Abstract:

The invention relates to a conjugate comprising a polymer, a biologically active molecule and cyclitol linking the polymer to the biologically active molecule. The invention also relates to an activated polymer composition comprising a polymer, an active functional group and a cyclitol linking the polymer to the active functional group.

Title: CYCLITOL LINKER POLYMER CONJUGATE
CYCLITOL LINKER POLYMER CONJUGATE

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application Serial Number 60/703,133 filed July 28, 2005, Attorney Docket No. EYE-034P, which is hereby incorporated in its entirety by reference.

FIELD OF THE INVENTION

The invention relates to conjugates comprising a polymer, a biologically active molecule and carbocyclic group linking the polymer to the biologically active molecule.

BACKGROUND OF THE INVENTION

Conjugating biologically active molecules with polymers, high molecular weight non-immunogenic and lipophilic compounds has been described to improve in vivo profiles of the biologically active molecules. Biologically active molecules, modified with polymers, high molecular weight non-immunogenic and lipophilic compounds, exhibit reduced immunogenicity/antigenicity and improved pharmacokinetic properties such as stability compared to unmodified versions. For example, U.S. Patent No. 6,011,020 discloses conjugating a high molecular weight non-immunogenic compound to an aptamer for improving the aptamer’s in vivo circulation half-life.

Activated polymers have been reacted with biologically active molecules having nucleophilic functional groups that serve as attachment sites. Polyalkylene glycols such as polyethylene glycol (PEG) are among the most widely used polymers. U.S. Patent No. 4,179,337 describes early models of coupling PEG to peptides or polypeptides. U.S. Patent No. 5,122,614 discloses that PEG molecules activated with a N-succinimide carbonate functional group can be attached under aqueous, basic conditions by a urethane linkage to an amine group of a polypeptide. U.S. Patent No. 5,932,462 describes activated multi-armed PEG molecules and multi-armed PEG conjugates. The multi-armed PEG conjugates contain two linear PEG units attached to a multi-functional central moiety such as lysine as the linker between PEG chains and a biologically active molecule.
There is a continuing need for new activated polymer derivatives useful for coupling to biologically active molecules and new conjugates comprising biologically active molecules and polymers.

SUMMARY OF THE INVENTION

The invention relates to a conjugate comprising a polymer, a biologically active molecule and cyclitol linking the polymer to the biologically active molecule.

The invention also relates to an activated polymer composition comprising a polymer, an active functional group and a cyclitol linking the polymer to the active functional group.

The invention also relates to methods of forming a conjugate comprising a polymer, a biologically active molecule and cyclitol linking the polymer to the biologically active molecule.

In one aspect, the invention relates to a compound having the formula:

\[
\begin{align*}
A_1 & \text{ is a biologically active moiety or an active functional group; } \\
R_{1a}, R_{1b}, R_{1c}, R_{1d} \text{ and } R_{1e} & \text{ are each independently selected from the group consisting of } -\text{OH}, -\text{NH}_2, \text{ and } -X_i-L_i-A_{i}; \\
A_2 & \text{ is selected from the group consisting of a hydrogen, a polymer, a biologically active moiety and an active functional group; } \\
L_i \text{ and } L_2 & \text{ are each independently selected from the group consisting of a bond and a spacer moiety; } \\
X_i \text{ and } X_2 & \text{ are each independently selected from the group consisting of } -\text{O}, -\text{S} \text{ and } -\text{NR}_2; \text{ and }
\end{align*}
\]

...
R₂ is selected from the group consisting of a hydrogen and a lower alkyl group.

The invention has several advantages. Cyclitols are versatile multifunctional moieties allowing the attachment of up to five polymers onto a broad spectrum of biologically-active molecules through a single linker unit. Cyclitols are also robust moieties that provide conjugates with a hydrolytically stable linkage.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1** is a schematic representation of a synthesis of an aptamer conjugate comprising two PEG moieties and an inositol linker.

**Figure 2** is a schematic representation of a synthesis of a trifunctional inositol linking agent.

**Figure 3** is a schematic representation of a synthesis of an aptamer conjugate comprising two PEG moieties and an inositol linker.

**Figure 4** is a graphical representation of the results of a VEGFR-I (Flt-I) inhibition assay using a PEGylated VEGF antagonist aptamer having an inositol linker (compound 1), pegaptanib (Mad), and a 5’-5’ capped anti-VEGF aptamer (MacII).

**Figure 5** is a schematic representation of the chemical structure of a PEGylated VEGF antagonist aptamer having an inositol linker (compound 1).

**Figure 6** is a schematic representation of the chemical structure of a PEGylated VEGF antagonist aptamer having an inositol linker (compound 10).
DETAILED DESCRIPTION OF THE INVENTION

The invention relates to a conjugate comprising a polymer, a biologically active molecule and cyclitol linking the polymer to the biologically active molecule.

The invention also relates to an activated polymer composition comprising a polymer, an active functional group and a cyclitol linking the polymer to the active functional group.

In one aspect, the composition has the formula:

\[
\begin{align*}
& R_{1a} \quad R_{1b} \\
R_{1c} \quad X_1 \quad L_1 \quad A_1 \\
R_{1d} \quad R_{1e}
\end{align*}
\]

wherein:

A] is a biologically active moiety or an active functional group;

\[ R_{i_1}, R_{i_2}, R_{i_3}, R_{i_4}, \text{ and } R_{i_5} \] are each independently selected from the group consisting of \(-\text{OH}, -\text{NH}_2\), and \(-\text{X}_1\)-\text{L}_1-\text{A}_1\);

A_2 is selected from the group consisting of a hydrogen, a polymer, a biologically active moiety and an active functional group;

L_1 and L_2 are each independently selected from the group consisting of a bond and a spacer moiety;

X_1 and X_2 are each independently selected from the group consisting of \(-\text{O}_-, -\text{S}_-\) and \(-\text{NR}_2^-, \text{ and}

R_2 is selected from the group consisting of a hydrogen and a lower alkyl group.

In one embodiment, A is a biologically active moiety. In another embodiment, A is a nucleic acid. In another embodiment, A is an aptamer. In another embodiment, A is an anti-VEGF aptamer.

In one embodiment, A is an active functional group. In another embodiment A is an electrophilic functional group. In another embodiment A is an active functional group comprising a carboxylic acid, carboxylic acid halide, carboxylic acid chloride, halogen,
N-succinimide carbonate, succinimidyl ester, 1-benzotriazolylcarbonate ester, N-hydroxymaleimidyl ester, vinyl sulfone, azalactone, cyclic amide thione carbonyl, imidazole carbonyl, isocyanate or isothiocyanate.

In one embodiment, at least one of Ri, Ru, Rb, Rd and Re is \(-X_2-L_2-A_2\). In another embodiment, at least two of Ri, Rib, Rb, Rd and Re are independently \(-X_2-L_2-A_2\). In another embodiment, at least three of Ri, Rib, Rb, Rd and Re are independently \(-X_2-L_2-A_2\). In another embodiment, at least four of Ri, Rib, Rb, Rd and Re are independently \(-X_2-L_2-A_2\). In another embodiment, each of Ri, Rn, Rib, Rb, Rd and Re are independently \(-X_2-L_2-A_2\).

In one embodiment, \(A_2\) is a polymer. In another embodiment, at least one \(A_2\) is a polymer. In another embodiment, \(A_2\) is a biologically active moiety. In another embodiment, \(A_2\) is an active functional group.

In one embodiment, \(L_1\) and \(L_2\) are each independently a spacer moiety represented by the formula:

\[-(CH_2)_n-(X_3)_m-(C(X_4))_p-(X_5)_q-(CH_2)_r-\]

wherein,

- \(n\) is 0 to 10;
- \(m\) is 0 or 1;
- \(p\) is 0 or 1;
- \(q\) is 0 or 1; and
- \(r\) is 0 to 10.

In another embodiment, \(L_1\) and \(L_2\) are each independently a spacer moiety represented by a formula selected from the group consisting of:

\[-(CH_2)_n-(C(X_4))_p-;\]
\[-(C(X_4))_p;\]
\[-(C(X_4)V(CH_2)V;\]
\[-(C(X_4)V;\] and
\[-(CH_2)_n-;\]

wherein \(n\), \(p\) and \(r\) are as described above.
In one embodiment, $X_1, X_2, X_3, X_4$ and $X_5$ are each independently selected from the group consisting of -O- and -NHr. In another embodiment, $X_1, X_2, X_3, X_4$ and $X_5$ are each -O-.

In another aspect, the conjugate has a formula selected from the group consisting of:

$$
\text{POLY} \xrightarrow{X_5} \text{C} \xrightarrow{(X_3)_m} \text{-(CH}_2_2)_{n} \xrightarrow{D} \text{POLY} \\
\text{POLY} \xrightarrow{X_5} \text{C} \xrightarrow{(X_3)_m} \text{-(CH}_2_2)_{n} \\
\text{POLY} \xrightarrow{X_5} \text{C} \xrightarrow{(X_3)_m} \text{-(CH}_2_2)_{n} \\
\text{POLY} \xrightarrow{X_5} \text{C} \xrightarrow{(X_3)_m} \text{-(CH}_2_2)_{n} \\
\text{POLY} \xrightarrow{X_5} \text{C} \xrightarrow{(X_3)_m} \text{-(CH}_2_2)_{n}
$$

wherein:

$D$ is a biologically active moiety;

POLY is a polymer;
each $X$, $X_4$ and $X_5$ are each independently selected from the group consisting of -O-, -S- and -NR$_2$;

$R_2$ is selected from the group consisting of a hydrogen and a lower alkyl group;

$m$ is 0 or 1; and

$n$ is an integer from 0 to 10.

In one embodiment, D is a nucleic acid. In another embodiment, D is an aptamer. In another embodiment, D is an anti-VEGF aptamer.

In one particular embodiment, the conjugate has a formula selected from the group consisting of:

\[
\tag{SEQ ID NO: 5} \]

wherein $z$ is about 450.
In another aspect, the activated polymer composition has a formula selected from the group consisting of:

\[
\text{POLY} \stackrel{X_5}{\rightarrow} \text{C} - \left( \text{X}_3 \right)_{m} - \left( \text{CH}_2 \right)_{n} \rightarrow \text{O} \rightarrow \left( \text{CH}_2 \right)_{n} - \left( \text{X}_5 \right)_{m} - \text{C} \rightarrow \text{G}
\]

wherein:

- \( G \) is a leaving group;
- \( \text{POLY} \) is a polymer;
- each \( X_3, X_4 \) and \( X_5 \) are each independently selected from the group consisting of \(-O-, -S-\) and \(-NR_2-\);
R is selected from the group consisting of a hydrogen and a lower alkyl group;

m is 0 or 1; and

n is an integer from 0 to 10.

In one embodiment, G is a leaving group selected from the group consisting of a halide, chloride, N-succinimide, i'-benzotriazole, N-hydroxymaleimide, azalactone, cyclic amide thione and imidazole.

In one embodiment, POLY is a high molecular weight polymer. In another embodiment, POLY is a polymer selected from the group consisting of polyether polyols, polysaccharides, polyesters, high molecular weight polyoxyalkylene, polyamides, polyurethanes, polysiloxanes, polyacrylates, polyols, polyvinylpyrrolidones, polyvinyl alcohols, polyanhydrides, carboxymethyl celluloses, other cellulose derivatives, chitosan, polyaldehydes and polyethers. In another embodiment, POLY is a polyalkylene glycol. In another embodiment, POLY is a polyethylene glycol (PEG).

In another embodiment, POLY is a PEG having a molecular weight from 5 kDa to 100 kDa. In another embodiment, POLY is a PEG having a molecular weight of about 20 kDa.

In one embodiment, each \( X_3, X_4 \) and \( X_5 \) are each independently selected from the group consisting of \(-\text{O}-\) and \(-\text{NH}-\).

In one embodiment, n is an integer from 0-6. In another embodiment, n is 5.

It is understood that in certain embodiments in which there is more than one polymer, each polymer can be the same or different.

It is understood that in certain embodiments in which there is more than one biologically active molecule, each biologically active molecule can be the same or different.

A "cyclitol", as referred to herein, is a cycloalkane comprising a hydroxyl group on one or more ring atoms. T. Hudlicky et al. provide a compilation of known cyclitols and their derivatives (Cyclitols and Their Derivatives: A Handbook of Physical, Spectral and Synthetic Data, T. Hudlicky and M. Cebulak, Wiley, John & Sons, Incorporated, 1993; which is incorporated herein by reference in its entirety).
In one embodiment, the cyclitol is a 6-membered cycloalkane. In one embodiment, the cyclitol is a 5-membered cycloalkane. In one embodiment, cyclitols are tri-, tetra-, penta- and hexa-hydroxy cyclohexanes. Cyclitol derivatives optionally comprise substituents including, but not limited to, alkyl, amino, thio, carbonyl or halogen substituents.

In one embodiment, the cyclitol is an inositol. Inositols include, but are not limited to myo-inositol, D-c/z/rø-inositol, L-c/zzVø-inositol, mwcø-ihositol, scyllo-inositol, /fo-inositol, e/?/-inositol, ciy-inositol and «eø-inositol. (See Inositol Phosphates and Derivatives Synthesis, Biochemistry, and Therapeutic Potential, Edited by A. B. Reitz, American Chemical Society, Oxford University Press, 1991, which is hereby incorporated by reference in its entirety.)

"Activated polymers", as referred to herein, are polymers that comprise active functional groups. Activated polymers can be used to couple with a biologically active molecule to form a conjugate.

An "active functional group" as referred to herein, is a functional group that can react readily with electrophilic or nucleophilic groups on other molecules. For example, as would be understood in the art, the term "active ester" would include those esters, such as N-hydroxysuccinimidyl carboxylate ester, that react readily with nucleophilic groups such as amines. Other examples of active esters include, but are not limited to, N-hydroxymaleimide carboxylate esters and 1-benzotriazoly carbonate esters.

Active functional groups may comprise a leaving group. Examples of suitable leaving groups include, but are not limited to, halides, N-hydroxy-succinimides, N-succinimides, 1-benzotriazols, N-hydroxymaleimides, cyclic amide thiones and imidazoles.

Examples of active functional groups that react with nucleophiles include, but are not limited to, a carboxylic acid, carboxylic acid halide, carboxylic acid chloride, N-succinimide carbonate, succinimidyl ester, 1-benzotriazoly carbonate ester, N-hydroxymaleimidylic ester, vinyl sulfone, azalactone, cyclic amide thione carbonyl imidazole, isocyanate and isothiocyanate.

Examples of active functional groups that react with electrophiles include, but are not limited to, primary amines, alcohols, hydrazine and hydrazide functional groups, acylhydrazides, carbazates* semicarbazates, and thiocarbazates.
For example, to couple a polyalkylene oxide to a nucleophile, one of the hydroxyl end-groups of the polyalkylene oxide is converted into an active functional group. This process is referred to as "activation" and the product is called an "activated polyalkylene oxide".

As used herein, the term "spacer" or "spacer moiety" refers to a moiety that covalently links the cyclitol together with the polymer, biologically active moiety or active functional group.

In one embodiment, the spacer is represented by the formula:

\[-(CH_2)_n-(X_3)_m-(C(X_4))_p-(X_5)_q-(CH_2)_r-\]

wherein:

- n is Oto 10
- m is 0 or 1;
- p is 0 or 1;
- q is Oto 1; and
- r is Oto 10.

In another embodiment, the spacer is represented by a formula selected from the group consisting of:

\[-(CH_2)_n-(C(X_4))_p-;\]
\[-(C(X_4))_p-(CH_2)_r-;\]
\[-(C(X_4))_p;\]
\[-(CH_2)_n-;\]

wherein: n, p and r are as described above.

A "lower alkyl group" is a C_1-C_8 straight chain or branched hydrocarbon or a C_3-C_8 cyclic hydrocarbon. Examples of lower alkyl groups include but are not limited to methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tertiary butyl, cyclopropyl, cyclobutyl, cyclopentyl and cyclohexyl groups.

The term "hydrolytically stable" or "non-hydrolyzable" bond or linkage is used herein to refer to bonds or linkages that are substantially stable in water or substantially do not react.
with water. For example, a hydrolytically stable linkage does not react under aqueous conditions for an extended period of time. A hydrolytically stable linkage may exist under aqueous conditions indefinitely.

The term "physiologically stable" bond or linkage is used herein to refer to bonds or linkages that are substantially stable against *in vivo* cleavage or hydrolysis, but may be also stable in the presence of other *in vitro* agents. A physiologically stable bond or linkage is hydrolytically stable and is stable to physiological processes in a cell, an organ, the skin, a membrane or elsewhere within the body of a patient. A physiologically stable linkage may exist under physiological conditions indefinitely.

An "esterase resistant" or "esterase stable" bond or linkage is stable in the presence of an esterase.

Although it is preferred that any bond or linkage will have reasonable stability under aqueous or physiological conditions, in certain aspects, the use of selectively hydrolyzable bonds or linkages is contemplated. Selectively hydrolyzable bonds or linkages still have reasonable stability in the circulation. "Selectively hydrolyzable" bonds or linkages include all linkages that are releasable, cleavable or hydrolyzable only or preferentially under certain conditions.

Examples of selectively hydrolyzable bonds include but are not limited to disulfide and trisulfide bonds and acid-labile bonds.

A "biologically active molecule", "biologically active moiety" or "biologically active agent" can be any substance which can affect any physical or biochemical properties of a biological organism, including but not limited to, viruses, bacteria, fungi, plants, animals, and humans. Biologically active molecules can include any substance intended for diagnosis, cure mitigation, treatment, or prevention of disease in humans or other animals, or to otherwise enhance physical or mental well-being of humans or animals. Examples of biologically active molecules include, but are not limited to, nucleic acids, nucleosides, oligonucleotides, aptamers, peptides, proteins, enzymes, small molecule drugs, dyes, lipids, cells, viruses, liposomes, microparticles and micelles. Classes of biologically active agents that are suitable for use with the invention include, but are not limited to, antibiotics, fungicides, anti-viral agents, anti-inflammatory agents, anti-tumor agents, cardiovascular agents, anti-anxiety agents, hormones, growth factors, steroidal agents, and the like.
By "treating" is meant the medical management of a patient with the intent that a cure, amelioration, stasis or prevention of a disease, pathological condition, or disorder will result. This term includes active treatment, that is, treatment directed specifically toward improvement of a disease, pathological condition, or disorder, and also includes causal treatment, that is, treatment directed toward removal of the cause of the disease, pathological condition, or disorder. In addition, this term includes palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, pathological condition, or disorder; preventive treatment, that is, treatment directed to prevention of the disease, pathological condition, or disorder; and supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the disease, pathological condition, or disorder. The term "treating" also includes symptomatic treatment, that is, treatment directed toward constitutional symptoms of the disease, pathological condition, or disorder.

In one embodiment, the method of the invention provides a means for suppressing or treating an ocular neovascular disorder. In some embodiments, ocular neovascular disorders amenable to treatment or suppression by the method of the invention include ischemic retinopathy, iris neovascularization, intraocular neovascularization, age-related macular degeneration, corneal neovascularization, retinal neovascularization, choroidal neovascularization, retinopathy of prematurity, retinal vein occlusion, diabetic retinal ischemia, diabetic macular edema, or proliferative diabetic retinopathy. In still another embodiment, the method of the invention provides a means for suppressing or treating psoriasis or rheumatoid arthritis in a patient in need thereof or a patient diagnosed with or at risk for developing such a disorder.

As used herein, the terms "neovascularization" and "angiogenesis" are used interchangeably. Neovascularization and angiogenesis refer to the generation of new blood vessels into cells, tissue, or organs. The control of angiogenesis is typically altered in certain disease states and, in many cases, the pathological damage associated with the disease is related to altered, unregulated, or uncontrolled angiogenesis. Persistent, unregulated angiogenesis occurs in a multiplicity of disease states, including those characterized by the abnormal growth by endothelial cells, and supports the pathological damage seen in these conditions including leakage and permeability of blood vessels.
By "ocular neovascular disorder" is meant a disorder characterized by altered or unregulated angiogenesis in the eye of a patient. Exemplary ocular neovascular disorders include optic disc neovascularization, iris neovascularization, retinal neovascularization, choroidal neovascularization, corneal neovascularization, vitreal neovascularization, glaucoma, pannus, pterygium, macular edema, diabetic retinopathy, diabetic macular edema, vascular retinopathy, retinal degeneration, uveitis, inflammatory diseases of the retina, and proliferative vitreoretinopathy.

A variety of anti-VEGF agents that inhibit the activity or production of VEGF, including aptamers and VEGF antibodies, are available and can be used in the methods of the present invention. Particular anti-VEGF agents are nucleic acid ligands of VEGF, such as those described in U.S. Patent Nos. 6,168,778; 6,147,204; 6,051,698; 6,011,020; 5,958,691; 5,817,785; 5,811,533; 5,696,249; 5,683,867; 5,670,637; and 5,475,096, hereby incorporated in their entirety by reference. One particular anti-VEGF agent is pegaptanib sodium.

Classes of biologically active agents include anti-infectives including, without limitation, antibiotics, antivirals, and antifungals; analgesics; antiallergenic agents; mast cell stabilizers; steroidal and non-steroidal anti-inflammatory agents; decongestants; anti-glaucoma agents including, without limitation, adrenergics, beta-adrenergic blocking agents, alpha-adrenergic blocking agonists, parasympathomimetic agents, cholinesterase inhibitors, carbonic anhydrase inhibitors, and protaglandins; antioxidants; nutritional supplements; angiogenesis inhibitors; antimetabolites; fibrinolytics; wound modulating agents; neuroprotective drugs; angiostatic steroids; mydriatics; cyclopegic mydriatics; miotics; vasoconstrictors; vasodilators; anticoagulant agents; anticancer agents; immunomodulatory agents; VEGF antagonists; immunosuppresant agents; and combinations and prodrugs thereof. Other biologically active agents include, but are not limited to, dyes, lipids, cells, viruses, liposomes, microparticles and micelles.

Examples of biologically active agents include, but are not limited to, nucleic acids, nucleosides, oligonucleotides, antisense oligonucleotides, RNA, DNA, siRNA, RNAi, aptamers, antibodies, peptides, proteins, enzymes, fusion proteins, porphyrins, and small molecule drugs. Examples of antibodies include, but are not limited to, VEGF antibodies bevacizumab (Avastin®) and ranizumab (Lucentis™); Genentech, San Francisco, CA.
Examples of aptamers include, but are not limited to, pegaptanib (Macugen®; (OSI) Eyetech, Inc., New York, NY). Examples of steroids include, but are not limited to, anecortave acetate
Administration of the compositions of the present invention may be administered by any suitable means that results in a concentration that is effective for the treatment of a neovascular disorder. Each composition, for example, may be admixed with a suitable carrier substance, and is generally present in an amount of 1-95% by weight of the total weight of the composition. The composition may be provided in a dosage form that is suitable for ophthalmic, oral, parenteral (e.g., intravenous, intramuscular, subcutaneous), rectal, transdermal, nasal, or inhalant administration. Accordingly, the composition may be in form of, e.g., tablets, capsules, pills, powders, granulates, suspensions, emulsions, solutions, gels including hydrogels, pastes, ointihents, creams, plasters, delivery devices, suppositories, enemas, injectables, implants, sprays, or aerosols. The pharmaceutical compositions containing a single antagonist or two or more antagonists may be formulated according to conventional pharmaceutical practice (see, e.g., Remington: The Science and Practice of Pharmacy, (20th ed.) ed. A.R. Gennaro, 2000, Lippincott Williams & Wilkins, Philadelphia, PA. and Encyclopedia of Pharmaceutical Technology, eds., J. Swarbrick and J. C. Boylan, 1988-2002, Marcel Dekker, New York).

It will be understood that pharmaceutically acceptable prodrugs of the biologically active agents disclosed herein are also included in the present invention and can be used in the compositions and methods disclosed herein.

It will be understood that pharmaceutically acceptable salts of the biologically active agents disclosed herein are also included in the present invention and can be used in the compositions and methods disclosed herein.

In one aspect of the invention, the anti-VEGF agent is provided in a controlled release formulation. Examples of controlled release microparticles are described in US Patent Provisional Application Serial No. 60/796,071 which is hereby incorporated by reference in its entirety.
In one embodiment the controlled release formulation comprises a biocompatible, biodegradable polymer selected from the group consisting of lactide polymers, lactide/glycolide copolymers, or poloxylene-polyoxypolyethylene copolymers.

A formulation of the invention may be used in the treatment of any eye disease. A formulation of the invention may also be used to direct an anti-VEGF agent to a particular eye tissue, e.g., the retina or the choroid. The anti-VEGF agent or combination of biologically active agents will be chosen based on the disease, disorder, or condition being treated. In addition to an anti-VEGF agent for a particular condition, other compounds may be included for secondary effects, for example, an antibiotic to prevent microbial growth. The amount and frequency of the dosage will depend on the disease, disorder, or condition being treated and the biologically active agent employed. One skilled in the art can make this determination.

The term "oligomer" refers to a polymer whose molecular weight is too low to be considered a polymer. Oligomers typically have molecular weights in the hundreds, but polymers typically have molecular weights in the thousands or higher.

The term "oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and inter-sugar (backbone) linkages. The term also includes modified or substituted oligomers comprising non-naturally occurring monomers or portions thereof, which function similarly.

Incorporation of substituted oligomers is based on factors including enhanced cellular uptake, or increased nuclease resistance and are chosen as is known in the art. The entire oligonucleotide or only portions thereof may contain the substituted oligomers.

As used herein, the term "aptamer" means any polynucleotide, or salt thereof, having selective binding affinity for a non-polynucleotide molecule via non-covalent physical interactions. An aptamer can be a polynucleotide that binds to a ligand in a manner analogous to the binding of an antibody to its epitope. The target molecule can be any molecule of interest. An example of a non-polynucleotide molecule is a protein. An aptamer can be used to bind to a ligand-binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein.

"Anti-VEGF aptamers" or "VEGF aptamers" are meant to encompass polynucleotide aptamers that bind to, and inhibit the activity of, VEGF. Such anti-VEGF aptamers may be
RNA aptamers, DNA aptamers or aptamers having a mixed \( (i.e., \) both RNA and DNA) composition. Such aptamers can be identified using known methods. For example, Systematic Evolution of Ligands by Exponential enrichment, or SELEX, methods can be used as described in U.S. Patent Nos. 5,475,096 and 5,270,163, each of which are incorporated herein by reference in its entirety. Anti-VEGF aptamers include the sequences described in U.S. Patent Nos. 6,168,778, 6,051,698, 5,859,228, and 6,426,335, each of which are incorporated herein by reference in its entirety. The sequences can be modified to include 5'-5' and/or 3'-3' inverted caps. (See Adamis, A.P. et al, published application No. WO 2005/014814, which is hereby incorporated by reference in its entirety).

Suitable anti-VEGF aptamer sequences of the invention include the nucleotide sequence GAAGAAUUGG (SEQ ID NO: 1); or the nucleotide sequence UUGGACGC (SEQ ID NO: 2); or the nucleotide sequence GUGAAUGC (SEQ ID NO: 3).

Examples of anti-VEGF aptamers include, but are not limited to:

(i) An anti-VEGF aptamer having the sequence:

\[
CGGAAUCAGUGAAUGCUUA'UACAUCCG
\]

(SEQ ID NO: 4, described in U.S. Patent No. 6,051,698, incorporated herein by reference in its entirety). Each C, G, A, and U represents, respectively, the naturally-occurring nucleotides cytidine, guanidine, adenine, and uridine, or modified nucleotides corresponding thereto; and preferably

(ii) An anti-VEGF aptamer having the sequence:

\[
C_mG_mA_rG_mA_rU_rG_mG_mA_rG_mU_rG_mC_rU_fA_mG_mU_fG_mA_mC_fA_mG_mU_fG_mG_mA_mA_mU_fG_mC_fU_fU_fA_mC_fA_mC_fG_m
\]

(SEQ ID NO: 5)

An example of a PEGylated aptamer is pegaptanib (EYEOOl; Mad) having the structure:

\[
\text{PEG-lysine linker-C}_mG_mA_rG_mA_rU_rG_mA_rG_mU_rG_mG_mA_rG_mU_rG_mA_mC_fA_mG_mU_fG_mC_fU_fU_fA_mC_fA_mG_m3'-3'\_Td
\]

(SEQ ID NO: 6).
An example of a 5'-5' capped anti-VEGF aptamer is EYE002 (MacII) having the structure:

\[
T_d -5'\text{-}5'\text{-CGmGmA}_mUCA_rA_r...n\text{ is greater than 3. In one embodiment, } n \text{ is from about 4 to about 4000. In another embodiment, } n \text{ is from about 4 to about 4000.}
\]

wherein "G_m" represents 2'-methoxyguanylic acid, "A_m" represents 2'-methoxyadenylylic acid, "C_f" represents 2'-fluorocytidylic acid, "U_f" represents 2'-fluorouridylic acid, "A_r" represents riboadenlylic acid, and "T_d" represents deoxyribothymidylic acid. (See Adamis, A.P. et al, published application No. WO 2005/014814, which is hereby incorporated by reference in its entirety.)

Examples of polymers of the invention include, but are not limited to, polyether polyols (such as polyethylene glycol), polysaccharides (such as carboxymethyl celluloses, other cellulose derivatives, glycosaminoglycans, hyaluronans, and alginates), polyesters, high molecular weight polyoxyalkylene ethers (such as PLURONIC®) polyamides, polyurethanes, polysiloxanes, polyacrylates, polyols, polyvinylpyrrolidones, polyvinyl alcohols, polyaldehydes, chitosan, polyaldehydes or polyethers.

In one embodiment, the polymers of the present invention are water soluble. In another embodiment the polymers are non-peptidic polymers. In another embodiment the polymers are non-nucleic acid polymers. In another embodiment the polymers are high molecular weight steric groups.

In one embodiment, the polymer is a polyether polyol. In one embodiment, the polymer is a polyalkylene glycol. In one embodiment, the polymer is a polyethylene glycol (PEG). The PEG may have a free hydroxyl group or may be alkylated. In one embodiment, the terminal end of the PEG not bound to the linker, cyclitol, active functional group or biologically active compound has a methoxy group. A PEG comprising a terminal methoxy group may be referred to as "mPEG". In another embodiment, the polyalkylene glycol is poly(propylene glycol) ("PPG") and copolymers thereof (e.g., copolymers of ethylene glycol and propylene glycol), terpolymers thereof, mixtures thereof, and the like.

The term "polyethylene glycol," "poly(ethylene glycol)" or "PEG" refers to any polymer of general formula \(H(\text{O0})_n\text{H}_2\text{CH}_2\text{j}_2\text{OH}\). In one embodiment, \(n\) is greater than 3. In one embodiment, \(n\) is from about 4 to about 4000. In another embodiment, \(n\) is from about
20 to about 2000. In one embodiment, n is about 450. In one embodiment, PEG has a molecular weight of from about 800 Daltons (Da) to about 100,000 Da. In further embodiments, the polyethylene glycol is a 20 kDa PEG, 40 kDa PEG, or 80 kDa PEG.

The average relative molecular mass of a polyethylene glycol is sometimes indicated by a suffixed number. For example, a PEG having a molecular weight of 4000 daltons (Da) may be referred to as "polyethylene glycol 4000". A PEG-conjugated product may be referred to as a PEGylated product.

The use of the term PEG is intended to be inclusive and not exclusive. It should be understood that other related polymers are also suitable for use in the practice of this invention. The term PEG includes polyethylene glycol in any of its forms, including bifunctional PEG, multi-armed PEG, forked PEG, branched PEG and pendent PEG (i.e. PEG or related polymers having one or more functional groups pendent to the polymer backbone.

PEG has several advantages. PEG is typically clear, colorless, odorless, soluble in water, stable to heat, inert to many chemical agents, does not hydrolyze or deteriorate, and is generally non-toxic. PEG is considered to be biocompatible. For example, PEG is capable of co-existence with living tissues or organisms without causing harm. More specifically, PEG is substantially non-immunogenic. For example, PEG does not tend to produce an immune response in the body. When attached to a molecule having some desirable function in the body, such as a biologically active agent, the PEG tends to mask the agent and can reduce or eliminate any immune response so that an organism can tolerate the presence of the agent. PEG conjugates tend not to produce a substantial immune response or cause clotting or other undesirable effects. Furthermore, the addition of soluble, high molecular weight steric groups, such as PEG, may improve the antagonist properties of an aptamer (see U.S. Application Serial No. 11/105,279, which is hereby incorporated by reference in its entirety).

In one embodiment, the polymer is a polysaccharide. Examples of polysaccharides, include but are not limited to, dextran, cellulose, chitqan, polyglucosamine and derivatives thereof. The reducing end of a polysaccharide is available for coupling to an amine group of a molecule by the Schiff-Base chemistry in conjugation. Polysaccharides may be attached to an amine such as an amino substituted cyclitol or an amine terminus of a biologically active compound by reductive animation.
In another embodiment, the polymer is dextran. The dextran can be a straight chain or branched dextran. In another embodiment the dextran is a carboxymethyl dextran (CMDex).

In another embodiment, the polymer is a cellulose derivative. In another embodiment the polymer is a carboxymethyl cellulose (CMC).

In another embodiment, the polymer is a polyaldehyde. In further embodiments, the polyaldehyde group may be either synthetically derived or obtained by oxidation of an oligosaccharide.

In another embodiment, the polymer is an alginate. In a particular embodiment, the alginate group is an anionic alginate group that is provided as a salt with a cationic counter-ion, such as sodium or calcium.

In another embodiment, the polymer is a polyester. In a particular embodiment, the polyester group may be a co-block polymeric polyesteric group.

In another embodiment, the polymer is a polylactic acid (PLA) or a polylactide-co-glycolide (PLGA). Suitable PLGA groups and methods for conjugating PLGA groups are found in J.H. Jeong et al., *Bioconjugate Chemistry* 2001, 12, 917-923; J.E. Oh et al., *Journal of Controlled Release* 1999, 57, 269-280 and J.E. Oh et al., US Patent No. 6,589,548; the contents of each are hereby incorporated by reference in their entirety.

In another embodiment, the polymer is a biologically active molecule. In another embodiment, the polymer is a nucleic acid, nucleoside, oligonucleotide, aptamer, peptides or protein.

In another embodiment, the polymer is a glycosaminoglycan, a hyaluronan, a hyaluronic acid (HA), an alginate, a high molecular weight polyoxyalkylene ether, a PLURONIC® (a block copolymer based on ethylene oxide and propylene oxide), a polyamide, a polyurethane, a polysiloxane, a polyacrylate, a polylvinlypyrrolidone, a polyvinyl alcohol, a polyanhydride, a polyether, a polycaprolactone or a polypeptide.

In another embodiment, the polymer is a dendron. The dendron may be composed of any combination of monomer and surface modifications. Examples of useful monomers include, but are not limited to, polyamidoamine (PAMAM). Examples of useful surface
modification groups include, but are not limited to, cationic ammonium, \( N \)-acyl, and \( N \)-carboxymethyl group. The dendron may be polyanionic, polycationic, hydrophobic or hydrophilic. In one particular embodiment, the dendron has about 1 to about 256 surface modification groups. In another particular embodiment, the dendron has about 4, 8, 16, 32, 64 or 128 surface modification groups.

The term "dendron" refers to a molecule representing half of a dendrimer structure. A dendron is typically constructed on one half of a dendrimer core or by cleavage of a dendrimer core after construction of the dendrimer. The dendron may be composed of any combination of monomer and surface modifications. Examples of useful monomers include, but are not limited to, polyamidoamine (PAMAM). Examples of useful surface modifications include, but are not limited to, cationic ammonium, \( 7V \)-acyl, and \( N \)-carboxymethyl modifications. Alternate surface modifications allow for vastly different properties. For example, the dendron may be polyanionic, polycationic, hydrophobic or hydrophilic. The dendron may be rationally tailored such that the precise number of monomers and surface modification groups are determined by the generation of the dendron (G1, G2, G3, G4, G5, and G6 possessing 4, 8, 16, 32, 64, and 128 groups respectively). Examples of dendron and dendrimer conjugation techniques are found in US Patent No. 5,714,166 and US Patent Application Publication Nos. 2005/0009988 and 2002/0123609; which are hereby incorporated by reference in their entirety. The construction of a dendron-biologically active molecule conjugate with 1:1 stoichiometry may be accomplished by reduction of the disulfide in a dendrimer that contains a cystamine core. This reduction results in the formation of a single orthogonal sulphydryl functionality that may be coupled to any biologically active molecule that has been modified such that it contains a single thiol-reactive group. This may be accomplished by reacting the amine-containing biologically active molecule with a bifunctional linker that contains an amine-reactive group on one terminus and a thiol-reactive group on the other terminus. Examples of disulfide-containing dendritic polymers and dendritic polymer conjugates are found in US Patent No. 6,020,457; which is hereby incorporated by reference in its entirety. Examples of star-like polymeric compounds having a polyol core that form micelles in solution are found in US Patent Application Publication No. 2004/0198641; which is hereby incorporated by reference in its entirety. In one embodiment of the present invention, the cyclitol moiety itself serves as the core for a dendron.
in another embodiment, the soluble, high molecular weight steric group is bovine serum albumin (BSA). The presence of free thiol on BSA permits the conjugation of amine-containing aptamer to BSA by employing a bifunctional linker that contains a thiol-reactive group on one terminus and an amine-reactive group on the other terminus.

The polymer backbone can be linear or branched. Branched polymer backbones are generally known in the art. Typically, a branched polymer has a central branch core moiety and a plurality of linear polymer chains linked to the central branch core. PEG is commonly used in branched forms that can be prepared by addition of ethylene oxide to various polyols, such as glycerol, glycerol oligomers, pentaerythritol and sorbitol. The central branch moiety can also be derived from several amino acids, such as lysine. The branched poly(ethylene glycol) can be represented in general form as $W(-PEG-OH)_x$ in which $W$ is derived from a core moiety, such as glycerol, glycerol oligomers, or pentaerythritol, and $x$ represents the number of arms.

Those of ordinary skill in the art will recognize that the foregoing list for polymers is by no means exhaustive and is merely illustrative, and that all polymeric materials having the qualities described above are contemplated.

Polymers of the present invention can have a molecular weight of from about 800 Da to about 3,000,000 Da. In one embodiment the polymers have a molecular weight of from about 20 kilodaltons (kDa) to about 1000 kDa. In another embodiment the polymers have a molecular weight from about 5 kDa to about 100 kDa. In one particular embodiment, the polymers have a molecular weight of about 20 kDa. In another particular embodiment, the polymers have a molecular weight of about 40 kDa. In another particular embodiment, the polymers have a molecular weight of about 80 kDa.

The polymers of the invention may be conjugated to nucleic acids. Conjugation of the polymer to a nucleic acid may be through the 5’ end of the nucleic acid, the 3’ end of the nucleic acid, or any position along the nucleic acid sequence between the 5’ and 3’ ends. Examples of suitable internal nucleic acid sequence positions for joining to the polymer (i.e., non 5’- or 3’-end positions) include an exocyclic amino group on a base, a 5-position of a pyrimidine nucleotide, an 8-position of a purine nucleotide, a hydroxyl group of a phosphate, or a hydroxyl group of a ribose group of the nucleic acid sequence.
The terminal activating group or the cyclitol can also include a spacer moiety. The spacer moiety can be located proximal to the polyalkylene oxide or proximal to the terminal activating group. The spacer moiety may be a heteroalkyl, alkoxy or alkyl group containing up to and including 18 carbon atoms or even an additional polymer chain. The spacer moieties can be added using standard synthesis techniques.

It will be understood that certain compounds of the invention may be obtained as different stereoisomers (e.g., diastereomers and enantiomers) and that the invention includes all isomeric forms and mixtures of the disclosed compounds (including pure isomers, and racemic mixtures thereof).

It will be understood that certain compounds of the invention may be obtained as different pharmaceutically acceptable salts. The term "pharmaceutically acceptable salt" refers to non-toxic acid addition salts and alkaline earth metal salts of the compounds of the present invention. The salts can be prepared in situ during the final isolation and purification of such compounds, or separately by reacting the free base or acid functions with a suitable organic acid or base, for example. Representative acid addition salts include, but are not limited to, hydrochloride, hydrobromide, sulfate, bisulfate, acetate, valerate, oleate, palmate, stearate, laurate, borate, benzoate, lactate, phosphate, tosylate, mesylate, citrate, maleate, fumarate, succinate, tartrate, glucoheptonate, lactobionate, lauryl sulfate salts and the like. Representative alkali and alkaline earth metal salts include, but are not limited to sodium, calcium, potassium and magnesium salts.

The invention also relates to methods of forming a conjugate comprising a polymer, a biologically active molecule and a cyclitol linking the polymer to the biologically active molecule.

An inositol may be prepared such that the positions that will bear PEG chains may be functionalized selectively.

Conduritols may be used as precursors to the inositol fragment. Synthesis of conduritols are described in Balci, Pure App. Chem. 1997, 69, 97-104; Subtseyaz et al., J. Chem. Soc. Chem. Commun. 1988, 1330-1331 and references therein; each of which are hereby incorporated by reference in their entirety.

An example of the preparation of inositol linked PEG-VEGF aptamer conjugate 1 is shown in Figure 1 and Example 3. Coupling of 2 to activated ester derivatives of 2OkDa
conjugate. Alternative conditions that contain aqueous buffer are optionally employed. A two-step, one-pot synthesis of 1 was accomplished with in-situ activation of compound 3 with an excess of $N,N,N',N'$-Tetramethyl-0-(N-succinimidyl)uronium tetrafluoroborate (TSU) followed by addition of aptamer.

Preparation of inositol linker 2 and inositol activated polymer 3 is shown in Figure 2 and Examples 1-2. Alkylation of myo-inositol-1,3,5-orthoformate gives compound 5. An element of symmetry in 5 allows for a simplified confirmation of regiochemistry by NMR and ultimately precludes the formation of diastereomeric products upon conjugation to an optically active API. Removal of the silyl protecting group from 5 gave intermediate 6. Alternative electrophiles may be used. The desired linker 2 is then prepared upon sequential deprotection of 7. This was accomplished by first refluxing in 0.1M methanolic HCl for 15min then refluxing in 0.4M methanolic hydrazine for 60min.

An example of the preparation of inositol linked PEG-VEGF aptamer conjugate 10 is shown in Figure 3 and Examples 4 and 5.

A receptor binding assay for determining the Ability of the VEGF aptamer conjugates of the present invention to inhibit VEGF binding to VEGF-R1 (Flt-I) is described in Example 6. Results of the assay are shown in Example 7. The ability of inositol-linked VEGF aptamer conjugate 1 to inhibit VEGF binding to VEGF-R1 (Flt-I) was compared to that of pegaptanib (Mad) and 5'-5' capped anti-VEGF aptamer, EYE002 (MacII). The inositol linked PEG-VEGF aptamer conjugate demonstrated activity that is indistinguishable from that of the pegaptanib.

**EXAMPLES**

The following examples serve to illustrate certain useful embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof. Alternative materials and methods can be utilized to obtain similar results.
Example 1

Synthesis of /wj/ø-Inositol Linker (2)

Compound 2 was prepared from the commercially available /wyo-inositol-1,3,5-orthoformate using the procedure as described in Carbohydrate Research 2000, 325, 313-320.

Sodium hydride (1.6g, 41mmoles of a 60% dispersion in mineral oil) was added to a stirring solution of 4 (2.5g, 8.2mmoles) in 75mL of anhydrous N-methylpyrrolidinone at O°C. The ice bath was immediately removed and the solution stirred for 30 minutes, at which time N-(3-bromopropyl)-phthalimide (11.0g, 41mmoles) and tetrabutylammonium iodide (3.0g, 8.2mmole) were added. The reaction was stirred for 18 hours at room temperature under an argon atmosphere then quenched by adding 10mL of ice-cold water drop-wise. The resulting solution was extracted with chloroform (3x15mL) and the organic layers pooled and concentrated in vacuo. The crude material was eluted from flash silica gel using 7:3 hexanes/ethyl acetate on a Biotage Flash+ chromatography system. 4.0g of compound 5 was isolated (72% yield).

A solution of tetrabutylammonium fluoride (TBAF) (500µL, 1M) in tetrahydrofuran (THF) was added to a solution of 5 (200mg, 295µmoles) in 2mL of 1,4-dioxane. This solution was stirred for 16 hours at room temperature then concentrated in vacuo. The resulting oil was purified by eluting from flash silica gel using 2:3 hexanes/ethyl acetate to give 52mg of compound 6 an oil (31%).

Sodium hydride (65mg, 1.61mmoles of a 60% dispersion in meinal oil) was added to a stirring solution of 6 (91.0mg, 161µmoles) in 4mL of anhydrous DMF at O°C. The ice bath was immediately removed and the solution was stirred for 30 min, at which time 8-bromooctanoic acid (144mg, 645µmoles) was added. The reaction was stirred for 24 hours at room temperature under an argon atmosphere then quenched by adding 5mL of water drop-wise at O°C. The pH of the resulting solution was adjusted to 4 with 1M HCl and the solution was extracted with chloroform (4x4mL). The organic layers were pooled and concentrated in vacuo. The crude material was eluted from flash silica gel using 1:1 hexanes/ethyl acetate (500mL) then 1:2 hexanes/ethyl acetate (500mL) then 100% ethyl acetate (750mL). Isolated yield of pure material was 35mg (31%).
Crude 7 was dissolved in 5mL of methanolic 0.1M HCl and refluxed for 15min. The resulting solution was concentrated *in vacuo* and co-evaporated thrice with 5mL of methanol. The resulting residue was re-dissolved in 40mL of methanol before 503µL of hydrazine was added. This solution was refluxed for 1h. The solution was then concentrated to one half of its original volume by collecting the distillate. The resulting solution was cooled and then concentrated *in vacuo*. The resulting material was chromatographed on a C-18 HPLC column (Waters Deltapak, 15µm, 300Å, 18.5min).

**Example 2**

**Synthesis of Activated PEG compound (3)**

To a solution of compound 2 (750µg, 1µmole) in 2mL of anhydrous dimethylformamide (DMF) was added mPEG-SPA (75mg, 3.7µmoles, Nektar Therapeutics LN PT-OIE-16) in 1mL of anhydrous dichloromethane (DCM) followed by 100µL of pyridine. The solution was stirred under an argon atmosphere for 28 hours at room temperature and then 8mL of diethyl ether (Et₂O) was added to precipitate all PEG species. The PEG precipitate was sedimented by centrifugation (5min at 1000 x g) and the solution decanted. The resulting pellet was re-dissolved in 4mL of DCM and again precipitated with 36mL of Et₂O and centrifuged (5min at 1000 x g) to pellet the PEG species. The PEG species were further dried *in vacuo* then purified on SEC (Shodex KW-804, isocratic 100% PBS as eluent). The 40kDa fraction was lyophilized to a white powder and analyzed by MALDI-MS.

**Example 3**

**Synthesis of Inositol-linked PEG-VEGF Aptamer conjugate (1)**

Compound 3 (2.1mg, 52.9nmoles) was dried by co-evaporation with toluene then dissolved in 420µL of DMSO. To this solution, stirring under and argon atmosphere, was added 16µL of a TSU solution (Img/mL in DMSO, 1.1 molar equivalents) then 1µL of a triethylamine (TEA) solution (8.9µL/mL in DMSO, 2.0 equivalents) and the reaction was stirred at room temperature for 1h. VEGF Aptamer (1mg) was added to the solution as a lyophilized powder and the reaction stirred for an additional hour. To this reaction mixture was added 1mg of TSU dissolved in 100µL of DMSO as 10µL aliquots every 10 minutes
until all the material was added. 5µl of TEA was added at the completion of the TSU additions.

Compound 1 was purified using an analytical HPLC (C-18, Hamilton PRP-I) using method "RPHPLC_ImLmin_PI3 A" (Solvent A: 10mM Ammonium Acetate, Solvent B: Acetonitrile). Two 100µL injections were done on the same column. The material that eluted at 25.6 minutes in each run was pooled, concentrated, then lyophilized to a white powder. This powder was re-dissolved in 50µL of PBS and the concentration of aptamer was determined by a Beer's law calculation (260nm) and confirmed against a standard curve of pegaptanib injections on the HPLC.

Example 4

Synthesis of Activated my\(\theta\)-Inositol Orthoformate Linker (8)

To a stirring solution of \(\text{my}\_\theta\text{-O}-\text{inositol-1,3,5-orthoformate (1.00g, 5.26mmoles)}\) in 20mL of N-methylpyrrolidinone at \(0^\circ\text{C}\) was added sodium hydride (1.05g, 26.3mmoles). The solution was stirred for 30 min. at \(0^\circ\text{C}\) then tetrabutylammonium iodide (10.0 mg) and allyl bromide (3.56mL, 42.1mmoles) were added to the solution. The solution remained in the ice bath at \(0^\circ\text{C}\) and warmed slowly thereafter as the ice melted. The solution remained stirring at ambient temperature for 14h and was then quenched by drop-wise addition of deionized water (20mL). The reaction mixture was extracted with chloroform (3x20mL), the organic layers were combined and the products concentrated at ~0.1mmHg and 30\(^\circ\)C. The crude oil was chromatographed on silica gel (Biotage 40S flash column) using a binary mixture of hexanes and ethyl acetate (85:15) to elute. The desired product was observed on silica TLC at Rf 0.3 using hexanes and ethyl acetate (85:15) to elute. 1.12g of the resulting di-allyl compound were recovered (3.62mmoles, 69% yield). IH-NMR and 13C-NMR, ESI-MS (neg ion mode) calcd for C16H22O6 310.14, found 311.3 (H+ adduct) and 333.2 (Na+ adduct).

To a stirring solution of the di-allyl compound (130mg, 419µmoles) in 15mL of 1,4-dioxane was added catecholborane.(2mL). The solution was stirred at reflux for 1h and was then quenched by drop-wise addition of an alkaline hydrogen peroxide solution (5mL of 1M NaOH and 5mL of 30% H2O2) at\(0^\circ\)C. The reaction was warmed to room temperature and stirred for 14h then extracted with chloroform (3x25mL), the organic layers were combined and the products concentrated in vacuo. The crude oil was chromatographed on silica gel
(Biotage 25+ flash column) using a binary mixture of hexanes and ethyl acetate (1:2) to elute. The desired product was observed on silica TLC at Rf 0.22 using hexanes and ethyl acetate (1:2) to elute. 38.8mg of the resulting borate compound were recovered. ESI-MS (neg ion mode) calcd for C_{10}H_{25}O_{9}: 371.20, found 371.23 (H+ adduct).

To a stirring solution of the borate compound (2 mg) in 2mL of deionized water and 2mL of IM NaOH, was added silver oxide (10mg). Acetonitrile (4mL) was then added and the solution stirred at room temperature for 2h. The solution was then filtered through a paper filter to remove any silver adducts that did not precipitate as a mirror on the walls of the glass flask. The filtrate was concentrated and analyzed by MS. ESI-MS (negative ion mode) calcd for C_{10}H_{22}O_{12}: 406.1 t. found 430.01 (Na+ adduct).

Example 5

Synthesis of Inositol-linked PEG-VEGF Aptamer Conjugate (10)

To a stirring solution of 8 (25 µmoles) in MES buffer (2.5mL, 0.14M, pH5.5) was added 20 kDa mPEG-NH2 (1.0g, 50µmoles) in acetonitrile (2.5mL) and solid EDC (95.8mg, 500µmoles). The solution was stirred for 14h at room temperature then diethyl ether (90mL) was added. The resulting precipitate was isolated and dried on a Buchner funnel. MALDI-MS indicated that this material is a mixture of 20kDa and 40kDa species (THAP - NH4Citrate matrix). This mixture of PEG species was dissolved in anhydrous DMSO (10mL) and DCC (100. mg) was added. The solution was stirred 14h under an argon atmosphere and at room temperature. PEG species were again precipitated by the addition of diethyl ether (90mL) and isolated on a Buchner funnel. The PEG adducts were dissolved in acetonitrile (8mL) then added to a solution of an anti-VEGF aptamer containing an amino-terminus (250mg) in borate buffer (6mL, 100mM, pH 8.5) then shaken for 14h. The resulting solution was concentrated then diluted to 9mL in deionized water. Analysis of this material by reverse phase HPLC demonstrated co-elution of a product with a pegaptanib standard.

Purification of compound 9 using multiple elutions from a C-18 HPLC yielded 8.5mg of the 50kDa product that co-elutes with pegaptanib.

Compound 9 is dissolved in methanolic 0.1M HCl and refluxed. The resulting solution is concentrated in vacuo and co-evaporated 5mL of methanol. The resulting material is chromatographed on a C-18 HPLC column (Waters Deltapak, 15µm, 300µ).
Example 6

**Receptor Binding Assay**

**Receptor Plate Coating**

For each set of binding experiment, one row (12 wells) of a 96-well Isoplate Plate is used. Each of the 12 wells is first coated with 2 picomole (300 nanograms (ng)) of anti-human IgG1 Fc fragment-specific antibody in 100 microliter (µL) of PBS at 4°C overnight. The next day, further protein binding in each well is blocked by washing with 300 µL of Super Block blocking buffer at room temperature for 3 times, 5 minutes each. Each well is then washed with 300 µL of binding buffer (PBS with 1 mM calcium chloride, 1 mM magnesium chloride, 0.01% HSA, PH 7.4) at room temperature twice. For KDR/Fc, 0.25 picomole (85 ng) of the chimeric receptor in 100 µL of binding buffer is added into the first 11 wells, whereas the twelfth well receive 0.5 picomole (118 ng) of human ICAM-1/Fc chimera protein as the background control well. For Flt-I/Fc, 0.125 picomole (30.8 ng) of the chimeric receptor in 100 µL of binding buffer each is added into the first 11 wells, whereas the background control well (#12) receive 0.5 picomole (118 ng) of human ICAM-1/Fc chimera protein. For neuropilin-1/Fc, 0.2 picomole (48 ng) of the chimeric receptor in 100 µL of binding buffer is added to all 12 wells. The chimeric receptors and human ICAM-1/Fc protein are captured onto the well by binding to the immobilized anti-human IgG1 Fc fragment-specific antibody in each well at room temperature for 2 to 3 hour. Each well is washed with 300 µL of binding buffer at room temperature to remove the free chimeric receptors and human ICAM-1/Fc protein.

**Preparation of 125I-VEGFi65-Pegaptanib binding mix**

A set of 10 five-fold dilutions of the Pegaptanib (tube #1 to #10) ranging from 1 µM (or 2 µM) to 0.512 picomolar (pM) (or 1.024 pM) are each mixed with about 0.01 µCi of 125I-VEGFi, in binding buffer (PBS with 1 mM calcium chloride, 1 mM Magnesium Chloride, 0.01% HSA, PH 7.4) in non-stick 1.5 mL microfuge tubes, in a total 100 µL final volume each. For tube #11 and #12, only 0.01 µCi of 125I-VEGFi, are added without any Pegaptanib and they are the positive and background controls, respectively. All 12 tubes are incubated at 37°C (for KDR and Flt-I) or at room temperature (for neuropilin-1) for 15 to 20 min to allow the binding of Pegaptanib to VEGF to reach equilibrium. The 100 µL binding mix from each tube is then applied to the corresponding well on the receptor-coated Isoplate. The plate is
incubated at 37°C (for KDR and Flt-1) or at room temperature (for neuropilin-1) for 2 to 3
hours to allow equilibrium binding to occur. The plate is washed 4 times with 300 µL/well of
binding buffer with (for KDR and neuropilin-1) or without (for Flt-1) 0.05% Tween 20, at
room temperature. The plate is air dried for about 10 min, and about 200 µl of scintillation
fluid is added to each well. The radioactivity of each well is determined by scintillation
counting. For experimental negative control, polyethylene glycol 40,000 MW (40 kDa PEG)
is used at identical molar concentration to replace the Pegaptanib in the binding assay,
following all the steps described above for Pegaptanib.

Determining effective concentration for 50% inhibition (IC50) of VEGF receptor binding

The 125I-VEGF165:receptor binding ratios in the wells are calculated as: number of
counts retained on the wells (#1 to #11) minus the background (well #12) divided by the
maximum binding (positive control, well #11) minus the background (well #12). The
resulting binding ratios at different pegaptanib concentrations are analyzed by using
nonlinear regression with the GraphPad PRISM program (one site competition), and the
resulting curve is used to determine the half-maximum inhibition (IC50) of pegaptanib in
inhibiting the receptor binding to VEGF165. Data from the experimental negative control
using PEG are analyzed by the same method.

Example 7

Comparative Inhibition of VEGF-RI (Flt-1)

The ability of cyclitol-linked VEGF aptamer conjugates to inhibit VEGF binding to
VEGF-R1 (Flt-1) was compared to that of lysine-linked VEGF aptamer conjugates and non-
sterically enhanced VEGF aptamer conjugates.

Using the assay as described in Example 6, the Flt-I receptor binding activity of
compound (1) was measured. For each sample the percent inhibition, IC50, IC50 range and R2
were determined. The assay of inositol-linked VEGF aptamer conjugate (1) was performed
in duplicate and labeled "Inositol Conjugate (1)-I" and "Inositol Conjugate (1)-2". The
results are shown in Table 1 and Figure 3. "Inositol Conjugate (1)-I" and "Inositol
Conjugate (1)-2" have an IC50 of 0.5890 nM and 0.8259 nM respectively. Mad has an IC50
of 0.4569 nM. MacII has an IC50 of 0.375 nM. The results indicate that the inositol-linked
VEGF aptamer conjugate (1) is as effective as the lysine-linked VEGF aptamer conjugate,
Pegaptanib (EYE-001, Mad), while both the inositol and lysine-linked VEGF aptamer
conjugates are much more effective in inhibiting VEGF binding than are non-enhanced VEGF aptamers such as EYE-002 (MacII).

Table 1.

<table>
<thead>
<tr>
<th></th>
<th>MacI</th>
<th>MacII</th>
<th>Inositol Conjugate (1)-1</th>
<th>Inositol Conjugate (1)-2</th>
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<tbody>
<tr>
<td>% Inhibition</td>
<td>74.51</td>
<td>20.79</td>
<td>73.36</td>
<td>72.93</td>
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<tr>
<td>IC_{50} (nM)</td>
<td>0.4569</td>
<td>0.375</td>
<td>0.5890</td>
<td>0.8259</td>
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<tr>
<td>IC_{50} (nM) Range</td>
<td>0.2436 to 0.8570</td>
<td>0.1568 to 0.8969</td>
<td>0.4082 to 0.8496</td>
<td>0.5640 to 1.209</td>
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<tr>
<td>R^2</td>
<td>0.9831</td>
<td>0.9682</td>
<td>0.9941</td>
<td>0.9937</td>
</tr>
</tbody>
</table>
Incorporation by reference

The patent and scientific literature referred to herein establishes knowledge that is available to those of skill in the art. All patents, patent applications, and published references cited herein are hereby incorporated by reference in their entirety.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific embodiments described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.
We claim:

1. A conjugate comprising a polymer, a biologically active molecule and a cyclitol group linking the polymer to the biologically active molecule.

2. The conjugate of claim 1, wherein the cyclitol group is a 6-membered cycloalkane, or a 5-membered cycloalkane.

3. The conjugate of claim 1, wherein the cyclitol group is selected from the group consisting of tri-, tetra-, penta- and hexa-hydroxy cyclohexanes.

4. The conjugate of claim 1, wherein the cyclitol group comprises a substituent selected from the group consisting of alkyl, amino, thio, carbonyl and halogen substituents.

5. The conjugate of claim 1, wherein the cyclitol group is an inositol.

6. The conjugate of claim 5, wherein the inositol is selected from the group consisting of myo-inositol, D-c/zz>ø-inositol, L-c/zzrø-inositol, muçco-inositol, scy/Yo-inositol, a//o-inositol, epz-inositol, cis-inositol and eeφ-inositol.

7. The conjugate of claim 1, wherein the biologically active molecule is selected from the group consisting of nucleic acids, nucleosides, oligonucleotides, aptamers, peptides, proteins, enzymes, small molecule drugs, dyes, lipids, cells, viruses, liposomes, microparticles and micelles.

8. The conjugate of claim 1, wherein the biologically active molecule is an oligonucleotide.

9. The conjugate of claim 1, wherein the biologically active molecule is an aptamer.

10. The conjugate of claim 1, wherein the biologically active molecule is an anti-VEGF aptamer.
11. The conjugate of claim 1, wherein the biologically active molecule is an anti-VEGF aptamer having the sequence:
\[ \text{C}_\text{G}G\text{mAmAmUfGnAmA}_{\text{d}}\text{U}_\text{f}G\text{mCfUfAmAnUfAmCfAm}_{\text{d}}\text{U}_\text{f}G\text{mCfGm} (\text{SEQ ID NO: 5}). \]

12. The conjugate of claim 1, wherein the polymer is water soluble.

13. The conjugate of claim 1, wherein the polymer is a non-peptidic polymer.

14. The conjugate of claim 1, wherein the polymer is a non-nucleic acid polymer.

15. The conjugate of claim 1, wherein the polymer is a high molecular weight steric group.

16. The conjugate of claim 1, wherein the polymer is selected from the group consisting of polyether polyols, polyethylene glycol, polysaccharides, carboxymethyl celluloses, cellulose derivatives, glycosaminoglycans, hyaluronans, alginites, polyesters, high molecular weight polyoxyalkylene ethers, polyamides, polyurethanes, polysiloxanes, polyacrylates, polyols, polyvinylpyrrolidones, polyvinyl alcohols, polyanhydrides, chitosan, polyaldehydes and polyethers.

17. The conjugate of claim 1, wherein the polymer has a molecular weight of from about 800 Daltons to about 100,000 Daltons.

18. The conjugate of claim 1, wherein the polymer has a molecular weight selected from the group consisting of about 20 kDa, 40 kDa, and 80 kDa.

19. A compound having the formula:

![Chemical structure](image)

wherein:

\[ A_{1} \] is a biologically active moiety or an active functional group;
R_{1a}, R_{1b}, Ric, Rid and R_{ie} are each independently selected from the group consisting of -OH, -NH$_2$, and -X$_2$-L$_2$-A$_2$;

A$_2$ is selected from the group consisting of a hydrogen, a polymer, a biologically active moiety and an active functional group;

L$_1$ and L$_2$ are each independently selected from the group consisting of a bond and a spacer moiety;

X$_1$ and X$_2$ are each independently selected from the group consisting of -O-, -S- and -NR$_2$-; and

R$_2$ is selected from the group consisting of a hydrogen and a lower alkyl group;

and pharmaceutically acceptable salts thereof.

20. The compound of claim 19, wherein A$_i$ is a biologically active moiety.

21. The compound of claim 19, wherein A$_i$ is an active functional group.

22. The compound of claim 19, wherein the active functional group is an electrophilic functional group.

23. The compound of claim 19 wherein the active functional group is an electrophilic functional group selected from the group consisting of a carboxylic acid, carboxylic acid halide, carboxylic acid chloride, halogen, iV-succinimide carbonate, succinimidyl ester, 1-benzotriazolylcarbonate ester, iV-hydroxymaleimidyl ester, vinyl sulfone, azalactone, cyclic amide thione carbonyl, imidazole carbonyl, isocyanate and isothiocyanate.

24. The compound of claim 19, wherein at least one of R$_{1a}$, R$_{ib}$, Rio, Rid and R$_{ic}$ is -X$_2$-L$_2$-A$_2$.

25. The compound of claim 19, wherein at least two of R$_{1a}$, R$_{ib}$, Ric, Rid and R$_{ic}$ are each independently -X$_2$-L$_2$-A$_2$. 
26. The compound of claim 19, wherein at least three of $R_{1a}$, $R_{ib}$, $R_{ie}$, Rid and $R_{le}$ are each independently $-X_2^-L_2^-A^-\_2$.

27. The compound of claim 19, wherein at least four of $R_{1a}$, $R_{n3}$, $R_{ib}$, $R_{id}$ and $R_{le}$ are each independently $-X_2^-L_2^-A^-\_2$.

28. The compound of claim 19, wherein each of $R_{1a}$, $R_{ib}$, $R_{ie}$, Rid and $R_{le}$ are each independently $-X_2^-L_2^-A^-\_2$.

29. The compound of claim 19, wherein $L_i$ and $L_2$ are each independently a spacer moiety represented by the formula:

$$-(CH_2)_n-(X_3)^m-(C(X_4))_p-(X_5)^q-(CH_2)_r^-, \text{ wherein,}$$

- $n$ is 0 to 10;
- $m$ is 0 or 1;
- $p$ is 0 or 1;
- $q$ is 0 or 1; and
- $r$ is 0 to 10.

30. The compound of claim 19, wherein $L_i$ and $L_2$ are each independently a spacer moiety represented by a formula selected from the group consisting of:

$$-(CH_2)V(C(X_4))V;$$
$$-(C(X_4))_p-(CH_2)_r^-;$$
$$-(CGX_4)V; \text{ and}$$

$$-(CH_2)V;$$

wherein $n$ is 0 to 10;

$p$ is 0 or 1; and

$r$ is 0 to 10.

31. The compound of claim 19, wherein $X_1$, $X_2$, $X_3$, $X_4$ and $X_5$ are each independently selected from the group consisting of $-O-$ and $-NH-$.

32. The compound of claim 19, wherein $X_1$, $X_2$, $X_3$, $X_4$ and $X_5$ are each $-O-$.
33. A compound having a formula selected from the group consisting of:

wherein:

D is a biologically active moiety;

POLY is a polymer;
X₃, X₄ and X₅ are each independently selected from the group consisting of -O-, -S- and -NR₂⁻;  

R₂ is selected from the group consisting of a hydrogen and a lower alkyl group;  

m is 0 or 1; and  

n is an integer from 0 to 10;  

and pharmaceutically acceptable salts thereof.

34. The compound of claim 33, wherein D is a nucleic acid.

35. The compound of claim 33, wherein D is an aptamer.

36. The compound of claim 33, wherein D is an anti-VEGF aptamer.

37. The compound of claim 33, wherein D is an anti-VEGF aptamer having the sequence:  

\[
\begin{align*}
C_f & G_m A_m A_m U_f U_f C_f G_m A_m A_m U_f U_f C_f G_m \ (SEQ \ ID \ NO: \ 5).
\end{align*}
\]

38. A compound having a formula selected from the group consisting of:

\[
\begin{align*}
\text{POLY} & \quad \text{X}_5 \quad \text{C} \quad (\text{X}_3)_m \quad \text{-(CH₂)}_n \quad \text{---O-HO---}\quad \text{OH} \quad \text{X}_4 \\
 & \quad \text{OH} \quad \text{O---(CH₂)}_n \quad \text{-(X₃)}_m \quad \text{---C---G} \\
\end{align*}
\]

\[
\begin{align*}
\text{POLY} & \quad \text{X}_5 \quad \text{C} \quad (\text{X}_3)_m \quad \text{-(CH₂)}_n \\
 & \quad \text{O} \quad \text{OH} \quad \text{O---(CH₂)}_n \quad \text{-(X₃)}_m \quad \text{---C---G} \\
\end{align*}
\]

\[
\begin{align*}
\text{POLY} & \quad \text{X}_5 \quad \text{C} \quad (\text{X}_3)_m \quad \text{-(CH₂)}_n \quad \text{---O---}\quad \text{OH} \quad \text{X}_4 \\
 & \quad \text{OH} \quad \text{O---(CH₂)}_n \quad \text{-(X₃)}_m \quad \text{---C---G} \\
\end{align*}
\]
wherein:

G is a leaving group;

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POLY is a polymer; 1

X₃, X₄ and X₅ are each independently selected from the group consisting of -0-, -S- and -NR₂⁻;

R₂ is selected from the group consisting of a hydrogen and a lower alkyl group;

m is 0 or 1; and

n is an integer from 0 to 10;

and pharmaceutically acceptable salts thereof.

39. The compound of claim 38, wherein G is a leaving group selected from the group consisting of a halide, chloride, iV-succinimide, 1-benzotriazole, iV-hydroxymaleimide, azalactone, cyclic amide thione and imidazole.
40. A compound having the structure:

\[ \text{C}_5 \text{G}_m \text{G}_m \text{A}_1 \text{A}_U \text{A}_U \text{G}_m \text{U}_2 \text{G}_m \text{A}_m \text{U}_2 \text{C}_1 \text{U}_1 \text{G}_m \text{C}_1 \text{U}_1 \text{A}_m \text{U}_1 \text{A}_m \text{U}_1 \text{C}_1 \text{C}_1 \text{G}_m \]

(SEQ ID NO: 5)

wherein \( z \) is about 450;

and pharmaceutically acceptable salts thereof.

41. A compound having the structure:

\[ \text{C}_5 \text{G}_m \text{G}_m \text{A}_1 \text{A}_U \text{A}_U \text{G}_m \text{U}_2 \text{G}_m \text{A}_m \text{U}_2 \text{C}_1 \text{U}_1 \text{G}_m \text{C}_1 \text{U}_1 \text{A}_m \text{U}_1 \text{A}_m \text{U}_1 \text{C}_1 \text{C}_1 \text{G}_m \]

(SEQ ID NO: 5)

wherein \( z \) is about 450;

and pharmaceutical acceptable salts thereof.
Figure 1

REPLACEMENT SHEET

SUBSTITUTE SHEET (RULE 26)
Figure 3
Figure 4
wherein z is about 450

Figure 5
wherein z is about 450

Figure 6