, polysulfone, polypropylene, poly(ethylene glycol), poly(propylene glycol), polyurethanes, ethylene vinyl acetate copolymers, collagen, poly isobutylene, ethylene vinyl alcohol copolymers, polyethylene polycarbonate, polycaprolactone, polylactide, polyglycolide, carbomers, polyesters, polyethers, polyanhydrides, polyacrylates, polyvinyl acetates, polyvinyl pyrrolidones, polysaccharides (such as hyaluronic acid, hydroxyalkylcelluloses, carboxyalkylcelluloses, or derivatives thereof), polyether, derivatives thereof and combinations thereof. The biocompatible polymers can be further modified by methods such as sulfation, sulfonation, deuteration, etc.

[0106] Polysaccharides useful as the biocompatible polymer include, but are not limited to, cellulose, carboxymethylcellulose, methyl cellulose, hydroxypropyl cellulose, chitin, glycosaminoglycans, chondroitin sulfate, hyaluronan (hyaluronic acid), heparin, heparan

sulfate, among others. In some embodiments, the biocompatible polymer can be a polysaccharide. In some embodiments, the biocompatible polymer can be a glycosaminoglycan. In some embodiments, the biocompatible polymer can be hyaluronic acid.

5 **[0107]** The biocompatible polymer of the present invention can be of any suitable molecular weight. For example, suitable biocompatible polymers can have a molecular weight of from about 0.1 MDa to about 3 MDa, or about 100 kDa to about 3,000 kDa. A polymer molecular weight can typically be expressed as the number average molecular weight (M_n) or the weight average molecular weight (M_w). The number average molecular
10 weight is the mathematical mean of the molecular masses of the individual macromolecules. The weight average molecular weight is influenced by larger molecules and so is a larger number than the number average molecular weight. The ratio of M_w/M_n is the polydispersity of the polymer and represents the breadth of molecular weights in the polymer sample. Reference to molecular weights in the present invention are to the weight average molecular
15 weight (M_w) unless stated otherwise.

[0108] Molecular weights useful for biocompatible polymer include, but are not limited to, from about 0.1 MDa to about 3 MDa, from about 0.1 MDa to about 2 MDa, from about 0.2 MDa to about 1.5 MDa, from about 0.8 MDa to about 3 MDa, from about 1 MDa to about 3 MDa, from about 1.5 MDa to about 3 MDa, or from about 1 MDa to about 2 MDa. In some
20 embodiments, the biocompatible polymer has a molecular weight of from about 0.1 MDa to about 3 MDa. In some embodiments, the biocompatible polymer has a molecular weight of from about 0.1 MDa to about 2 MDa. In some embodiments, the biocompatible polymer has a molecular weight of from about 0.2 MDa to about 1.5 MDa. In some embodiments, the biocompatible polymer has a molecular weight of from about 0.8 MDa to about 3 MDa. The
25 biocompatible polymer can have a molecular weight of about 0.1 MDa, or 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, or about 3 MDa. In some embodiments, the biocompatible polymer has a molecular weight of at least about 0.85 MDa. In some embodiments, the biocompatible polymer has a molecular weight of about 0.9 MDa.
30 In some embodiments, the biocompatible polymer has a molecular weight of at least about 1 MDa. In some embodiments, the biocompatible polymer has a molecular weight of about 2 MDa.

[0109] The biocompatible polymer can have a molecular weight of from about 2 kDa to about 750 kDa per peptide, or 5 kDa to about 600 kDa per peptide, from about 5 kDa to 500 kDa, from about 5 kDa to about 400 kDa, from about 5 kDa to about 300 kDa, from about 5 kDa to about 200 kDa, to about 5 kDa to about 100 kDa, from about 5 kDa to about 50 kDa, from about 5 kDa to about 40 kDa, from about 5 kDa to about 30 kDa, from about 5 kDa to about 20 kDa, or from about 5 kDa to about 10 kDa per peptide. The biocompatible polymer can have a molecular weight per peptide of about 5 kDa, or 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or about 100 kDa.

10 Conjugates

[0110] The peptide-polymer conjugates of the present invention can include any suitable combination of peptide and biocompatible polymer where the molar ratio of peptide to polymer is at least 5:1. Representative molar ratios of peptide to biocompatible polymer useful in the present invention include from 5:1 to about 1000:1, from 5:1 to about 500:1, from 5:1 to about 400:1, from about 10:1 to about 500:1, from about 10:1 to about 400:1, from about 10:1 to about 300:1, from about 10:1 to about 200:1, from about 10:1 to about 100:1, from about 20:1 to about 100:1, from about 30:1 to about 100:1, from about 50:1 to about 100:1, from about 10:1 to about 50:1, from about 20:1 to about 50:1, or from about 30:1 to about 50:1. Other molar ratios of peptide to biocompatible polymer useful in the present invention include from about 50:1 to about 500:1, from about 50:1 to about 400:1, from about 50:1 to about 300:1, or from about 50:1 to about 200:1. Representative molar ratios of peptide to biocompatible polymer include about 10:1, or 15:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1, 60:1, 70:1, 80:1, 90:1, 100:1, 125:1, 150:1, 175:1, 200:1, 250:1, 300:1, 350:1, 450:1, 500:1, 550:1, 600:1, 650:1, 700:1, 750:1, 800:1, 850:1, 900:1, 950:1 or about 1000:1. In some embodiments, subscript n is an integer of from 10 to 400. In some embodiments, subscript n is an integer of from 10 to 100. In some embodiments, subscript n is an integer of from 50 to 100.

[0111] The conjugates of the peptide of biocompatible polymer of the present invention can have longer diffusion half-lives compared to the unconjugated peptide. For example, the conjugate can have a diffusion half-life of at least 2 times longer than that of the peptide, or 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, or at least 100 times longer than that of the peptide. The diffusion half-life of the conjugate can be from about 2 to about

100 times longer than the peptide, or from about 2 to about 50, from about 10 to about 100, from about 25 to about 100, from about 50 to about 100 times longer than the peptide. In some embodiments, the diffusion half-life of the conjugate is at least about 2 times longer than the peptide. In some embodiments, the diffusion half-life of the conjugate is from about 2 to about 100 times longer than the peptide.

[0112] The conjugates of the present invention can also have longer intra-articular half-lives compared to the unconjugated peptide. For example, the conjugate can have an intra-articular half-life that is at least 20% longer than the unconjugated peptide, or at least 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900 or 1000% longer than the unconjugated peptide. The intra-articular half-life of the conjugate can be from about 20% to about 1000% longer than the unconjugated peptide, or from about 100% to about 1000%, or from about 100% to about 500%, or from about 100% to about 300% longer than the unconjugated peptide. In some embodiments, the intra-articular half-life of the conjugate is at least about 20% longer than the peptide. In some embodiments, the intra-articular half-life of the conjugate is from about 20% to about 1000% longer than the peptide.

[0113] In some embodiments, the biocompatible polymer has a molecular weight of from about 0.8 MDa to about 3 MDa; and each peptide has a molecular weight of from about 5 kDa to about 50 kDa; wherein each peptide is covalently linked to the polymer, and the molar ratio of the peptides to the polymer in the conjugate is at least about 10:1. In some embodiments, the biocompatible polymer has a molecular weight of from about 0.8 MDa to about 2 MDa; and each peptide has a molecular weight of from about 5 kDa to about 50 kDa; wherein each peptide is covalently linked to the polymer, and the molar ratio of the peptides to the polymer in the conjugate is at least about 10:1. In some embodiments, the biocompatible polymer has a molecular weight of from about 1 MDa to about 2 MDa; and each peptide has a molecular weight of from about 5 kDa to about 50 kDa; wherein each peptide is covalently linked to the polymer, and the molar ratio of the peptides to the polymer in the conjugate is at least about 10:1. In some embodiments, the biocompatible polymer has a molecular weight of from about 1 MDa to about 2 MDa; and each peptide has a molecular weight of from about 5 kDa to about 50 kDa; wherein each peptide is covalently linked to the polymer, and the molar ratio of the peptides to the polymer in the conjugate is at least about 20:1. In some embodiments, the biocompatible polymer has a molecular weight of about 2 MDa; and each peptide has a molecular weight of from about 5 kDa to about 50 kDa;

wherein each peptide is covalently linked to the polymer, and the molar ratio of the peptides to the polymer in the conjugate is at least about 50:1.

[0114] A drug that exhibits greater intravitreal residence time may be preferred by the patient as well relative to a drug product that must be administered more frequently for an equivalent therapeutic function. While the intravitreal injection is usually performed under topical anesthesia and is generally not regarded as painful, it is burdensome for the patient. It must be performed by a clinician, and thus an office visit is required for each administration of the drug. There is typically short-term irritation and blurred vision due to increased tearing. There may also be short term changes to the appearance of the eye at the vicinity of the injection site. Finally, progressive and irreversible disease damage can occur when the patient delays treatment, and less frequent opportunities for delayed treatment corresponds to better overall long-term efficacy. Thus, patients would likely exhibit a preference for an equivalent therapy that would require fewer intravitreal injections.

[0115] The need for less frequent injections would also be preferable from the physician's perspective. The intravitreal injections must be performed by an ophthalmologist, and thus this procedure can occupy a substantial portion of their clinic time. The number of patients that are receiving the intravitreal therapy in their practice can be limited by the frequency that each patient must receive the intravitreal injections. Less frequent injection would increase the number of patients that are able to receive the method of therapy. A longer acting drug would also be preferable to a depot or long-term drug delivery device, as these typically require a longer implantation procedure and access to a procedure room, which may offset the benefits of less frequent administration for the clinician.

[0116] A conjugate comprising a biologically active polypeptide and a biocompatible polymer exhibits a half-life in the vitreous that is greater than the half-life in the vitreous of the biologically active polypeptide not conjugated to the biocompatible polymer. The increased half-life of the conjugate in the vitreous confers certain advantages, including, e.g., reduced burden on the patient; reduced number and/or frequency of administrations; increased safety; decreased incidence of infection; increased patient compliance; and increased efficacy. In addition, a conjugate as described herein allows use of polypeptides for treatment of ocular disorders, which polypeptides would not, in unconjugated form, be retained in the eye for a time period suitable for therapy.

[0117] In some embodiments, an effective amount of a conjugate is an amount that is effective to inhibit pathological angiogenesis in the eye of the individual. For example, in some cases, an effective amount of a conjugate is an amount that, when administered in one or more doses, is effective to inhibit pathological angiogenesis in the eye of the individual by
5 at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, or at least 80%, or more than 80%, compared to the degree of pathological angiogenesis in the eye in the absence of treatment with the conjugate, or before treatment with the conjugate.

[0118] In some embodiments, an effective amount of a conjugate is an amount that is
10 effective to reduce intraocular pressure in the eye of the individual. For example, in some cases, an effective amount of a conjugate is an amount that, when administered in one or more doses, is effective to reduce intraocular pressure by at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, or at least 80%, or more than 80%, compared to the intraocular pressure in the eye in the absence
15 of treatment with the conjugate, or before treatment with the conjugate.

[0119] In some embodiments, an effective amount of a conjugate is an amount that is effective to reduce macular edema in the eye of the individual. For example, in some cases, an effective amount of a conjugate is an amount that, when administered in one or more doses, is effective to reduce macular edema by at least 10%, at least 15%, at least 20%, at
20 least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, or at least 80%, or more than 80%, compared to the level of macular edema in the eye in the absence of treatment with the conjugate, or before treatment with the conjugate.

[0120] In some embodiments, an effective amount of a conjugate is an amount that is effective to increase visual acuity in an eye of the individual. For example, in some cases, an
25 effective amount of a conjugate is an amount that, when administered in one or more doses, is effective to increase visual acuity in an eye of the individual by at least at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 2-fold, at least 2.5-fold, at least 5-fold, or at least 10-fold, or more than 10-fold, compared to the visual acuity in the eye in the absence of
30 treatment with the conjugate, or before treatment with the conjugate.

[0121] In some embodiments, an effective amount of a conjugate is an amount that is effective to inhibit progression of an ocular disease in an individual. For example, in some

cases, an effective amount of a conjugate is an amount that, when administered in one or more doses, is effective to inhibit progression of an ocular disease in the individual by at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, or more, compared to the progression in the absence of treatment with the conjugate, or before treatment with the conjugate.

[0122] For example, in some cases, an effective amount of a conjugate is an amount that, when administered in one or more doses, is effective to inhibit progression of non-exudative ARMD to exudative ARMD or to inhibit progression of non-exudative ARMD to a more severe form. In some embodiments, an effective amount of a conjugate is an amount that is effective to inhibit progression of early ARMD (AREDS 2) to intermediate ARMD (AREDS 3) or to advanced ARMD (AREDS 4). In some embodiments, an effective amount of a conjugate is an amount that is effective to inhibit progression of intermediate ARMD (AREDS 3) to advanced ARMD (AREDS 4).

[0123] In some embodiments, an effective amount of a conjugate is an amount that is effective to enhance a biological activity of a retinal cell, e.g., where the retinal cell is a photoreceptor, a retinal ganglion cell, a Muller cell, a bipolar cell, an amacrine cell, a horizontal cell, or a retinal pigmented epithelium cell.

[0124] In some embodiments, a conjugate comprising a biologically active polypeptide and a biocompatible polymer exhibits a half-life in the vitreous of from about 12 hours to about 24 hours, from about 1 day to about 3 days, from about 3 days to about 7 days, from one week to about 2 weeks, from about 2 weeks to about 4 weeks, or from about 1 month to about 6 months.

[0125] In some embodiments, a conjugate comprising a biologically active polypeptide and a biocompatible polymer exhibits a therapeutically efficacious residence time in the vitreous of from about 12 hours to about 24 hours, from about 1 day to about 3 days, from about 3 days to about 7 days, from one week to about 2 weeks, from about 2 weeks to about 4 weeks, from about 1 month to about 3 months, or from about 3 months to about 6 months.

[0126] The biological activity of a polypeptide conjugated to the polymer substrate is enhanced relative to the activity of the polypeptide in soluble form, e.g., compared to the activity of the polypeptide not conjugated to the polymer. In some embodiments, the biological activity of the polypeptide of a polypeptide-polymer conjugate is at least about 25%, at least about 50%, at least about 75%, at least about 2-fold, at least about 5-fold, at

least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 75-fold, at least about 100-fold, at least about 200-fold, at least about 500-fold, or at least about 1000-fold, or more than 1000-fold, greater than the biological activity of the polypeptide in soluble (unconjugated) form.

[0127] In some embodiments, the biological activity of the polypeptide of a suitable polypeptide-polymer conjugate is at least about 25%, at least about 50%, at least about 75%, at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 75-fold, at least about 100-fold, at least about 200-fold, at least about 500-fold, or at least about 1000-fold, or more than 1000-fold, greater than the biological activity of the polypeptide in when conjugated to the polymer at a 1:1 molar ratio.

[0128] In some embodiments, the biological activity of the polypeptide of a suitable polypeptide-polymer conjugate is at least about 25%, at least about 50%, at least about 75%, at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 75-fold, at least about 100-fold, at least about 200-fold, at least about 500-fold, or at least about 1000-fold, or more than 1000-fold, greater than the biological activity of the polypeptide when present in admixture with the polymer.

[0129] In some embodiments, the half-maximal effective concentration (EC₅₀) of the polypeptide of a subject polypeptide-polymer conjugate is at least about 10%, at least about 25%, at least about 50%, at least about 75%, at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 75-fold, at least about 100-fold, at least about 200-fold, at least about 500-fold, or at least about 1000-fold, or more than 1000-fold, lower than the EC₅₀ of the polypeptide in soluble (unconjugated form).

[0130] In some embodiments, the half-maximal inhibitory concentration (IC₅₀) of the polypeptide of a subject polypeptide-polymer conjugate is at least about 10%, at least about 25%, at least about 50%, at least about 75%, at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 75-fold, at

least about 100-fold, at least about 200-fold, at least about 500-fold, or at least about 1000-fold, or more than 1000-fold, lower than the IC₅₀ of the polypeptide in soluble (unconjugated form).

5 [0131] Whether the biological activity of the polypeptide of a polypeptide-polymer conjugate is increased relative to the biological activity of the polypeptide in soluble (unconjugated) form is readily determined using an appropriate assay(s) for the biological activity.

10 [0132] The molar ratio of the polypeptide to the polymer can vary from about 5:1 to about 100:1, e.g., from about 5:1 to about 7:1, from about 7:1 to about 10:1, from about 10:1 to about 12:1, from about 12:1 to about 15:1, from about 15:1 to about 20:1, from about 20:1 to about 25:1, from about 25:1 to about 30:1, from about 30:1 to about 35:1, from about 35:1 to about 40:1, from about 40:1 to about 45:1, from about 45:1 to about 50:1, from about 50:1 to about 60:1, from about 60:1 to about 70:1, from about 70:1 to about 80:1, from about 80:1 to about 90:1, or from about 90:1 to about 100:1.

15 [0133] For example, where a polypeptide polymer conjugate comprises a polypeptide that inhibits angiogenesis (e.g., the polypeptide is an anti-angiogenic polypeptide), in some embodiments, the anti-angiogenic polypeptide of a polypeptide-polymer conjugate inhibits angiogenesis by at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least 20 about 75%, at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 75-fold, at least about 100-fold, at least about 200-fold, at least about 500-fold, or at least about 1000-fold, or more than 1000-fold, or more, compared to the degree of inhibition of angiogenesis by the anti-angiogenic polypeptide 25 when present in admixture with the polymer, when in soluble (unconjugated) form, or when conjugated to the polymer at a 1:1 molar ratio.

IV. PHARMACEUTICAL COMPOSITIONS

[0134] In some embodiments, the present invention provides a pharmaceutical composition including a conjugate of the present invention and a pharmaceutically acceptable excipient.

A. Formulation

[0135] For preparing pharmaceutical compositions from the conjugates of the present invention, pharmaceutically acceptable carriers can be either solid or liquid. Solid form preparations include powders, cachets, and dispersible granules. A solid carrier can be one or more substances, which may also act as diluents, binders, preservatives, disintegrating agents, or an encapsulating material. Details on techniques for formulation and administration are well described in the scientific and patent literature, see, e.g., the latest edition of Remington's Pharmaceutical Sciences, Maack Publishing Co, Easton PA ("Remington's").

[0136] In powders, the carrier is a finely divided solid, which is in a mixture with the finely divided active component. In tablets, the active component is mixed with the carrier having the necessary binding properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain from 5% or 10% to 70% of the conjugates of the present invention.

[0137] Liquid form preparations include solutions, suspensions, and emulsions, for example, water or water/propylene glycol solutions. For parenteral injection, liquid preparations can be formulated in solution in aqueous polyethylene glycol solution.

[0138] Aqueous solutions suitable for oral use can be prepared by dissolving the conjugates of the present invention in water and adding suitable colorants, flavors, stabilizers, and thickening agents as desired. Aqueous suspensions suitable for oral use can be made by dispersing the finely divided active component in water with viscous material, such as natural or synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia, and dispersing or wetting agents such as a naturally occurring phosphatide (e.g., lecithin), a condensation product of an alkylene oxide with a fatty acid (e.g., polyoxyethylene stearate), a condensation product of ethylene oxide with a long chain aliphatic alcohol (e.g., heptadecaethylene oxycetanol), a condensation product of ethylene oxide with a partial ester derived from a fatty acid and a hexitol (e.g., polyoxyethylene sorbitol mono-oleate), or a condensation product of ethylene oxide with a partial ester derived from fatty acid and a hexitol anhydride (e.g., polyoxyethylene sorbitan mono-oleate). The aqueous suspension can also contain one or more preservatives such as ethyl or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents and one or more sweetening agents, such as sucrose, aspartame or saccharin. Formulations can be adjusted for osmolality.

[0139] Also included are solid form preparations, which are intended to be converted, shortly before use, to liquid form preparations for oral administration. Such liquid forms include solutions, suspensions, and emulsions. These preparations may contain, in addition to the active component, colorants, flavors, stabilizers, buffers, artificial and natural sweeteners, dispersants, thickeners, solubilizing agents, and the like.

[0140] Oil suspensions can be formulated by suspending the conjugates of the present invention in a vegetable oil, such as arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin; or a mixture of these. The oil suspensions can contain a thickening agent, such as beeswax, hard paraffin or cetyl alcohol. Sweetening agents can be added to provide a palatable oral preparation, such as glycerol, sorbitol or sucrose. These formulations can be preserved by the addition of an antioxidant such as ascorbic acid. As an example of an injectable oil vehicle, see Minto, J. Pharmacol. Exp. Ther. 281 :93-102, 1997. The pharmaceutical formulations of the invention can also be in the form of oil-in- water emulsions. The oily phase can be a vegetable oil or a mineral oil, described above, or a mixture of these. Suitable emulsifying agents include naturally-occurring gums, such as gum acacia and gum tragacanth, naturally occurring phosphatides, such as soybean lecithin, esters or partial esters derived from fatty acids and hexitol anhydrides, such as sorbitan mono-oleate, and condensation products of these partial esters with ethylene oxide, such as polyoxyethylene sorbitan mono-oleate. The emulsion can also contain sweetening agents and flavoring agents, as in the formulation of syrups and elixirs. Such formulations can also contain a demulcent, a preservative, or a coloring agent.

[0141] The compositions of the present invention can also be delivered as microspheres for slow release in the body. For example, microspheres can be formulated for administration via intradermal injection of drug-containing microspheres, which slowly release subcutaneously (see Rao, J. Biomater Sci. Polym. Ed. 7:623-645, 1995; as biodegradable and injectable gel formulations (see, e.g., Gao Pharm. Res. 12:857-863, 1995); or, as microspheres for oral administration (see, e.g., Eyles, J. Pharm. Pharmacol. 49:669-674, 1997). Both transdermal and intradermal routes afford constant delivery for weeks or months.

[0142] In another embodiment, the compositions of the present invention can be formulated for parenteral administration into a body cavity such as the intra articular space of a joint. The formulations for administration will commonly comprise a solution of the compositions of the present invention dissolved in a pharmaceutically acceptable carrier. Among the

acceptable vehicles and solvents that can be employed are water and Ringer's solution, an isotonic sodium chloride. In addition, sterile fixed oils can conventionally be employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid can
5 likewise be used in the preparation of injectables. These solutions are sterile and generally free of undesirable matter. These formulations may be sterilized by conventional, well known sterilization techniques. The formulations may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents, e.g., sodium acetate, sodium chloride, potassium
10 chloride, calcium chloride, sodium lactate and the like. The concentration of the compositions of the present invention in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight, and the like, in accordance with the particular mode of administration selected and the patient's needs. For IV administration, the formulation can be a sterile injectable preparation, such as a sterile injectable aqueous or
15 oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally-acceptable diluent or solvent, such as a solution of 1,3-butanediol.

[0143] In another embodiment, the formulations of the compositions of the present
20 invention can be delivered by the use of liposomes which fuse with the cellular membrane or are endocytosed, i.e., by employing ligands attached to the liposome, or attached directly to the oligonucleotide, that bind to surface membrane protein receptors of the cell resulting in endocytosis. By using liposomes, particularly where the liposome surface carries ligands specific for target cells, or are otherwise preferentially directed to a specific organ, one can
25 focus the delivery of the compositions of the present invention into the target cells in vivo. (See, e.g., Al-Muhammed, J. Microencapsul. 13:293-306, 1996; Chonn, Curr. Opin. Biotechnol. 6:698-708, 1995; Ostro, Am. J. Hosp. Pharm. 46: 1576-1587, 1989).

[0144] Lipid-based drug delivery systems include lipid solutions, lipid emulsions, lipid
dispersions, self-emulsifying drug delivery systems (SEDDS) and self-microemulsifying drug
30 delivery systems (SMEDDS). In particular, SEDDS and SMEDDS are isotropic mixtures of lipids, surfactants and co-surfactants that can disperse spontaneously in aqueous media and form fine emulsions (SEDDS) or microemulsions (SMEDDS). Lipids useful in the formulations of the present invention include any natural or synthetic lipids including, but not

limited to, sesame seed oil, olive oil, castor oil, peanut oil, fatty acid esters, glycerol esters, Labrafil®, Labrasol®, Cremophor®, Solutol®, Tween®, Capryol®, Capmul®, Captex®, and Peceol®.

B. Administration

5 **[0145]** The conjugates and compositions of the present invention can be delivered by any suitable means, including oral, parenteral and topical methods. In some embodiments, the delivery method is intra-articular.

10 **[0146]** The pharmaceutical preparation is preferably in unit dosage form. In such form the preparation is subdivided into unit doses containing appropriate quantities of the conjugates and compositions of the present invention. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as packeted tablets, capsules, and powders in vials or ampoules.

15 **[0147]** The conjugates and compositions of the present invention can be co-administered with other agents. Co-administration includes administering the conjugate or composition of the present invention within 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 20, or 24 hours of the other agent. Co-administration also includes administering simultaneously, approximately simultaneously (e.g., within about 1, 5, 10, 15, 20, or 30 minutes of each other), or sequentially in any order. Moreover, the conjugates and compositions of the present invention can each be administered once a day, or two, three, or more times per day so as to provide the preferred dosage level per day.

20 **[0148]** In some embodiments, co-administration can be accomplished by co-formulation, i.e., preparing a single pharmaceutical composition including the conjugates and compositions of the present invention and any other agent. Alternatively, the various components can be formulated separately.

25 **[0149]** The conjugates and compositions of the present invention, and any other agents, can be present in any suitable amount, and can depend on various factors including, but not limited to, weight and age of the subject, state of the disease, etc. Suitable dosage ranges include from about 0.1 mg to about 10,000 mg, or about 1 mg to about 1000 mg, or about 10 mg to about 750 mg, or about 25 mg to about 500 mg, or about 50 mg to about 250 mg. Suitable dosages also include about 1 mg, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900 or 1000 mg. The composition can also contain other compatible

therapeutic agents. The conjugates described herein can be used in combination with one another, with other active agents known to be useful in modulating a glucocorticoid receptor, or with adjunctive agents that may not be effective alone, but may contribute to the efficacy of the active agent.

5 V. METHODS OF OCULAR TREATMENT

[0150] Ocular disorders that can be treated using a method of the present disclosure include, but are not limited to, macular degeneration, choroidal neovascularization, macular edema, retinal neovascularization, proliferative vitreoretinopathy, glaucoma, and ocular inflammation.

- 10 [0151] Ocular diseases that can be treated using a method of the present disclosure include, but are not limited to, acute macular neuroretinopathy; Behcet's disease; choroidal neovascularization; diabetic uveitis; non-infectious uveitis; histoplasmosis; macular degeneration, such as acute macular degeneration, non-exudative age related macular degeneration and exudative age related macular degeneration; edema, such as macular edema,
- 15 cystoid macular edema and diabetic macular edema; multifocal choroiditis; ocular trauma which affects a posterior ocular site or location; ocular tumors; retinal disorders, such as central retinal vein occlusion, diabetic retinopathy (including proliferative diabetic retinopathy and diabetic macular edema), proliferative vitreoretinopathy (PVR), retinal arterial occlusive disease, retinal detachment, uveitic retinal disease; sympathetic ophthalmia;
- 20 Vogt Koyanagi-Harada (VKH) syndrome; uveal diffusion; a posterior ocular condition caused by or influenced by an ocular laser treatment; posterior ocular conditions caused by or influenced by a photodynamic therapy; photocoagulation, radiation retinopathy; epiretinal membrane disorders; branch retinal vein occlusion; anterior ischemic optic neuropathy; non-retinopathy diabetic retinal dysfunction; retinoschisis; retinitis pigmentosa; glaucoma; Usher syndrome, cone-rod dystrophy; Stargardt disease (fundus flavimaculatus); inherited macular
- 25 degeneration; chorioretinal degeneration; Leber congenital amaurosis; congenital stationary night blindness; choroideremia; Bardet-Biedl syndrome; macular telangiectasia; Leber's hereditary optic neuropathy; retinopathy of prematurity; and disorders of color vision, including achromatopsia, protanopia, deuteranopia, and tritanopia.
- 30 [0152] In some embodiments, the ocular disease is glaucoma, retinitis pigmentosa, macular degeneration, retinoschisis, Leber's Congenital Amaurosis, diabetic retinopathy, achromatopsia, or color blindness. In some embodiments, the ocular disorder is macular

degeneration, choroidal neovascularization, retinal neovascularization, proliferative vitreoretinopathy, glaucoma, or ocular inflammation. In some embodiments, the ocular disorder is macular degeneration, choroidal neovascularization, retinal neovascularization, proliferative vitreoretinopathy, diabetic retinopathy, glaucoma, or ocular inflammation.

- 5 **[0153]** Subjects suitable for treatment with a method of the present disclosure include individuals who have been diagnosed as having an ocular disease or disorder, e.g., any of the above-listed ocular diseases or disorders. Subjects suitable for treatment with a method of the present disclosure include individuals who have been treated for an ocular disease or disorder, and who have failed to respond to the treatment.
- 10 **[0154]** Individuals suitable for treatment with a method of the present disclosure include individuals with reduced visual acuity due to an ocular disease or disorder. Individuals suitable for treatment with a method of the present disclosure include individuals with abnormally high ocular pressure due to an ocular disease or disorder. Individuals suitable for treatment with a method of the present disclosure include individuals with pathological
- 15 angiogenesis in an eye due to an ocular disease or disorder.

- [0155]** Visual acuity can be measured using, for example, a Snellen chart, a Bailey-Lovie chart, a decimal progression chart, a Freiburg visual acuity test, a measurement of minimum angle of resolution (MAR), Best Corrected Visual Acuity (BCVA) test, log of the Minimum Angle of Resolution (LogMAR) test, etc. Metamorphopsia (visual distortion) may be
- 20 measured using an Amsler chart. Contrast sensitivity may be measured using a Pelli-Robson chart. Diagnostic studies include, but are not limited to, standard ophthalmologic examination of the fundus, stereo biomicroscopic examination of the macula, intravenous fundus fluorescein angiography, fundus photography, indocyanine green video-angiography, and optical coherence tomography. A subject displaying an abnormality on one or more of these
- 25 diagnostic studies (e.g., a subject that falls outside a range that is considered normal for a healthy eye) may be treated in accordance with the present disclosure. For example, subjects may be classified as having early, intermediate, or advanced ARMD in accordance with the classification scheme used in the Age-Related Eye Diseases Study. A subject falling into any of the categories described therein, may be treated in accordance with a method of the present
- 30 disclosure.

[0156] The conjugates useful in the method of treating an ocular disease or disorder can have any suitable vitreous half-life. For example, the vitreous half-life can be from about 12

hours to about 24 hours, from about 1 day to about 3 days, from about 3 days to about 7 days, from one week to about 2 weeks, from about 2 weeks to about 4 weeks, or from about 1 month to about 6 months. In some embodiments, the vitreous half-life of the conjugate is at least 2 weeks.

5 **[0157]** In some embodiments, the subject is a human.

[0158] The conjugates useful in the method of treating an ocular disease or disorder can be administered at any suitable interval. For example, the conjugate can be administered at least once a day, or at least once every 2, 3, 4, 5, 6 or 7 days, or at least once every 1, 2, 3 or 4 weeks, or at least once every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 months. In some
10 embodiments, the conjugate can be administered once every two months, once every three months, once every 6 months, or once a year. In some embodiments, the conjugate can be administered once every two months. In some embodiments, the conjugate can be administered once every three months. In some embodiments, the conjugate can be administered once every 6 months. In some embodiments, the conjugate can be administered
15 once a year.

[0159] The conjugates useful in the method of treating an ocular disease or disorder can have any suitable vitreous half-life. For example, the vitreous half-life of the conjugate can be at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, at least about 30-fold, at least about 40-fold, at least
20 about 50-fold, at least about 75-fold, at least about 100-fold, at least about 200-fold, at least about 500-fold, or at least about 1000-fold, or more than 1000-fold, greater than the vitreous half-life of the biologically active polypeptide not conjugated to the biocompatible polymer. In some embodiments, the vitreous half-life of the conjugate is at least 2-fold greater than the half-life of the biologically active polypeptide not conjugated to the biocompatible polymer.
25 In some embodiments, the vitreous half-life of the conjugate is at least 5-fold greater than the half-life of the biologically active polypeptide not conjugated to the biocompatible polymer.

VI. METHODS OF JOINT TREATMENT

[0160] The present invention provides a method of treating a disease or disorder in an articular joint using a peptide-polymer conjugate of the present invention. In some
30 embodiments, the present invention provides a method of treating a disease or disorder in an

articular joint, the method comprising injecting into the articular joint an effective amount of a conjugate of the present invention.

[0161] The present invention also provides methods of treating disease and disorders of the joint tissues using the conjugates of the present invention. Examples of diseases and disorders of the joint tissues include, but are not limited to rheumatoid arthritis, wear-related osteoarthritis, age-related osteoarthritis, post-traumatic osteoarthritis, psoriatic arthritis, and aseptic implant loosening, joint effusion, ankylosing spondylitis, bursitis, gout, reactive, arthritis, synovitis, and avascular necrosis. In some embodiments, the disease or disorder is rheumatoid arthritis, wear-related osteoarthritis, age-related osteoarthritis, post-traumatic osteoarthritis, psoriatic arthritis, and aseptic implant loosening, joint effusion, ankylosing spondylitis, bursitis, gout, reactive arthritis, synovitis, or avascular necrosis.

[0162] Many polypeptides are used as drugs to attenuate immune cell function have substantial utility in treating many joint disorders. Joint tissues are particularly susceptible to injury and disease because the typical cellular responses to these assaults, i.e., upregulating of inflammatory mediators, is also a signal to encourage catabolism of articular cartilage and resorption of the underlying bone tissues. Degeneration of the articular surfaces encourages the worsening of damage to the joint tissues and further up regulation of inflammatory mediators. Over time, these mechanisms generate a feed-forward loop that results in cumulative damage to the joint tissues.

[0163] Any joint of the human or animal body can be treated using the methods and conjugates of the present invention. Representative joints include, but are not limited to, fibrous joints, cartilaginous joints, synovial joints, facet joints, synarthrosis joints, amphiarthrosis joints, and diarthrosis joints. The joints can be simple joints having two articulation surfaces, a compound joint having three or more articulation surfaces, or complex joints having two or more articulation surfaces and an articular knee or meniscus. Anatomical joints that can be treated using the conjugates and methods of the present invention include, but are not limited to, hand joints including the fingers, elbow joints, wrist joints, shoulder joints, joints of the sternum and clavicle, vertebral joints, jaw and skull joints, pelvic and hip joints, knee joints, ankle joints and foot joints including the toes. The joints can also be classified as a plane joint, ball and socket joint, hinge joint, pivot joint, condyloid joint and saddle joint. The conjugates and methods of the present invention can be used to

treat the tissues of the joint, including, but not limited to, connective tissue, cartilage, articulation surfaces, synovial cavities, meniscus, and others.

[0164] Examples of drugs that are designed to attenuate immune cell function include antibodies that can interfere with Tumor Necrosis Factor- α and IL-1 β , IL-6, or interferon- γ .

5 Other examples include selective antibody inhibitors of T cell and B cell function. These antibodies may be monoclonal IgG antibodies, IgG antibody fragments, single chain scFv antibodies, single-domain heavy-chain VHH antibodies, or engineered antibody-like scaffolds such as adnectins, affibodies, anticalins, DARPins, and engineered Kunitz-type inhibitors. Other examples also include receptor decoys of immunomodulatory cytokines
10 such as Tumor Necrosis Factor- α and IL-1 β , IL-6, or interferon- γ .

[0165] One common side effect of using anti-inflammatory drugs such as those listed above is a higher risk of infection. Because they attenuate the body's immune responses, the immune system becomes impaired to fight bacteria, viruses, and parasites. Therefore, the benefits of systemic use of these drugs needs to be weighed carefully against the risks
15 associated with systemic immune suppression. In the case of diseases where the whole body is affected by a hyperimmune disorder, such as rheumatoid arthritis, systemic use of immune attenuating drugs may be justified. However, for conditions effecting only one or a limited number of joints, the system risk of infection often does not justify the systemic use of these drugs.

20 [0166] As an alternative, intra-articular (IA) administration of immune modulating drugs has been proposed to prevent or inhibit the long-term effects of inflammation that are associated with osteoarthritis. However, these drugs are rapidly cleared out of the joint space and do not provide adequate duration of therapy after IA administration. After IA injection, the half-life of anti-inflammatory proteins in the synovium is short (<1.5 hours). This is
25 evident from clinical studies where inflammation inhibitors, including infliximab and etanercept, have been administered by IA injection in humans for a variety of joint disorders. Some of these studies report a significant reduction in joint inflammation, but acknowledge that frequent (e.g. weekly) administration was required for a successful outcome. Thus, IA anti-inflammatory therapy using existing drugs would be limited by high costs and the
30 inconvenience of frequent IA dosing. Clearly, methods to extend anti-inflammatory drug bioactivity within the synovial fluid are needed to enable this therapeutic approach for treating joint disorders.

[0167] The primary symptoms associated with joint disorders are pain, effusion, limited range of motion, and pathological remodeling of the joint anatomy. Efficacy for a treatment to treat joint disorders may include a reduction in pain as measured by a generalized assessment, such as the visual assessment score. Efficacy may also be determined based on an improved score using a system that is specific to a particular joint disorder, such as the WOMAC score for osteoarthritis, the ACR20 for rheumatoid arthritis, the Psoriatic Arthritis Quality of Life for psoriatic arthritis, or the SASSS for ankylosing spondylitis. Efficacy may also be measured using a functional output, such as an increase in pain free walking distance or an increase in the range of joint motion. Efficacy may also be measured based on radiographic evidence showing restoration of normal joint anatomy.

[0168] The methods of treating a disease or disorder in an articular joint can use a peptide-polymer conjugate of the present invention having a longer diffusion half-life than the peptide. For example, the conjugate can have a diffusion half-life of at least 2 times longer than that of the peptide, or 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, or at least 100 times longer than that of the peptide. The diffusion half-life of the conjugate can be from about 2 to about 100 times longer than the peptide, or from about 2 to about 50, from about 10 to about 100, from about 25 to about 100, from about 50 to about 100 times longer than the peptide. In some embodiments, the diffusion half-life of the conjugate is at least about 2 times longer than the peptide. In some embodiments, the diffusion half-life of the conjugate is from about 2 to about 100 times longer than the peptide.

[0169] The methods of treating a disease or disorder in an articular joint can use a peptide-polymer conjugate of the present invention having a longer intra-articular half-life longer than the peptide. For example, the conjugate can have an intra-articular half-life that is at least 20% longer than the unconjugated peptide, or at least 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900 or 1000% longer than the unconjugated peptide. The intra-articular half-life of the conjugate can be from about 20% to about 1000% longer than the unconjugated peptide, or from about 100% to about 1000%, or from about 100% to about 500%, or from about 100% to about 300% longer than the unconjugated peptide. In some embodiments, the intra-articular half-life of the conjugate is at least about 20% longer than the peptide. In some embodiments, the intra-articular half-life of the conjugate is from about 20% to about 1000% longer than the peptide.

[0170] The conjugate can be administered at any suitable frequency or amount as discussed above. In some embodiments, the conjugate is injected into the articular joint no more than about once a month. In some embodiments, the conjugate is injected into the articular joint from about once a month to once every 6 months. In some embodiments, the conjugate is
5 injected into the articular joint once every 2 months or once every 3 months.

A. Osteoarthritis

[0171] In 2015, an estimated 7.75 million Americans experienced symptoms of osteoarthritis (OA) that could be associated with a known joint injury. Post-traumatic OA (PTOA) accounts for at least 15% of all OA cases, although it is assumed many other OA
10 diagnoses may also be related to a prior joint trauma. Due to a lack of disease modifying therapies, joint replacement surgery is often the only treatment option to eliminate the associated discomfort and restore mobility. However, PTOA is often diagnosed in younger patients, for whom joint replacement is not a viable option. Overall, the cost of treating these PTOA patients exceeds \$4B in health care costs each year.

[0172] Short-term inhibition of injury-related inflammation will limit the long-term symptoms of PTOA. Many types of joint injury have been associated with PTOA, including dislocations, ligament tears, meniscal damage, and intra-articular fractures. Although the initial damage may be acute, the injury is sufficient to initiate a cascade of inflammatory mediators. The resulting chronic whole-joint inflammation can encourage catabolism of the
20 articular cartilage, resulting in further tissue damage that accumulates over time and presents as PTOA. $\text{TNF}\alpha$ and $\text{IL-1}\beta$ have well-known roles in mediating joint inflammation. These cytokines interact to promote destruction of cartilage, which occurs by both downregulating the expression of the cartilage matrix components and upregulating the expression of matrix metalloproteinases (MMPs). $\text{TNF}\alpha$ also stimulates osteoclast recruitment, and induces
25 apoptosis of bone-forming osteoblasts in inflammatory environments, which contributes to the erosion of articular cartilage tissues. $\text{TNF}\alpha$ and $\text{IL-1}\beta$ are compelling targets for mitigating the inflammatory response to joint injury. Inhibiting these key acute inflammatory cytokines in the joint environment has been proposed for early intervention to stall the progression of PTOA.

B. Inflammation due to immune response to intra-articular microparticles

[0173] Wear occurring between the articular surfaces of a joint can generate particles at the micron scale that drive joint inflammation and osteolysis. Wear particles may be generated due to abrasion between endogenous surfaces, such as ossified cartilage lesions, osteophytes (bone spurs), or exposed subchondral bone lesion. This type of wear particle generation occurs frequently in later stage of OA, resulting in severe joint pain and immobility. This additional inflammatory response accelerates the rate of joint tissue degeneration in OA.

[0174] Wear particles may also be formed between the surfaces of an artificial joint. In 2015, more than 7 million Americans were living with an implanted artificial joint. Nearly 250,000 of these individuals will eventually require a revision surgery due to osteolysis of the bone surrounding the device, eventually resulting in device loosening and failure.

[0175] Wear-related inflammation stems from the foreign body response to otherwise inert microparticles shed from the articulating surfaces. Macrophages inside the synovial lining readily recognize wear microparticles as foreign bodies, release pro-inflammatory factors that recruit other active immune cells to the synovium, and stimulate osteoclast expansion while simultaneously inhibiting bone formation. Thus, sustained inflammation triggers a feed-forward cycle where cartilage degeneration and osteolysis leads to more abrasions between articulating surfaces and more movement and physical stress that in turn produces more particles.

[0176] In some embodiments, the peptide modulates the activity of immune cell function. In some embodiments, the peptide inhibits tumor necrosis factor- α , interleukin-1 β , interleukin-6, or interferon- γ . In some embodiments, the peptide inhibits tumor necrosis factor- α .

[0177] Tumor necrosis factor (TNF α) is a compelling target for controlling the foreign body response. TNF α has a well-known role in mediating joint inflammation. TNF α also stimulates osteoclast recruitment, and induces apoptosis of bone-forming osteoblasts in inflammatory environments, leading to osteolysis of subchondral bone. Inhibition of TNF α using a systemically-administered receptor antagonist (etanercept) has been shown to reduce bone resorption induced by wear particles in mice, although the risks associated with systemic anti-TNF α are not generally regarded as acceptable for localized conditions. As an

alternative, IA anti-TNF α therapy has been proposed to prevent or inhibit the osteolytic response to intra-articular wear particle

[0178] In some embodiments, the peptide is a monoclonal IgG antibody, an IgG antibody fragment, a single-chain variable region antibody, a single-domain heavy chain antibody, an
5 adnectin, an affibody, an anticalin, a DARPin, a Kunitz-type inhibitor, or a receptor decoy.

[0179] The methods of the present invention include a peptide-polymer conjugate comprising a biocompatible polymer having a molecular weight of from about 0.1 MDa to about 2 MDa; and a plurality of peptides each having a molecular weight of from about 5 kDa to about 100 kDa, wherein each peptide is covalently linked to the polymer, wherein
10 there is from about 50 kDa of polymer to about 5 kDa of polymer for every peptide, and wherein the molar ratio of peptides to polymer is at least 5:1.

[0180] In some embodiments, the conjugate comprises a peptide having the CDRs according to SEQ ID NO:3 to SEQ ID NO:5:

DHSGYTYTIG (SEQ ID NO:3),

15 ARIYWSSGNTYYADSVKG (SEQ ID NO:4), and

RDGIPT (SEQ ID NO:5).

[0181] In some embodiments, the conjugate comprises a peptide having an amino acid sequence according to SEQ ID NO:1:

QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGKEREFVARIY
20 WSSGNTYYADSVKGRFAISRDIKNTVDLTMNNLEPEDTAVYYCAARDGIPTSR
SVNYWGQGTQVTVSS (SEQ ID NO:1).

[0182] In some embodiments, the conjugate comprises a peptide having an amino acid sequence according to SEQ ID NO:2:

SNAQVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGKEREFVA
25 RIYWSSGNTYYADSVKGRFAISRDIKNTVDLTMNNLEPEDTAVYYCAARDGIPTSR
SVESVNYWGQGTQVTVSSPSTPPTPSTPPGGC (SEQ ID NO:2).

[0183] The conjugates of the present invention are well-positioned to ameliorate inflammation that occurs near joint to inhibit subsequent cartilage degeneration and osteolysis. These conjugates have been designed to exhibit biophysical attributes that are

matched to the macromolecules of the synovial fluid that is retained within the joint. In addition, conjugating multiple copies of the bioactive polypeptide is sufficient to increase their potency by engaging multivalent interactions with their targets. Thus, this invention is well suited to enable methods of administering long-acting drugs locally to articulating joints for the treatment of various diseases.

[0184] One example target market for bioconjugates drugs is the ~25% of patients who experience chronic inflammation and effusion following joint injury and are thus at risk for developing PTOA. Options for these patients are currently limited to systemic analgesia and local corticosteroid treatment. Failure to resolve the prolonged inflammatory phase can lead to catabolism of articular cartilage and result in further injury that accumulates over time. A treatment based on anti-inflammatory bioconjugates and designed for administration every three months (or even less frequently) could mitigate the effect of long-term joint inflammation, thereby reducing pain and delaying or preventing the need for costly surgeries. These benefits would likely outweigh the downsides of repeated IA injections (as many as 4 per year), which may include risk of infection, inconvenience for the patient, and procedure costs.

[0185] Another example target market for bioconjugates drugs are patients who experience chronic inflammation and pain due to the development of calcified cartilage lesions, bone spurs, or subchondral bone lesions. Although surgical repair may eliminate the acute cause of the pain, existing inflammation, inflammation due to surgery, and inflammation to additional wear particles will accelerate joint damage and degeneration. Long-term options for these patients are currently limited to systemic analgesia and local corticosteroid treatment. Failure to resolve the prolonged inflammatory phase can lead to failure of the articulating surface, which requires joint replacement surgery to return the patient back to physical activity. A treatment based on anti-inflammatory bioconjugates and designed for administration every three months (or even less frequently) could mitigate the effect of long-term joint inflammation, thereby reducing pain and delaying or preventing the need for costly surgeries. These benefits would likely outweigh the downsides of repeated IA injections (as many as 4 per year), which may include risk of infection, inconvenience for the patient, and procedure costs.

[0186] Another example target market for bioconjugates drugs is the ~25% of patients with artificial joints who experience pain and effusion following joint replacement but do not

demonstrate evidence of peri-implant infection to account for these symptoms. Options for these patients are currently limited to analgesia and clinical monitoring until subsequent osteolysis leads to device failure. A treatment based on anti-inflammatory bioconjugates designed for administration every three months or less frequent administration could mitigate the response to wear particles, thereby reducing pain and delaying or preventing the need for costly revision surgeries. These benefits would likely outweigh the downsides of chronic IA injections (as many as 4 per year), which may include risk of infection, inconvenience for the patient, and procedure costs.

[0187] Multivalent antibody conjugates are well-positioned to ameliorate inflammation that occurs due to joint injury or exposure to wear particles and inhibit the subsequent catabolic tissue damage. In addition to exhibiting high potency, the conjugates can be engineered with specific macromolecular properties will be retained within the joint. By conjugating an anti-inflammatory peptide to HyA that is sufficiently large to be retained with the synovium, the bioactive half-life of the conjugated antibody may be significantly extended compared to equivalent unconjugated antibodies.

VII. EXAMPLES

Abbreviations

HA/HyA: hyaluronic acid

EMCH: N-ε-maleimidocaproic acid hydrazide

MP2H: 1-[3-({[2-(3-Hydrazino-3-oxopropoxy)ethoxy]methyl}amino)-3-oxopropyl]-1*H*-pyrrole-2,5-dione

MP77H (MP3400H): Maleimide PEG₇₇ Hydrazide, Maleimide 3.4 kDa PEG-CO-NHNH₂

BMPH: N-(β-maleimidopropionic acid) hydrazide

MHPH: 5-Maleimido-2-hydraziniumpyridine hydrochloride

n-AEM: n-aminoethylmaleimide

APN-PEG4-amine: 3-{*p*-[3-(2-{2-[2-(2-Aminoethoxy)ethoxy]ethoxy}ethoxy)propionylamino]phenyl}propiononitrile

APN-amine: 3-(*p*-Aminophenyl)propiononitrile

APN-C4-amine- 3-[*p*-(4-Aminobutyrylamino)phenyl]propionitrile

MP2A: 1-(3-{2-[2-(3-Hydrazino-3-oxopropoxy)ethoxy]ethylamino}-3-oxopropyl)-1*H*-pyrrole-2,5-dione or Maleimide-PEG₂-amine

MP3A: 1-[3-(2-{2-[2-(2-Aminoethoxy)ethoxy]ethoxy}ethylamino)-3-oxopropyl]-1*H*-pyrrole-2,5-dione

sNHS: N-hydroxysulfosuccinimide

HOBt: hydroxybenzotriazole

MVP: multivalent peptide polymer conjugate

Materials and Methods

10 **[0188]** The peptides and conjugates of the present invention can be prepared according to the methods described in WO 2017/100470 and PCT Application No. PCT/US19/21460 (WO2019/173777), each of which is incorporated herein in its entirety.

Example 1. Modification of Hyaluronic Acid with Hydrazide Linkers

15 **[0189]** To obtain diacylhydrazine linked heterobifunctional crosslinker modification of hyaluronic acid or other acid containing sugar aiming for a thiol reactive valency of ~10-400 (depending on crosslinker), suspend 830 kDa hyaluronic acid in 0.1 M 2-(N-morpholino)ethanesulfonic acid buffer pH 5.7 at 4 mg/mL by gentle rotation or mixing with nutation overnight at RT. To 3 mg (3.6 nmol, amount will vary based on polymer composition and MW) of HA in solution is added 50-500 equivalents hydroxybenzotriazole (HOBt) hydrate or N-hydroxysulfosuccinimide (s-NHS) as a 5-20 mg/mL stock solution in DMSO or buffer, followed by 50-1000- equivalents of hydrazide-X-thiol reactive linker in DMSO or buffer (10-25 mg/mL stock), and finally 9500 equivalents of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) as a 1 g/mL stock in 0.1 M MES buffer pH 5.7 and bring final volume to 1 mL with buffer. Mix with gentle pipetting between 20 each reagent addition. React at room temperature for 2 h with nutating mixer. After 2 h, 25 purify the thiol reactive biopolymer using 7 kDa MWCO 5 mL Zeba desalting spin column equilibrated with 10% v/v glycerol pH 6.5 DPBS 0.01% v/v polysorbate 20. Elute product into clean conical tube using centrifuge at RT, elution time ~25-45 minutes. Use immediately for reaction with thiol or aliquot and flash freeze on dry ice.

[0190] Alternatively, reaction pH or equivalents of hydrazide linker, HOBt or sNHS, and EDC can be altered higher or lower in order to increase or decrease the number of thiol reactive small molecule linkers covalently linked per biopolymer (valency). Activated biopolymer intermediate can also be purified away from reactants using size exclusion chromatography, other desalting columns, tangential flow filtration, ion exchange chromatography, dialysis, or alcohol/acetone precipitation. Linkers that are >1000 Da will not be able to be purified by desalting and will require one of the other methods listed above.

Table 1. Intermediates

Intermediate	Linker	HOBt eq.	Linker eq.	EDC eq.	[Maleimide] (μM)	Maleimide Valency assuming no HA loss	Linker reaction efficiency based on valency
1	EMCH	50	500	9500	777.6	206	41%
2	EMCH	50	500	9500	656.9	192	38%
3	BMPH	50	500	9500	825.2	202	40%
4	MP2H	50	500	9500	349	90	18%
5*	EMCH	500	1000	9500	638	204	20%
6	MP77H	50	500	9500	172	62	12%
7**	MHPH	50	500	9500			0%
8	MP2H	50	500	9500	227	83	17%
9	EMCH	50	500	9500	520	179	36%
10	EMCH	50	500	9500	810.75	252	50%
11	MP2H	50	500	9500	155.17	49	10%
12	BMPH	50	500	9500	806.42	258	52%
13	MP2H	50	500	9500	177.5	48	10%
14	MP2H	50	1000	9500	353.19	93	9%
15	MP2H	50	1000	9500	476	137	14%
16	EMCH	50	250	9500	418	122	49%
17	MP2H	50	100	9500	44.49	13	13%
18	MP2H	50	250	9500	96.81	30	12%
19	MP2H	50	500	9500	219.12	67	13%
20	MP2H	50	1000	9500	399.64	138	14%
21	EMCH	50	50	9500	93.5	28	56%
22	EMCH	50	100	9500	178.3	54	54%
23	EMCH	50	250	9500	380.3	115	46%
24	EMCH	50	500	9500	709.7	208	42%
38	MP2H	50	500	9500	236	72	14%
39	EMCH	50	500	9500	811	248	50%
40	EMCH	50	500	9500			

41	MP2H	50	500	9500			
42***	MP2H	50	500	9500	827	171	

*Denotes use of sNHS in reaction. ** denotes product gelled upon thawing. *** denotes valency and maleimide concentration determined by UV.

Table 2. Intermediates

Intermediate	Linker	Maleimide Valency
30	MP3A	30
28	n-AEM	27
5	EMCH	204
6	MP77H	62
7	MHPH	ND
29	APN-C4-amine	66
31	MP2A	44
4	MP2H	90

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Example 2. Modification of Hyaluronic Acid with Amine Linkers

[0191] To obtain amide linked heterobifunctional crosslinker modification of hyaluronic acid or other acid containing sugar, aiming for a thiol reactive valency of ~2-200 (depending on crosslinker), suspend 830 kDa hyaluronic acid in 0.1 M 2-(N-morpholino)ethanesulfonic acid buffer pH 6.5 at 4 mg/mL by gentle rotation or mixing with nutation overnight at RT. To 10 3 mg (3.6 nmol) of HA in solution is added 9500 equivalents of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) as a 1 g/mL stock in 0.1 M MES buffer pH 6.5, followed by 50-500 equivalents N-hydroxysulfosuccinimide (sNHS) or hydroxybenzotriazole (HOBt) hydrate as a 5-20 mg/mL stock solution in buffer or DMSO, 15 and finally 1000 equivalents of amine-X-thiol reactive linker in DMSO (10-25 mg/mL stock) or 1:1 DMSO:0.1 M sodium bicarbonate for HCl salt, and bring final volume to 1 mL with buffer. Mix with gentle pipetting between each reagent addition. React at room temperature for 2 h with nutating mixer. After 2 h, purify the thiol reactive biopolymer using 7 kDa MWCO 5 mL Zeba desalting spin column equilibrated with 10% v/v glycerol pH 6.5 DPBS 20 0.01% v/v polysorbate 20. Elute product into clean conical tube using centrifuge at RT, elution time ~25-45 minutes. Use immediately for reaction with thiol or aliquot and flash freeze on dry ice.

- [0192] Alternatively, equivalents of amine linker, HOBt or sNHS, and EDC, along with reaction pH, can be altered higher or lower in order to increase or decrease the number of thiol reactive small molecule linkers covalently linked per biopolymer (valency). Activated biopolymer intermediate can also be purified away from reactants using size exclusion chromatography, other desalting columns, tangential flow filtration, ion exchange chromatography, dialysis, or alcohol/acetone precipitation.

Table 3. Intermediates

Intermediate	Linker	sNHS eq.	Linker eq.	EDC eq.	[Maleimide] (μM)	Maleimide Valency assuming no HA loss	Linker reaction efficiency based on valency
26*	n-AEM	50	500	9500	96.8	27	5%
27	MP3A	500	1000	9500	182	59	6%
28	n-AEM	500	1000	9500	389	99	10%
29	APN C4 amine	500	1000	9500	180	55	6%
30	MP3A	500	1000	9500	85	30	3%
31	MP2A	500	1000	9500	151	44	4%
32	APN PEG4 amine	500	1000	9500	220	55	6%
33	APN amine	500	1000	9500	15	5	1%
34	n-AEM	500	1000	9500	102.8	24	2%
35	MP2A	500	1000	9500	146.87	47	5%
36	n-AEM	500	1000	9500	144.7	45	5%
37	MP3A	500	1000	9500	127.02	42	4%

* Denotes reaction ran with HOBt

10 **Example 3. Linker per biopolymer determination using UV absorbance spectrum or Ellman's reaction**

- [0193] The average number of linkers covalently attached to the biopolymer backbone on a per biopolymer basis (valency) was determined by taking the UV spectra of purified, activated intermediate samples. A 1200 μM linker solution in the same buffer the final activated intermediate will be purified into was made and serial diluted one to one six times for a total of seven samples and a blank. The UV spectra is collected of each standard (200-324 nm, 2 nm step) using Biotek take 3 trio plate as a sample holder, analyzing samples in duplicate or quadruplicate. The slope of the standard curve generated by this data will give

the product of molar absorptivity and pathlength for the linker in appropriate buffer across any wavelength of interest. A second UV spectra standard curve was generated using the biopolymer (i.e. 4 mg/mL 830 kDa hyaluronic acid, high purity) in same buffer using the buffer the final activated intermediate will be purified into. This solution was serial diluted
5 one to one six times for a total of seven samples and a blank. The UV spectra is collected of each standard (200-324 nm, 2 nm step) using Biotek take 3 trio plate as a sample holder, analyzing samples in duplicate or quadruplicate. The slope of the standard curve generated by this data will give the product of molar absorptivity and pathlength for the biopolymer in appropriate buffer at different wavelengths. The plot of each of these solutions' absorbance
10 values at 204 nm and 230 nm vs concentration was used to determine molar absorptivity*pathlength for the two molecules, where the absorptivity for the biopolymer is essentially zero 230 nm. Unknown samples must be in same buffer used to generate standard curves. The absorbance values at 204 and 230 nm were determined for unknown intermediates. This data was used to calculate unknown linker and biopolymer concentrations
15 for Zeba purified intermediates by first calculating the linker concentration using the 230 nm standard curve generated for the linker. This concentration was then used to calculate the absorbance contribution of the linker at 204 nm. The difference between the total absorbance at 204 and the absorbance contribution from the linker at 204 nm was used to calculate the biopolymer concentration. From this information the v total number of covalently bound
20 linkers per biopolymer or valency can be approximated, but this does not give information on the number of intact/thiol reactive maleimides.

[0194] Additionally, the number of thiol reactive groups per polymer can be calculated using a modified version of the colorimetric Ellman's assay. N-ethylmaleimide (NEM) standards was made using six 1:1 serial dilutions of a 400 μ M NEM stock and these solutions
25 were added to a well plate in triplicate. Thiol reactive intermediate samples were prepared in triplicate using two dilutions for six replicates per sample. Sodium 2-mercaptoethanesulfonate (MESNA) was added to all samples at a final concentration of 400 μ M and the reaction was allowed to proceed 2 h at RT with orbital shaking (~200 rpm). After this time, Ellman's reagent (5,5-dithio-bis-(2-nitrobenzoic acid) was added to each sample for
30 a final concentration of 0.5 mg/mL to initiate a colored product resulting from Ellman's reagent reaction with unreacted MESNA. The Ellman's reaction was allowed to proceed for 15 min with orbital shaking, after which the plate was analyzed for absorbance at 412 nm.

The NEM standard curve allows for calculation of thiol reactive molecules, assuming that the molecules react with thiols at the same rate as NEM.

Table 4. Linker Valency

Intermediate	Thiol reactive linker	Linker Valency (UV)	Intact maleimide valency (Ellman's)
1	EMCH	315	206
3	BMPH		204
38	MP2H	170	72
27	MP3A	150	59
26	n-AEM	77	23
7	MHPH	Gelled	Gelled
101	MP2H	143	109
102	MP3A		48
103	MP3A	149	51
29	Amine-C4-APN		46

5 **Example 4. Purity Determination**

[0195] UV absorbance spectra were used to determine intermediate purity after subsequent Zeba purifications and to determine the number of Zeba spin column purifications needed to remove all reactants/byproducts from the activated biopolymer. Spectra from crude reaction mixtures were compared to spectra of samples after one or two Zeba purification column passes. The UV spectra for one and two Zeba pass samples overlaid identically, demonstrating that the spectra were the same and no change occurred between the first and second Zeba purification step. This confirmed that there was no change in UV signal of intermediate between 1st and 2nd Zeba spin column pass using 0.5 mL Zeba column, to demonstrate that one Zeba purification is enough to remove reactants from intermediate synthesis. Reverse phase HPLC can also be used to assess purity of intermediates. A reverse phase HPLC analysis of intermediate before and after Zeba column purification was performed using either an Agilent AdvanceBio RP-mAb-SB-C8 or Phenomenex Onyx Monolithic C18 column and a mobile phase consisting of a gradient of acetonitrile 0.1% TFA in 60 mM ammonium formate, which allowed for sufficient separation between product and reactant/reaction byproduct peaks. HPLC seems to show that there may be residual reactant (likely EDC) in small amounts but this needs to be further verified.

Example 5. Preparation of Peptide-Polymer Conjugates

[0196] To obtain bioconjugates with peptides conjugated to a range of intermediates synthesized using different small molecule linkers, a fixed concentration of peptide was combined with the polymer in PBS targeting ~0.5-2 protein equivalents per maleimide or targeting a fixed number of peptides per polymer and allowed to react at either 4 °C or ambient temperature for at least 4 and 2 hours respectively with rotation or nutating mixing (most reactions are ran at RT to improve solubility). In some cases before the conjugation reaction, 10-100 equivalents of a reducing agent such as DTT or TCEP HCl can be added per protein equivalent to reduce any disulfide bringing between peptides. This can be removed from the protein solution prior to conjugation by a desalting column or buffer exchange or can be added to the conjugation reaction directly in the form of TCEP immobilized on polymeric beads. During the conjugation reaction, one or more of the following was added to improve the reaction efficiency: 0.5-10 mM EDTA to minimize free thiol oxidation, tween20, carbohydrate, cosolvent, or glycerol to stabilize protein and/or help reduce non-specific interactions between protein and activated biopolymer, increased or decreased salt concentration to stabilize protein and/or help reduce non-specific interactions between protein and activated biopolymer. Unreacted peptide was removed from the peptide-polymer conjugates by one or more of the following methods: dialysis with 50-1000 kDa MWCO against an appropriate buffer (pH should be >1 unit above or below the pI of peptide) for two times for 4 hours each and once for at least 4 hours at 4 °C-room temperature, tangential flow filtration against DPBS pH 6-8, or 50 mM tris 150 mM NaCl pH 8-8.5 with EDTA and tween or other additives like trehalose, depending on peptide, FPLC polishing using a size exclusion column, FPLC polishing with an affinity chromatography column designed to bind the polymer component of the conjugate, or selective precipitation of the conjugates. If reaction efficiency is high enough (e.g., <4% unreacted protein present) purification may not be necessary.

[0197] Alternatively, to each solution of activated polymer, the peptide was added at a suitable peptide:polymer molar feed ratio and Tween-20 to a final concentration of 0.01%. The solution was allowed to react for 2 hours while agitating by rotation (~5 RPM) or nutation at ambient temperatures. Unreacted peptides were removed by were removed by dialysis using 100 kDa MWCO membranes against each of the following buffer solutions in sequence: First, phosphate buffered saline or equivalent tris buffered saline (pH depends on peptide) with 0.01% Tween-20 for at least 4 hours, second phosphate buffered saline with

0.01% Tween-20 overnight, and phosphate buffered saline with 0.01% Tween-20 for 4 hours at 4 °C or RT. Additives like tween20, EDTA, and carbohydrates can be added to enhance protein stability.

[0198] For peptide-polymer conjugate formation using commercially available antibodies, the antibody (2-10 mg/mL) was first activated with 5-20 molar equivalents of 2-iminothiolane (Traut's reagent) in order to generate free thiols on the antibody surface through a one hour ambient reaction at ambient with nutation. The activated antibody was purified using 0.5 mL 7 kDa MWCO Zeba columns. Thiol displaying antibodies can also be generated by partial or full reduction of the antibody using a reducing agent such as TCEP. After purification, the activated antibody was mixed with the EMCH or MP2H intermediate at 0.5-2 equivalents per maleimide and allowed to react at ambient for 2-3 hours mixing with nutation. The antibody conjugate reaction was then diluted to 800 µL and purified using 1000 kDa MWCO dialysis cassette and dialyzed against an appropriate buffer (pH should be >1 unit above or below the pI of peptide) for two times for 4 hours each and once for at least 4 hours at 4 °C-room temperature. Alternate purification strategies are tangential flow filtration against DPBS pH 6-8, or 50 mM tris 150 mM NaCl pH 8-8.5 with EDTA and tween or other additives like trehalose, depending on peptide, FPLC polishing using a size exclusion column, FPLC polishing with an affinity chromatography column designed to bind the polymer component of the conjugate, or selective precipitation of the conjugates. If reaction efficiency is high enough (e.g., <4% unreacted protein present) purification may not be necessary.

[0199] To synthesize conjugates containing low molecular weight peptides (<5000 Da; see SEQ ID NO:20), the peptides were brought up in DMSO:buffer mixes to 10 mg/mL, reduced using 20 equivalents (TCEP) for 60 minutes at RT to produce free thiols or reduced using TCEP bound on beads for ease of purification of peptides <1500 Da. The peptides reduced with soluble TCEP were purified using 0.5 mL 7 kDa MWCO Zeba desalting columns. The purified activated peptides were mixed with intermediates at a ratio of 2-5 peptide equivalents per maleimide and allowed to react at RT for 2 h. In some cases, 0.5 equivalents of TCEP per peptide were added to keep the peptides containing disulfides from crosslinking through disulfide formation between two peptides or reaction of one peptide with two different polymer intermediates. Unreacted peptide was removed from the peptide-polymer conjugates by one or more of the following methods: dialysis with 50-100 kDa MWCO against an appropriate buffer (pH should be >1 unit above or below the pI of peptide, and

may require a cosolvent like DMSO to keep conjugate soluble) for two times for 4 hours each and once for at least 4 hours at 4 °C-room temperature, desalting column with 40 kDa MWCO, tangential flow filtration against DPBS pH 6-8, or 50 mM tris 150 mM NaCl pH 8-8.5 with EDTA and tween or other additives like trehalose or cosolvents, depending on peptide, FPLC polishing using a size exclusion column, FPLC polishing with an affinity chromatography column designed to bind the polymer component of the conjugate, or selective precipitation of the conjugates. If reaction efficiency is high enough (e.g., <4% unreacted protein present) purification may not be necessary.

[0200] Verification of conjugation was determined using either SDS PAGE or HPLC size exclusion chromatography (SEC) to assess conjugate formation and percent unreacted protein after purification. Densitometry against a standard curve was used to quantify unreacted monomer in SDS PAGE analysis of purified conjugates stained with Coomassie blue or a fluorescent stain like SYBR Ruby. SEC peak areas at 280 nm were used to determine percent unreacted protein that remained the purified conjugates. For SEC, the conjugate was filtered prior to analysis to remove particles and analyzed using a Shodex OHpak LB-804 or 806 column or Phenomenex PolySep-6000 with DPBS as the mobile phase to get baseline trace at 280 nm. Additionally, SEC can be combined with multiangle light scattering (MALS) to determine the purified peptide-polymer conjugate radius of gyration and molecular weight.

[0201] The sequences were prepared accordingly to standard methods, obtained from various commercial sources, or provided by third parties. For example, SEQ ID NO:18 and SEQ ID NO:19 were provided by Hybrigenics Services.

Table 5. Reaction Efficiency of Conjugates

Protein	Linker	Intermediate	Conjugate number	Peptide conc (mg/mL)	Valency	Conjugate Reaction Efficiency (including monomeric VHH)
aVEGF BI VHH	EMCH	1	1*	0.08	14	13%
aVEGF BI VHH	EMCH	2	2*	ND	ND	ND
aVEGF BI VHH	BMPH	3	3*	ND	ND	ND
aVEGF BI VHH	MP2H	4	4	0.38	72	68%
aVEGF BI VHH	MP3A	25	5	0.17	35	33%
aVEGF BI VHH	n-AEM	26	6	0.26	50	48%

aVEGF DARPin	MP2H	8	7	0.75	22	29%
aVEGF DARPin	MP3A	30	8	0.27	6	23%
aVEGF DARPin	MP2A	31	9	0.45	12	23%
aVEGF DARPin	EMCH	9	10	0.46	18	12%
aVEGF E1-1 VHH	EMCH	9	11	0.6	104	58%
aVEGF E1-1 VHH	MP2H	8	13	0.46	51	62%
aVEGF E1-1 VHH	MP3A	30	14	0.29	29	97%
aVEGF E1-1 VHH	MP2A	31	15	0.27	28	64%
aTNFa FLAG VHH	MP2A	35	16	0.89	22	47%
aTNFa FLAG VHH	n-AEM	36	17	0.96	23	51%
aTNFa FLAG VHH	MP3A	37	18	1.03	23	55%
aTNFa FLAG VHH	EMCH	10	19	0.42	28	11%
aTNFa FLAG VHH	MP2H	11	20	1.23	30	61%
aTNFa FLAG VHH	BMPH	12	21	0.44	30	12%
aTNFa FLAG VHH	MP2H	13	22	0.95	29	60%
aTNFa FLAG VHH	MP2H	14	23	1.76	77	83%
G5-1 aVEGF VHH	MP2H	15	24	0.372	52	74%
G5-1 aVEGF VHH	EMCH	16	25	0.411	53	75%
aEGFR VHH	MP3A	27	26	1.83	62	39%
aEGFR VHH	n-AEM	28	27	1.92	68	43%
aEGFR VHH	EMCH	5	28*	0.35	7	4%
aEGFR VHH	APN C4 amine	29	29	1.16	22	14%
aVEGF DARPin	MP2H	38	30	1.07	50	74%
aVEGF DARPin	EMCH	39	31***	1.07	<179	<72%
aVEGF DARPin	MP2H	42	40	1.33	68	97%
Cy7-aVEGF DARPin	MP2H		41****	0.52	45	50%

* Denotes reaction that precipitated during conjugate formation. ** Denotes viscous product that took >2x as long to filter. *** Denotes high percentage (>70%) of unreacted protein skewing valency and reaction efficiency. **** Denotes fluorescently tagged protein.

5 **Example 6. Peptide-Polymer Binding Affinity**

[0202] Binding affinity verification of the conjugates' bioactivity can be determined using one or more of the following methods. One example, BioLayer interferometry (BLI; ForteBio Octet Red) was used to determine the binding affinity of each peptide and peptide-polymer conjugate to the target. Target peptides modified with biotin are adsorbed onto a glass BLI

probe covalently modified with streptavidin surface layer. The probe with bound target molecules are then placed in solutions containing known concentrations of either the peptide-polymer conjugate or peptide alone. Laser light is passed down the length of the BLI probe, and the interference generated by the peptide or conjugate binding to the target on the probe tip can be correlated directly to the mass of peptide or conjugate bound. The k-on binding constant can be determined by collecting the interference data over time during target binding. The probe is then placed in a solution of buffer without conjugate or peptide. As the peptide or conjugate dissociate from the BLI probe, the laser light interference will reverse allowing for the calculation of the k-off constant. Together, this method allows BLI to measure the binding affinity (k_d) for each peptide and conjugate to its target.

[0203] Another example method for determining conjugate binding are using a plate-based colorimetric ELISA or cell-based assays such as DiscoverX assay (EuroFins). In the DiscoverX assay, target peptide (i.e VEGF) binds to a modified cell surface receptor resulting in receptor dimerization that activates an enzyme. This enzyme allows for quantitation of peptide binding by chemiluminescence. The conjugate or peptide of interest is incubated in a solution containing the cells and target peptide. Competitive binding between the target peptide and either the cell receptor or conjugate/peptide of interest results in varying degrees of receptor activation within the cells. The strength of binding and dissociation constants are quantified by addition of the chemiluminescent substrate and the resulting intensity of the chemiluminescence is collected and correlated to the amount of free target peptide available to dimerize the cell surface receptors, allowing for calculation of EC50/IC50. Generally, better bioactivity is observed for conjugates synthesized using hydrophilic linkers. In all cases, the peptide polymer conjugate has improved binding kinetics compared to unreacted protein.

Table 6. BLI Dissociation Constant of Conjugates

Protein	Linker	Intermediate	Conjugate number	Protein conc (mg/mL)	Protein valency	Dissociation constant by BLI (nM)
aVEGF BI VHH	EMCH	1	1*	0.08	14	
aVEGF BI VHH	EMCH	2	2*	ND	ND	
aVEGF BI VHH	BMPH	3	3*	ND	ND	
aVEGF BI VHH	MP2H	4	4	0.38	72	0.011
aVEGF BI VHH	MP3A	25	5	0.17	35	
aVEGF BI VHH	n-AEM	26	6	0.26	50	0.012

aVEGF DARPin	MP2H	8	7	0.75	22	<0.001
aVEGF DARPin	MP3A	30	8	0.27	6	5.99
aVEGF DARPin	MP2A	31	9	0.45	12	1.381
aVEGF DARPin	EMCH	9	10	0.46	18	0.397
aVEGF E1-1 VHH	EMCH	9	11	0.6	104	1.763
aVEGF E1-1 VHH	MP2H	8	13	0.46	51	0.678
aVEGF E1-1 VHH	MP3A	30	14	0.29	29	1.31
aVEGF E1-1 VHH	MP2A	31	15	0.27	28	1.918
aTNFa FLAG VHH	MP2A	35	16	0.89	22	1.335
aTNFa FLAG VHH	n-AEM	36	17	0.96	23	0.566
aTNFa FLAG VHH	MP3A	37	18	1.03	23	0.886
aTNFa FLAG VHH	EMCH	10	19	0.42	28	0.239
aTNFa FLAG VHH	MP2H	11	20	1.23	30	<0.001
aTNFa FLAG VHH	BMPH	12	21	0.44	30	0.274
aTNFa FLAG VHH	MP2H	13	22	0.95	29	<0.001
aTNFa FLAG VHH	MP2H	14	23	1.76	77	<0.001
G5-1 aVEGF VHH	MP2H	15	24	0.372	52	0.568
G5-1 aVEGF VHH	EMCH	16	25	0.411	53	0.732
aEGFR VHH	MP3A	27	26	1.83	62	
aEGFR VHH	n-AEM	28	27	1.92	68	
aEGFR VHH	EMCH	5	28*	0.35	7	
aEGFR VHH	APN C4 amine	29	29	1.16	22	
aVEGF DARPin	MP2H	38	30	1.07	50	
aVEGF DARPin	EMCH	39	31***	1.07	<179	
aVEGF DARPin	MP2H	42	40	1.33	68	0.0109
Cy7-aVEGF DARPin	MP2H		41****	0.52	45	

* Denotes reaction that precipitated during conjugate formation. *** Denotes high percentage of unreacted protein (>70%). **** Denotes fluorescently tagged peptide

Example 7. Stability Determination

- 5 **[0204]** Several methods can be used to assess MVP stability initially and with time and storage at different temperatures. Turbidity was determined via visible light absorbance/scatter at ~660-700 nm and used to compare relative stabilities where higher turbidity indicates lower stability. Solution turbidity based on 660-700 nm absorbance increases with decreasing conjugate stability. A qualitative measure of turbidity or the
- 10 presence of precipitate/aggregates was done by visual analysis. In many cases, aggregates and precipitates are visible by eye but can also be analyzed via microscope images or light scattering methods, which can be used to verify aggregation and assess aggregate size.

[0205] Stability and initial conjugate size was also assessed using HPLC size exclusion chromatography (SEC). This method was also used to analyze MVP formation, relative size by retention time, and percent unreacted protein after purification. To assess stability via SEC, MVP was filtered to remove particles and analyzed using a Shodex OHpak LB-804 or 806 column or Phenomenex PolySep-6000 with DPBS as the mobile phase to get baseline trace at 280 nm. After various time points samples were removed and analyzed using the same SEC method. Increases in retention time and peak width relative to the baseline sample indicated degradation. In addition, decreases in MVP peak area and/or increases in monomer and dimer protein species peak area also indicate MVP degradation. Percent conjugate loss was quantified by comparing peak area differences with time. The SEC stability analysis has also been coupled with MALS to quantify molecular weight and valency changes of the conjugate with age at different temperatures.

Table 7. Conjugate Stability

Conjugate	MVP valency	MVP stability	MVP filterability
1	14	Precipitated mid-reaction	some loss of UV signal after filtering
3	CNA	Precipitated mid-reaction	some loss of UV signal after filtering
4	72	no visible precipitation	no loss of UV signal after filtering
15	28	no visible precipitation	no loss of UV signal after filtering
5	35	no visible precipitation	no loss of UV signal after filtering
6	50	no visible precipitation	some loss of UV signal after filtering
28	7	Precipitated mid-reaction	ND
26	62	no visible precipitation	ND
27	68	no visible precipitation	ND
38	75	no visible precipitation	some loss of UV signal after filtering
39	18	Precipitated mid-reaction	CNA
34	52	no visible precipitation	no loss of UV signal after filtering
35	110	no visible precipitation	no loss of UV signal after filtering

CNA – could not analyze

ND – not determined

Example 8. Determination of Filterability

[0206] To determine the filterability of the peptide polymer conjugates generated with various small molecule linkers, the product was subject to spin filtration (i.e. cellulose acetate, 0.22 μ m) or syringe filtration. For both methods, a sample was saved pre filtration for

5 UV analysis and spectral comparison before and after filter. For spin filtration, the sample was loaded in the spin filter undiluted, pre-concentrated using a 100 kDa MWCO spin concentrator, or diluted 1:1 with buffer and centrifuged to elute. The filtered eluent absorbance spectrum was taken and plotted over the pre filtration absorbance spectrum to check for losses using change in signal or concentration changes calculated using the peptide

10 extinction coefficient. The percent loss was calculated based on direct protein signal at 280 nm before and after filtration, or calculated concentration before and after filtration. In some cases, the purified conjugate or conjugate reaction was diluted 1:1 prior to purification or filtration. For syringe-based filtration, the plunger was removed from small volume luer lock syringe and a 0.45-5 μ m syringe filter was loaded on the luer lock syringe. The sample to be

15 filtered was pipetted into bottom of syringe. To filter the sample the plunger was reinserted into the syringe and the sample was pushed through the filter and captured in clean tube. This process is repeated using a 0.2 μ m luer lock syringe filter with fresh syringe for sterile filtration. The filtered eluent absorbance spectrum was taken and plotted over the pre filtration absorbance spectrum to check for losses. The percent loss was calculated based on

20 protein change in protein signal at 280 nm before and after filtration.

Table 8. Recovery Data for Conjugates

Protein	Linker	Intermediate	Conjugate number	Peptide conc pre filtration (mg/mL)	Protein valency	Recovery upon 0.2 μ m filtration
aVEGF BI VHH	EMCH	1	1*	0.08	14	73%
aVEGF BI VHH	EMCH	2	2*	ND	ND	69%
aVEGF BI VHH	BMPH	3	3*	ND	ND	72%
aVEGF BI VHH	MP2H	4	4	0.38	72	103%
aVEGF BI VHH	MP3A	25	5	0.17	35	102%
aVEGF BI VHH	n-AEM	26	6	0.26	50	83%
aVEGF DARPin	MP2H	8	7	0.75	22	
aVEGF DARPin	MP3A	30	8	0.27	6	
aVEGF DARPin	MP2A	31	9	0.45	12	
aVEGF DARPin	EMCH	9	10	0.46	18	
aVEGF E1-1 VHH	EMCH	9	11	0.6	104	97%

aVEGF E1-1 VHH	MP2H	8	13	0.46	51	99%
aVEGF E1-1 VHH	MP3A	30	14	0.29	29	100%
aVEGF E1-1 VHH	MP2A	31	15	0.27	28	95%
aTNFa FLAG VHH	MP2A	35	16	0.89	22	99%
aTNFa FLAG VHH	n-AEM	36	17	0.96	23	92%
aTNFa FLAG VHH	MP3A	37	18	1.03	23	96%
aTNFa FLAG VHH	EMCH	10	19	0.42	28	86%
aTNFa FLAG VHH	MP2H	11	20	1.23	30	94%
aTNFa FLAG VHH	BMPH	12	21	0.44	30	72%
aTNFa FLAG VHH	MP2H	13	22	0.95	29	102%
aTNFa FLAG VHH	MP2H	14	23	1.76	77	95%
G5-1 aVEGF VHH	MP2H	15	24	0.372	52	97%
G5-1 aVEGF VHH	EMCH	16	25	0.411	53	34%
aEGFR VHH	MP3A	27	26	1.83	62	
aEGFR VHH	n-AEM	28	27	1.92	68	
aEGFR VHH	EMCH	5	28*	0.35	7	
aEGFR VHH	APN C4 amine	29	29	1.16	22	
aVEGF DARPin	MP2H	38	30	2.76	50	92%
aVEGF DARPin	EMCH	39	31***	3.11	<179	
aVEGF DARPin	MP2H	42	40	1.33	68	
Cy7-aVEGF DARPin	MP2H		41****	0.52	45	100%

Filtration recovery for peptide-polymer conjugates filtered using 0.2 μm . * denotes reactions that precipitated during conjugate formation. ** denotes viscous product that took >2x as long to filter. *** denotes high percentage of unreacted protein in sample (>70%). **** denotes fluorescently tagged protein

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Example 9. Synthesis of High Valency Peptide-Polymer Conjugates

- [0207] 830 kDa HA intermediates were synthesized using either EMCH or MP2H to display a range of maleimides per HA backbone by varying the equivalents of linker to HA using the methods described above. This allowed for synthesis of anti-TNFa peptide-polymer conjugates with a varying peptide valencies using the methods described above. These conjugates were analyzed using methods listed above to assess the relationship between conjugate peptide valency/potency and handling properties, reaction efficiency, maximal achievable valency, and binding kinetics of the resulting conjugates synthesized using either a hydrophobic or hydrophilic linker.
- 10
- 15 [0208] In this experiment it was found that the EMCH product was limited in maximum achievable valency and filtration recovery compared to MP2H due to the instability of high valency peptide protein conjugates based on EMCH (max valency 75). The MP2H conjugates

- (max valency 110) demonstrated overall better handling properties, filterability, and reaction efficiency while also being sterilizable via filtration at higher drug loads than the EMCH. The ability to sterilize the higher valency MP2H conjugates by filtration makes MP2H an improved drug candidate compared to the EMCH conjugates with regards to production process scaling. Since high valency EMCH conjugates are unfilterable, EMCH conjugate synthesis requires time consuming sterile processing to get the highest achievable drug load, but these processes do not scale to clinical scale drug production. MP2H conjugates allow for higher peptide valencies per molecule, which provides equivalent or improved bioactivity compared to the EMCH conjugates that are limited in drug loading by solubility properties.
- The improved stability of the MP2H conjugates also allows for generation of higher final concentration therapeutic solutions, allowing for a larger drug load per dose.

Table 9. Increased Drug Valencies of Conjugates

Protein	Linker	Intermediate	Conjugate	Protein conc before filtration (mg/mL)	Protein valency	Recovery upon 0.2 μ m filtration	Conjugate Reaction Efficiency (including monomeric VHH)	Dissociation constant by BLI
aH aTNFa VHH	MP2H	17	32	0.41	10	102%	63%	54.99
aH aTNFa VHH	MP2H	18	33	0.7	22	101%	68%	2.388
aH aTNFa VHH	MP2H	19	34	1.3	52	105%	69%	0.123
aH aTNFa VHH	MP2H	20	35	1.91	110	101%	73%	<0.001
aH aTNFa VHH	EMCH	21	36	0.82	15	102%	48%	0.968
aH aTNFa VHH	EMCH	22	37	1.48	28	100%	46%	0.017
aH aTNFa VHH	EMCH	23	38	3.39	75	88%	57%	<0.001
aH aTNFa VHH	EMCH	24	39*	0.7				

Reaction yield, filtration result and binding properties peptide polymer conjugates synthesized using MP2H or EMCH linkers with a range of maleimide and peptide valencies.

* denotes precipitated reaction

Example 10. Intra-Articular Half-Life

[0209] A well-known rat model was used for evaluating the clearance rate of proteins from joints to measure the IA half-life of Anti-TNF bioconjugates (Arthritis Rheum. 1999;42(10):2094). For this assay, rats were anesthetized and the knees on their hind limbs prepared for sterile injection. Using a 30 G needle, an injection was made through synovial membrane of each knee joint, and a 40 μ L injection of sterile, buffered was made into the synovial fluid. In each right knee, the injections also contained either an anti-inflammatory peptide, or an anti-inflammatory peptide at an equivalent concentration of total peptide. Generally, peptides used for this experiment have been tagged with near-infrared fluorophore (e.g., Alexa Fluor 750) using routine peptide tagging methods. At various time points for up to 10 days after the inject, the rats were imaged using an *in vivo* imaging system (e.g., Perkin Elmer IVIS Spectrum) to determine the intensity of fluorescence signal (e.g., average radiant efficiency) at the knees. Each left knee was used as a contralateral imaging control. Near infrared reporters can be detected in rat knees with high sensitivity using an *in vivo* imaging system which will enable detection down to picogram amounts of protein in the joint. The half-life of each treatment was determined following IA injection using established exponential decay calculations for optical *in vivo* imaging (Pharmaceutical research. 2013;30(1):257). Therefore, the peptide concentration was used to estimate the intra-articular half-life of each peptide or conjugate within the joints after administration. The synovial fluid can be collected at the end of the experiment for proteomic analysis via mass spectroscopy to measure the final concentration of the peptides in the knee joint.

[0210] Prior to administration, peptides were tagged with Alexa Fluor 750 or alternate near-infrared fluorescent probe (ThermoFisher) following the manufacturers protocol. Briefly, peptides were mixed with Sulfo-Cy7 or AF750- NHS ester at a 2:1 ratio of probe:peptide. The probe was allowed to react with the peptides for 1 hour at room temperature, and then quenched with by adding 1 part 1.5M Tris to each 10 parts of reaction solution. The peptides were purified using NAP-10 desalting columns and eluting with PBS, pH 7.0.

[0211] In one set of experiments (FIG. 7), the intra-articular half-life of anti-TNF α VHH antibodies (n=10) was compared to those of anti-inflammatory bioconjugates made from anti-TNF α VHH conjugated to 2000 kDA HyA with EMCH linker (n=4), and anti-TNF α VHH conjugated to 2000 kDA HyA with Mal-PEG2-hydrazide linker (n=4). The half-life of either

bioconjugates made 2000 kDa HyA were significantly longer than the unconjugated VHH. In this experiment, the bioconjugates made with the Mal-PEG2-hydrazise linker exhibited approximately 30% longer retention time in the articular joint compared to the bioconjugates made with EMCH, despite both conjugates having the same size biopolymer component.

5 **Example 11. Intravitreal Half-Life**

[0212] A rabbit model can be used for determination of intravitreal half-life of the protein-polymer conjugates compared to unreacted peptide and analyzed via LCMS or radiant efficiency. For LCMS half life determination, animals received equimolar doses of either the peptide polymer conjugates or peptide alone by 50- μ L intravitreal injections in each eye. On
 10 days 1, 4, 10, 20, 60 and 90, 3 rabbits were sacrificed from each group and enucleated eyes were prepared for LCMS quantitation of intravitreal peptide and conjugate concentrations after trypsin digestion. The LCMS concentration measurement method is not subject to interference by tissue sample matrix components and can quantify peptides in the tryptic digests unique to the anti-VEGF peptide down to 2 nM, which is sufficient to detect more
 15 than 10 intravitreal half-lives.

[0213] For generation of the data in FIG. 17, fluorescently tagged peptide or peptide protein conjugate were synthesized and administered intravitreally in rabbits to determine intravitreal half-life. Prior to administration, peptides were tagged with a near-infrared probe following the manufacturers protocol. Briefly, peptides were mixed with Sulfo-Cy7 or
 20 AF750- NHS ester at a 2:1 ratio of probe:peptide. The probe was allowed to react with the peptides for 1 hour at room temperature, and then quenched with by adding 1 part 1.5M Tris to each 10 parts of reaction solution. The peptides were purified using NAP-10 desalting columns and eluting with PBS, pH 7.0. Intravitreal injections of peptide or peptide polymer conjugate was injected into rabbit eyes at day 0. Rabbits were euthanized at noted times post
 25 intravitreal injection and the entire ocular globe was removed and snap frozen in liquid nitrogen. While frozen, the entire vitreous, retina and aqueous humor were dissected and placed into black 24 well plates. The Total Radiant Efficiency [p/s] / [μ W/cm²] of each individual tissue sample was imaged using an IVIS Spectrum imager (Perkin Elmer) with excitation/emission of 740/800 nm using tissue with 1 second exposure from uninjected
 30 control eyes as a blank. The intravitreal half-lives were then calculated using the total radiant efficiency linear regression analysis.

- [0214] In this study it was observed that the peptide-polymer conjugates extend the intravitreal half live by two to three-fold compared to peptide alone. It has been demonstrated that for the protein polymer conjugate technology, higher valency results in higher bioactivity of the resulting molecule. Since the hydrophilic MP2H linker intermediates enable the synthesis of higher valency, higher concentration, better handling conjugates compared to EMCH and other hydrophobic linkers, the higher achievable valency of these conjugates will result in a maximum drug load to generate equivalent or greater bioactivity *in vivo*. The higher drug concentration allotted by the hydrophilic linkers will allow for higher doses achieve for the final drug product compared to the hydrophobic linkers. Additionally, hydrophobic linker-based conjugates used previously did not have the ability to be filtered for *in vivo* studies, so sterile processing methods had to be adopted to synthesize these conjugates. Sterile processing methods are timely and will not scale to large scale manufacturing processes, which will be required for clinical studies and drug manufacture.
- [0215] Although the foregoing invention has been described in some detail by way of illustration and Example for purposes of clarity of understanding, one of skill in the art will appreciate that certain changes and modifications may be practiced within the scope of the appended claims. In addition, each reference provided herein is incorporated by reference in its entirety to the same extent as if each reference was individually incorporated by reference. Where a conflict exists between the instant application and a reference provided herein, the instant application shall dominate.

SEQUENCES

SEQ ID NO:1 (Anti-TNFα Single-Domain Heavy-chain (VHH) Antibody)

QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGKER
 EFVARIYWSSGNTYYADSVKGRFAISRDIKNTVDLTMNNLEPEDTAVYY
 5 CAARDGIPTSRSVESYNYWGQGTQVTVSS

SEQ ID NO:2 (Anti-TNFα Single-Domain Heavy-chain (VHH) Antibody)

SNAQVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGKER
 EFVARIYWSSGNTYYADSVKGRFAISRDIKNTVDLTMNNLEPEDTAVYY
 CAARDGIPTSRSVESYNYWGQGTQVTVSSSPSTPPTPSPSTPPGGC

10 **SEQ ID NO:3**

DHSGYTYTIG,

SEQ ID NO:4

ARIYWSSGNTYYADSVKG

SEQ ID NO:5

15 RDGIPT

SEQ ID NO:6 (Anti-TNFα Affibody)

SNACGGGVDN KFNKEVGWAF GEIGALPNLN ALQFRAFIIS LWDDPSQSAN 50
 LLAEAKKLND AQAPK 65

SEQ ID NO:7 (Anti-TNFα Single-Domain Heavy-chain (VHH) Antibody)

20 SNAQVQLQES GGGLVQPGGS LRLSCAASGR TFS DHSGYTY TIGWFRQAPG 50
 KREFVARIY WSSGNTYYAD SVKGRFAISR DIAKNTVDLT MNNLEPEDTA 100
 VYYCAARDGI PTSRSVESYN YWGQGTQVTV SSSPSTPPTP SPSTPPGGCD 150
 DDDKHHHHHH DYKDDDDK 168

SEQ ID NO:8 (Anti-TNFα designed ankyrin repeat protein (DARPin))

25 SNADLGKKLL EVARAGQDDE VRILMANGAD VNAADHQSFT PLHLYAIFGH 50
 LEIVEVLLKN GADVNASDWH GNTPLHLAAW IGHLEIVEVL LKYGADV NAT 100
 DHSGSTPLHL AATLGHLEIV EVLLKYGADV NAQDKFGKTA FDISIDNGNE 150
 DLAEILQKAA GGGSGGGSC 169

SEQ ID NO:9 (Anti-IL-1B Single-Chain (scFv) Antibody)

SNAEIVMTQS PSTLSASVGD RVIITCQASQ SIDNWLSWYQ QKPGKAPKLL 50
 IYRASTLASG VPSRFSGSGS GAFTLTISS LQPDDFATYY CQNTGGGVSI 100
 AFGQGTKLTV LGGGGGSGGG GSGGGGSGGG GSEVQLVESG GGLVQPGGSL 150
 5 RLSCTASGFS LSSAAMAWVR QAPGKGLEWV GIIYDSASTY YASWAKGRFT 200
 ISRDTSKNTV YLQMNSLRAE DTAVYYCARE RAIFSGDFVL WGQGTLLTVS 250
 SSPSTPPTPS PSTPPGGC 268

SEQ ID NO:10 (Soluble interleukin receptor 2 (sILR2))

HTGAARSCRFRGRHYKREFR LEGEPVALRC PQVPYWLWAS VSPRINLTWH 50
 10 KNDSARTVPG EEETRMWAQD GALWLLPALQ EDSGTYVCTT RNASYCDKMS 100
 IELRVFENTD AFLPFISYPQ ILTLSTSGVL VCPDLSEFTR DKTDVKIQWY 150
 KDSLILDKDN EKFLSVRGTT HLLVHDALE DAGYYRCVLT FAHEGQQYNI 200
 TRSIELRIKK KKEETIPVII SPLKTISASL GSRLTIPCKV FLGTGTPLTT 250
 MLWWTANDTH IESAYPGGRV TEGPRQEYSE NNENYIEVPL IFDPVTREDL 300
 15 HMDFKCVVHN TLSFQTLRTT VKESPSTPPT PSPSTPPGGC 340

SEQ ID NO:11 Anti-(mouse)TNFa Single-Domain Heavy-chain (VHH) Antibody

SNAQVQLQDS GGGLVQAGGS LRLSCAASGG TFSSIIMAWF RQAPGKERE 50
 VGAVSWSGGT TVYADSVLGR FEISRDSARK SVYLQMNSLK PEDTAVYYCA 100
 ARPYQKYNWA SASYNVWGQG TQVTVSSSPS TPPTPSPSTP PGGCDDDDKH 150
 20 HHHHH 155

SEQ ID NO:12

26 gsklk dpelslkgqtq hmqegqtlh lqcrgeaahk
 61 wslpeevske serlsitksa cgrgkqfcs tltlntaqan htgfyckyl avptskkkt
 121 esaiyiflod tgrpfvems eipeihmte grelvipcrv tspnitvtlk kfpldtlipd
 181 gkriidark gfiisnatyk eiglitceat vngilyktny lthrgntii dvqiatprpv
 241 kllrghltvl nctattplnt rvqmtwsypd eknkrasvrr ridganshan ifyavltidk
 301 eqnkdkglyt crvrqgpfk svntsvhiyd kafitvkhk qqvletvagk rsyrlsmkvk
 361 afpapevwi kdgipateks aryltrgyal iikdvteeda gnytilsik qsnvfknlta
 421 tllvavkpqi yekavssfpd palypigserq iltctaygip qptikwfwbp cnhnhsearc
 481 dfcanneeaf ildadsnmgm riesitqrma iiegknkmae tlvvadsria giyiclasnk
 541 vgtvgrnisf yitdvpngfb vnlekmpteg edlklactvn kfilyrdvtwi llrtvnnrtm
 601 hysiskqkma itkehsitln ltimvslqd sgtyacrarn vytgeeilqk keitirdqea
 661 pylirnladh tvaiessttl dchangvpep qitwfknnhk iqgepgiilg pgsstlifier
 721 vteedegyyh ckatnqkgav essayltvqg tsdksnlcli tltctovaat lfwllitlfi

SEQ ID NO:13

```

26          gsklk dpelslkgtq himqagqtlh lqcrgeaahk
61 wslpemvske serlsitkea cgrngkqfcs tltlntaqan htgfysckyl avptskkket
121 esaiyifisd tgrpfvemye eipeihhmt grlvipcrv tsnitvtik kfpldtlipd
181 gkriiwdark gfiisnatyk eiglhtceat vnglyktny lthrgntii dvqistprpv
241 kllrghltvl notattplnt rvqmtwsypd eknkrasvrr ridqnsshan ifysvitidk
301 mqnkdkglyt crvrsqpsfk svntsvhi

```

SEQ ID NO:14

```

142          ipeihhmt grlvipcrv tsnitvtik kfpldtlipd
181 gkriiwdark gfiisnatyk eiglhtceat vnglyktny lthrgntii dvqistprpv
241 kllrghltvl notattplnt rvqmtwsypd eknkrasvrr ridqnsshan ifysvitidk
301 mqnkdkglyt crvrsqpsfk svntsvhi

```

5 SEQ ID NO:15 sFlt sequence

```

MVSYNWTGVLLCALLSCLLLTGSSSGSKLKDPFLSLKSTQHIMQAGQTLHLQCKGFAAHNWSLPENV
SKESERLSITKSACGRNGKQPCSTILNLTAGANHTGPHYCKYLAVFTSKKKKETESAIYIFISDTGRF
FVEMYSEIPEIITHMTEGRELVIPCRVTSPNITVTLKKFPLDTLIPDGKRIIWDARKGFIISNATYKE
IGLLTCEATVNGHLYKTNYLTHRGNTIIVDVQISTPRPVKLLRGHTLVNLCTATITPLNTRVQMTWSY
PDERNKRASVRRRIDQNSHANIFYSVLTIDKMQNKDKGLYTCRVRSQPSFKSVNTSVHLYDKAFIT
VKKCCDDDDKHHHHHH

```

SEQ ID NO:16 scFv anti-VEGF antibody

```

MEIWMQSFQ TLEASVGRV ITCQASSII HSWLAWYQK PGRASFLLIY 50
LASTLASGVF SRFSSSSGGA EFTLTISLIQ PDDFATYYCQ NVYLASTNGA 100
HFGQSTELTV LGGGGGGGGG GGGGGGGGGG GSEYQLVESG GGLVQPGGSL 150
RLCTASSFS LIDYYMTWY RQAPGKGLW VGFIDPDGSP YYATWAKRF 200
TISBNSKNT LYIQNSLRP EDTAVYYCAQ GDRNSGGLD IWQQTLYTV 250
SSSTPTPTP SPSTPPGGCD DDKHHHHHH 280

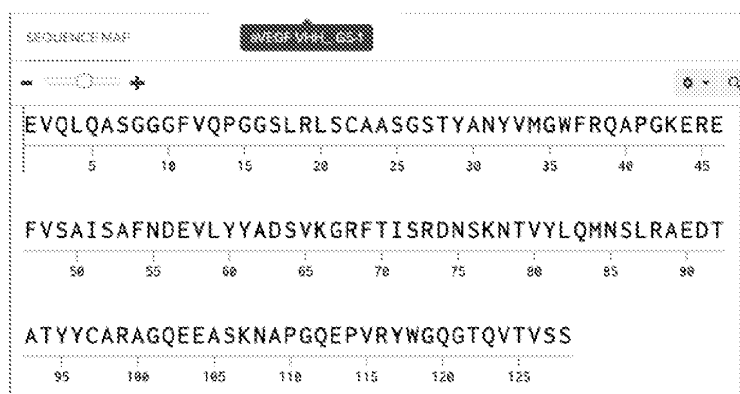
```

SEQ ID NO:17 anti-VEGF VHH

```

SNADYQLVES GGLVQPGGS LELSCAASGR TFSYNGWTF RQAPKEREK 50
VVAISNGGYK YDAVSLEGRF TISBNAKNT VYLQINSLEP EDTAVYYCAS 100
SRAYGSSRLR LADTYEYNGQ GTLVTVSSSP STPTPTSPST FPGGCDGDDK 150
HHHHHH 180

```

SEQ ID NO:18 anti-VEGF VHH E1-1**SEQ ID NO:19 anti-VEGF VHH G5-1**

5

SEQ ID NO:20 natriuretic peptide C-type

Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys [Disulfide Bridge: 6-22]

10 SEQ ID NO:21 anti-VEGF DARPin

SNAGSDLDKKLLEAARAGQDDEVRLMANGADVNARDSTGWTPLHLAAPWGHPEI
 VEVLLKNGADVNAADFQGWTPHLHLAAVGHLEIVEVLLKYGADVNAQDKFGKTAF
 DISIDNGNEDLAEILQKAAGGGSGGGSC