MATERIALS AND METHODS FOR CONJUGATING A WATER SOLUBLE FATTY ACID DERIVATIVE TO A PROTEIN

FIELD OF THE INVENTION

[0001] The present invention relates to materials and methods for conjugating a water soluble fatty acid derivative to a protein.

BACKGROUND OF THE INVENTION

[0002] A variety of molecules and/or compounds have been described for conjugating to therapeutic proteins in order to increase the half-life of the conjugated therapeutic proteins following administration to a patient (Veronese FM and Mero A, BioDrugs 2008;22:315-29; Gregoriadis G et al., Int J Pharm 2005; 300:125-30; and Shechter Y et al.; International Journal of Peptide Research and Therapeutics 2007; Vol 13 :105-17).

[0003] Fatty acids (FA) can be conjugated to therapeutic proteins to form longer-acting derivatives. This principle for prolongation of protein or peptide half-life is based on the fact that FA can bind to human serum albumin (HSA; also referred to as albumin binding probes). The association of a FA with human serum albumin in the blood stream can lead to a substantial prolongation of the half-life of the therapeutic protein as it will recycle together with albumin through the neonatal Fc receptor. FA and derivatives thereof (e.g., corresponding methyl esters) have shown similar albumin-binding properties (Spector AA, J Lipid Res 1975;16:165-79).

[0004] One prominent example for this longer-acting principle is insulin detemir (Levemir®) from Novo Nordisk. In insulin detemir, the carboxyl group of a FA is covalently coupled to the ε-amino group of a lysine residue of the insulin protein (see, e.g., US 5,866,538; US 6,011,007; and US 6,869,930). Other research groups have described similar approaches (Shechter Y et al., Bioconj Chem 2005;16:913-20; and Sasson K et al., J Control Release 2010;142:214-20). For example, these groups describe a releasable FMOC system containing an active NHS ester for coupling to amino groups of proteins. The difference however is that in this concept the FA is linked to the protein via a functional group in ω-position thereby rendering the carboxyl group intact. Thus, prolonged-acting prodrugs can be

prepared that bind to human serum albumin yet dissociate over time as the FMOC system undergoes slow hydrolysis under physiological conditions (Sasson K et al., J Control Release 2010;142:214-20).

[0005] In addition to fatty acids, the preparation of conjugates by forming a covalent linkage between the water soluble polymer and the therapeutic protein can be carried out by a variety of chemical methods. PEGylation of polypeptide drugs protects them in circulation and improves their pharmacodynamic and pharmacokinetic profiles (Harris and Chess, Nat Rev Drug Discov. 2003;2:214-21). The PEGylation process attaches repeating units of ethylene glycol (polyethylene glycol (PEG)) to a polypeptide drug. PEG molecules have a large hydrodynamic volume (5–10 times the size of globular proteins), are highly water soluble and hydrated, non-toxic, non-immunogenic and rapidly cleared from the body. PEGylation of molecules can lead to increased resistance of drugs to enzymatic degradation, increased half-life in vivo, reduced dosing frequency, decreased immunogenicity, increased physical and thermal stability, increased solubility, increased liquid stability and reduced aggregation. The first PEGylated drugs were approved by the FDA in the early 1990s. Since then, the FDA has approved several PEGylated drugs for oral, injectable and topical administration.

[0006] Polysialic acid (PSA), also referred to as colominic acid (CA), is a naturally occurring polysaccharide. It is a homopolymer of N-acetylneuraminic acid with $\alpha(2\rightarrow8)$ ketosidic linkage and contains vicinal diol groups at its non-reducing end. It is negatively charged and a natural constituent of the human body. It can easily be produced from bacteria in large quantities and with pre-determined physical characteristics (US Patent No. 5,846,951). Because the bacterially-produced PSA is chemically and immunologically identical to PSA produced in the human body, bacterial PSA is non-immunogenic, even when coupled to proteins. Unlike some polymers, PSA acid is biodegradable. Covalent coupling of colominic acid to catalase and asparaginase has been shown to increase enzyme stability in the presence of proteolytic enzymes or blood plasma. Comparative studies in vivo with polysialylated and unmodified asparaginase revealed that polysialylation increased the half-life of the enzyme (Fernandes and Gregoriadis, Int J Pharm. 2001;217:215-24).

[0007] Coupling of PEG-derivatives to peptides or proteins is reviewed by Roberts et al. (Adv Drug Deliv Rev 2002;54:459-76). One approach for coupling water soluble polymers to therapeutic proteins is the conjugation of the polymers via the carbohydrate moieties of the protein. Vicinal hydroxyl (OH) groups of carbohydrates in proteins can be easily oxidized

with sodium periodate (NaIO4) to form active aldehyde groups (Rothfus and Smith, J Biol Chem 1963; 238:1402-10; van Lenten and Ashwell, J Biol Chem 1971;246:1889-94). Subsequently the polymer can be coupled to the aldehyde groups of the carbohydrate by use of reagents containing, for example, an active hydrazide group (Wilchek M and Bayer EA, Methods Enzymol 1987;138:429-42). A more recent technology is the use of reagents containing aminooxy groups which react with aldehydes to form oxime linkages (WO 96/40662, WO2008/025856).

[0008] Additional examples describing conjugation of a water soluble polymer to a therapeutic protein are described in WO 06/071801 which teaches the oxidation of carbohydrate moieties in von Willebrand factor and subsequent coupling to PEG using hydrazide chemistry; US Publication No. 2009/0076237 which teaches the oxidation of rFVIII and subsequent coupling to PEG and other water soluble polymers (e.g. PSA, HES, dextran) using hydrazide chemistry; WO 2008/025856 which teaches oxidation of different coagulation factors, e.g. rFIX, FVIII and FVIIa and subsequent coupling to e.g., PEG, using aminooxy chemistry by forming an oxime linkage; and US Patent No. 5,621,039 which teaches the oxidation of FIX and subsequent coupling to PEG using hydrazide chemistry.

[0009] Notwithstanding the above materials and methods for protein conjugation, new materials and methods are desired that, for example, allow manipulation and preparation of stable protein conjugates. Although fatty acids can provide the benefit of binding HSA, fatty acids are often difficult to manipulate in an aqueous environment and can be released or removed from its protein binding partner over time.

SUMMARY

[0010] The present disclosure provides materials and methods for conjugating polymers and water soluble fatty acid derivatives to proteins that improves the protein's pharmacodynamic and/or pharmacokinetic properties while minimizing the costs associated with the various reagents and the health risks to the patient recipients when the conjugation reaction is catalyzed by a nucleophilic catalyst. The present disclosure provides materials and methods for conjugating water soluble fatty acid derivatives to proteins in an aqueous solution, thereby producing stable protein conjugates wherein the fatty acid derivatives are not released over time.

[0010a] In a first aspect of the present disclosure, there is provided a water soluble fatty acid derivative comprising a fatty acid or fatty acid ester attached to a water soluble linker, said fatty acid derivative stably attached to a therapeutic protein, wherein the water soluble linker comprises a water soluble polymer, at least one first functional group attached to the therapeutic protein, wherein the first functional group is an aminooxy group, and a second functional group attached to the fatty acid or fatty acid ester, wherein the second functional group is an aminooxy group.

[0010b] In a second aspect of the present disclosure, there is provided a method of preparing a fatty acid derivative as defined above comprising:

a) oxidizing an ω-hydroxy group on a fatty acid to generate an aldehyde group on the fatty acid; and

b) coupling a water soluble linker comprising an active aminooxy group to the aldehyde group to form a stable oxime linkage;

wherein said fatty acid derivative is water soluble;

wherein the ω-hydroxy group is oxidized by an oxidation reagent selected from the group consisting of: Dess Martin periodinane reagent, Tempo reagent, oxalyl chloride/DMSO, tetrapropylammoniumperruthenate (TPAP) and crome VI reagents (Collins reagent, pyridinium chloro chromate (PCC) and pyridinium dichromate);

wherein the fatty acid is a saturated fatty acid or unsaturated fatty acid.

[0010c] In a third aspect of the present disclosure, there is provided a method of preparing a fatty acid derivative as defined above comprising:

a) esterifying a carboxyl group on a fatty acid to generate an ester on the fatty acid;

b) activating an ω-hydroxy group on a fatty acid to generate a mesyl group on the fatty acid of step a); and

c) coupling a water soluble linker comprising an active aminooxy group by substituting the mesyl group of step b) thereby forming a stable oxyimine-methylene bond;

wherein said fatty acid derivative is water soluble;

wherein the carboxyl group is esterified by an esterifying agent selected from the group consisting of: acetyl chloride, methanol in the presence of acid, ethanol in the presence of acid, diazomethane, and methyliodide;

wherein the ω-hydroxy group is activated by an activating agent selected from the group consisting of: mesyl chloride, tosyl chloride and nosyl chloride; and

wherein the fatty acid is a saturated fatty acid or unsaturated fatty acid.

[0010d] In a fourth aspect of the present disclosure, there is provided a method of preparing a conjugated therapeutic protein comprising contacting an oxidized carbohydrate moiety on the therapeutic protein with a fatty acid derivative as defined above under conditions that allow conjugation;

said carbohydrate moiety oxidized by incubation with a buffer comprising an oxidizing agent selected from the group consisting of sodium periodate (NaIO4), lead tetraacetate (Pb(OAc)4) and potassium perruthenate (KRuO4);

wherein an oxime linkage is formed between the oxidized carbohydrate moiety and the active aminooxy group on the fatty acid derivative;

and wherein said oxime linkage formation is catalyzed by a nucleophilic catalyst selected from the group consisting of aniline, o-amino benzoic acid, m-amino benzoic acid, p-amino benzoic acid, sulfanilic acid, o-aminobenzamide, o-toluidine, m-toluidine, p-toluidine, o-anisidine, m-anisidine, and p-anisidine.

[0011] In one embodiment of the present disclosure, a water soluble fatty acid derivative is provided comprising a fatty acid or fatty acid ester attached to a water soluble linker, said fatty acid derivative stably attached to a therapeutic protein. In another embodiment, the fatty acid derivative binds human serum albumin (HSA) in vitro or in vivo. In still another embodiment, the fatty acid derivative – therapeutic protein conjugate has increased half-life relative to a native therapeutic protein. In yet another embodiment, an aforementioned fatty acid derivative comprises a saturated fatty acid or unsaturated fatty acid. In a related embodiment, the fatty acid is a saturated fatty acid. In yet another embodiment, the fatty acid is a branched chain fatty acid.

[0012] Various lengths of fatty acids in the fatty acid derivatives are contemplated. In one embodiment, an aforementioned fatty acid derivative is provided wherein the fatty acid comprises a chain length between C10 and C24, including synthetic fatty acids with odd carbon numbers. In one embodiment, an aforementioned fatty acid derivative is provided wherein the fatty acid comprises a chain length selected from the group consisting of: C10, C12, C14, C16, C18, C20, C20, C22 and C24. In another embodiment, the fatty acid has a chain length selected from the group consisting of C14, C16 and C18. In still another the fatty acid has a chain length selected from the group consisting of C13, C15 and C17.

[0013] In still another embodiment, an aforementioned fatty acid derivative is provided wherein the fatty acid is attached to the water soluble linker at a group on the fatty acid selected from the group consisting of: terminal carboxyl group and ω group. In another embodiment, the fatty acid is attached to the water soluble linker at the ω group. In still another embodiment, the ω group is selected from the group consisting of: hydroxyl, amino, thio, and carboxyl.

[0014] In one embodiment of the present disclosure, an aforementioned fatty acid derivative is provided wherein the fatty acid is 16-hydroxyhexadecanoic acid.

[0015] In another embodiment, a fatty acid derivative is provided wherein the fatty acid ester is selected from the group consisting of: methyl ester and ethyl ester. In one embodiment, the fatty acid ester is 16-hydroxyhexadecanoic acid methyl ester.

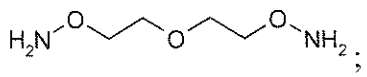
[0016] Various water soluble linkers are contemplated in the present disclosure. In one embodiment, an aforementioned fatty acid derivative is provided wherein the water soluble linker comprises a water soluble polymer and at least one functional group attached to the therapeutic protein. In one embodiment, the functional group attached to the therapeutic

protein has the ability to impart a negative or positive charge, thereby making the linker water soluble. In still another embodiment, the functional group is selected from the group consisting of a sulfo group, carboxyl group, hydroxyl group, amino group, amido group, maleimido group, aminooxy group and hydrazide group. In one embodiment, the functional group is an aminooxy group.

[0017] Numerous water soluble polymers are contemplated in the present disclosure. In one embodiment, an aforementioned fatty acid derivative is provided wherein the water soluble polymer, which is integral part of the linker, is selected from the group consisting of: polyethylene glycol (PEG), branched PEG, polysialic acid (PSA), hydroxyalkyl starch (HAS), hydroxyethyl starch (HES), carbohydrate, polysaccharides, pullulane, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, starch, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG), polyoxazoline, polyacryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate, polyvinylpyrrolidone, polyphosphazene, polyoxazoline, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, poly(1-hydroxymethylethylene hydroxymethylformal) (PHF), and 2-methacryloyloxy-2'-ethyltrimethylammoniumphosphate (MPC). In still another embodiment, the water soluble polymer is PEG.
Various lengths of water soluble polymers are also contemplated herein. In one embodiment, a fatty acid derivative is provided wherein the water soluble polymer comprises a chain length selected from the group consisting of O3, O5, O7, O9, O11, O13 and O15.

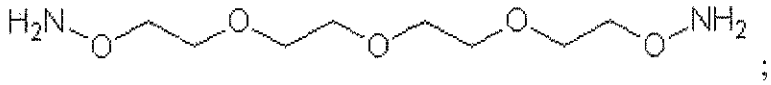
[0018] In one embodiment, a fatty acid derivative is provided wherein the water soluble linker is selected from the group consisting of:

3-oxapentane-1,5-dioxyamine



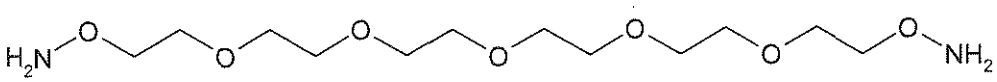
;

3,6,9-triaoxaundecane-1,11-dioxyamine



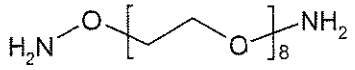
;

3,6,9,12,15-penatoxaheptadecane-1,17-dioxyamine;



; and

3,6,9,12,15,18,21-heptaoxatricosane-1,23-dioxyamine

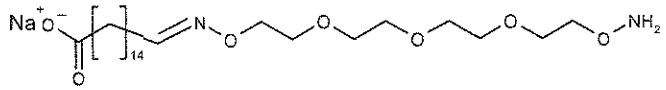


[0019] In another embodiment of the present disclosure, a fatty acid derivative is provided wherein the fatty acid derivative is stably attached to the therapeutic protein by an oxime linkage. In another embodiment, the oxime linkage is formed between an oxime group on the water soluble linker and an aldehyde group of an oxidized carbohydrate on the therapeutic protein.

[0020] In yet another embodiment of the present disclosure, a fatty acid derivative is provided wherein the fatty acid derivative is stably attached to the therapeutic protein by a maleimide group on the water soluble linker to a free sulfhydryl group on the therapeutic protein. In still another embodiment, the fatty acid derivative is stably attached to the therapeutic protein by an N-hydroxysuccinimide ester on the water soluble linker to a free amino group on the therapeutic protein.

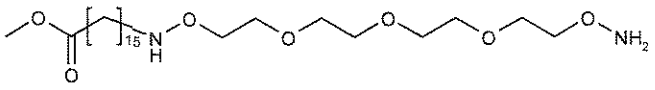
[0021] In one embodiment of the present disclosure, a fatty acid derivative is provided wherein the fatty acid derivative is selected from the group consisting of:

a) 16-(2-(2-(2-(2-Aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyimino)-hexadecanoic acid sodium salt of the formula:



; and

b) 16-(2-(2-(2-(2-Aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexadecanoic acid methyl ester,



[0022] Various therapeutic proteins are contemplated in the present disclosure. In one embodiment, an aforementioned fatty acid derivative is provided wherein the therapeutic protein is selected from the group consisting of: Factor IX (FIX), Factor VIII (FVIII), Factor VIIa (FVIIa), von Willebrand Factor (VWF), Factor FV (FV), Factor X (FX), Factor XI (FXI), Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF), ADAMTS 13 protease, IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, colony stimulating factor-1 (CSF-1), M-CSF, SCF, GM-CSF, granulocyte colony stimulating factor (G-CSF), EPO, interferon-alpha (IFN-alpha), consensus interferon, IFN-beta, IFN-gamma, IFN-omega, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-31, IL-32 alpha, IL-33, thrombopoietin (TPO), Ang-1, Ang-2, Ang-4, Ang-Y, angiopoietin-like polypeptide 1 (ANGPTL1), angiopoietin-like polypeptide 2 (ANGPTL2), angiopoietin-like polypeptide 3 (ANGPTL3), angiopoietin-like polypeptide 4 (ANGPTL4), angiopoietin-like polypeptide 5 (ANGPTL5), angiopoietin-like polypeptide 6 (ANGPTL6), angiopoietin-like polypeptide 7 (ANGPTL7), vitronectin, vascular endothelial growth factor (VEGF), angiogenin, activin A, activin B, activin C, bone morphogenic protein-1, bone morphogenic protein-2, bone morphogenic protein-3, bone morphogenic protein-4, bone morphogenic protein-5, bone morphogenic protein-6, bone morphogenic protein-7, bone morphogenic protein-8, bone morphogenic protein-9, bone morphogenic protein-10, bone morphogenic protein-11, bone morphogenic protein-12, bone morphogenic protein-13, bone morphogenic protein-14, bone morphogenic protein-15, bone morphogenic protein receptor IA, bone morphogenic protein receptor IB, bone morphogenic protein receptor II, brain derived neurotrophic factor, cardiotrophin-1, ciliary neutrophic factor, ciliary neutrophic factor receptor, cripto, cryptic, cytokine-induced neutrophil chemotactic factor 1, cytokine-induced neutrophil, chemotactic factor 2α, cytokine-induced neutrophil chemotactic factor 2β, β endothelial cell growth factor, endothelin 1, epidermal growth factor, epigen, epiregulin, epithelial-derived neutrophil attractant, fibroblast growth factor 4, fibroblast growth factor 5, fibroblast growth factor 6, fibroblast growth factor 7, fibroblast growth factor 8, fibroblast growth factor 8b, fibroblast growth factor 8c, fibroblast growth factor 9, fibroblast growth factor 10, fibroblast growth factor 11, fibroblast growth factor 12, fibroblast growth factor 13, fibroblast growth factor 16, fibroblast growth factor 17, fibroblast growth factor 19, fibroblast growth factor 20, fibroblast growth factor 21, fibroblast growth factor acidic, fibroblast growth factor basic, glial cell line-derived neutrophic factor receptor α1, glial cell line-derived neutrophic factor receptor α2, growth related protein, growth related protein α, growth related protein β, growth related protein γ, heparin binding

epidermal growth factor, hepatocyte growth factor, hepatocyte growth factor receptor, hepatoma-derived growth factor, insulin-like growth factor I, insulin-like growth factor receptor, insulin-like growth factor II, insulin-like growth factor binding protein, keratinocyte growth factor, leukemia inhibitory factor, leukemia inhibitory factor receptor α, nerve growth factor nerve growth factor receptor, neuropoietin,neurotrophin-3, neurotrophin-4, oncostatin M (OSM), placenta growth factor, placenta growth factor 2, platelet-derived endothelial cell growth factor, platelet derived growth factor, platelet derived growth factor A chain, platelet derived growth factor AA, platelet derived growth factor AB, platelet derived growth factor B chain, platelet derived growth factor BB, platelet derived growth factor receptor α, platelet derived growth factor receptor β, pre-B cell growth stimulating factor, stem cell factor (SCF), stem cell factor receptor, TNF, TNF0, TNF1, TNF2, transforming growth factor α, transforming growth factor β, transforming growth factor β1, transforming growth factor β1.2, transforming growth factor β2, transforming growth factor β3, transforming growth factor β5, latent transforming growth factor β1, transforming growth factor β binding protein I, transforming growth factor β binding protein II, transforming growth factor β binding protein III, thymic stromal lymphopoietin (TSLP), tumor necrosis factor receptor type I, tumor necrosis factor receptor type II, urokinase-type plasminogen activator receptor, phospholipase-activating protein (PUP), insulin, lectin ricin, prolactin, chorionic gonadotropin, follicle-stimulating hormone, thyroid-stimulating hormone, tissue plasminogen activator, IgG, IgE, IgM, IgA, and IgD, α-galactosidase, β-galactosidase, DNAse, fetuin, leutinizing hormone, estrogen, insulin, albumin, lipoproteins, fetoprotein, transferrin, thrombopoietin, urokinase, integrin, thrombin, leptin, Humira (adalimumab), Prolia (denosumab), Enbrel (etanercept), a protein in Table 1, or a biologically active fragment, derivative or variant thereof. In another embodiment, the therapeutic protein is FVIIa. In yet another embodiment, the therapeutic protein is FVIII. In still another embodiment, the therapeutic protein is FIX.

[0023] Methods of preparing fatty acid derivatives are also contemplated herein. In one embodiment, a method of preparing a fatty acid derivative described herein is provided comprising: a) oxidizing a ω-hydroxy group on a fatty acid to generate an aldehyde group on the fatty acid; and b) coupling a water soluble linker comprising an active aminooxy group to the aldehyde group to form a stable oxime linkage, wherein the fatty acid derivative is water soluble. In one embodiment, the aforementioned method is provided wherein the ω-hydroxy group is oxidized by an oxidation reagent selected from the group consisting of: Dess Martin

periodinane reagent, Tempo reagent, Swern oxidation with oxalyl chloride/DMSO, tetrapropylammoniumperruthenate (TPAP), chrome VI reagents such as Collins reagent, pyridinium chloro chromate (PCC), and pyridinium dichromate. In still another embodiment, the oxidation reagent is Dess Martin periodinane.

[0024] In another embodiment, an aforementioned method is provided wherein the fatty acid is a saturated fatty acid or unsaturated fatty acid. In still another embodiment, the fatty acid is a saturated fatty acid.

[0025] In yet another embodiment of the present disclosure, an aforementioned method is provided wherein the fatty acid is a branched chain fatty acid.

[0026] According to another embodiment, an aforementioned method is also provided wherein the fatty acid comprises a chain length between C10 and C24, including synthetic fatty acids with odd carbon numbers. In one embodiment, an aforementioned fatty acid derivative is provided wherein the fatty acid comprises a chain length selected from the group consisting of: C10, C12, C14, C16, C18, C20, C20, C22 and C24. In another embodiment, the fatty acid has a chain length selected from the group consisting of C14, C16 and C18. In still another the fatty acid has a chain length selected from the group consisting of C13, C15 and C17.

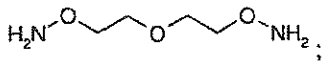
[0027] In still another embodiment, an aforementioned method is provided wherein the water soluble linker comprises a water soluble polymer and at least one aminooxy group.

[0028] Numerous water soluble polymers are contemplated in the present disclosure for use in an aforementioned method. In one embodiment, an aforementioned method is provided wherein the water soluble polymer is selected from the group consisting of: polyethylene glycol (PEG), branched PEG, polysialic acid (PSA), hydroxyalkyl starch (HAS), hydroxyethyl starch (HES), carbohydrate, polysaccharides, pullulane, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, starch, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG), polyoxazoline, polyacryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate, polyvinylpyrrolidone, polyphosphazene, polyoxazoline, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, poly(1-hydroxymethylethylene hydroxymethylformal) (PHF), and 2-methacryloyloxy-2'-ethyltrimethylammoniumphosphate (MPC). In one embodiment, the water soluble polymer is PEG.

[0029] Various lengths of water soluble polymers are also contemplated. In one embodiment, an aforementioned method is provided wherein the water soluble polymer comprises a chain length selected from the group consisting of O5, O7, O9, O11, O13 and O15.

[0030] In still another embodiment, an aforementioned method is provided wherein the water soluble linker is selected from the group consisting of:

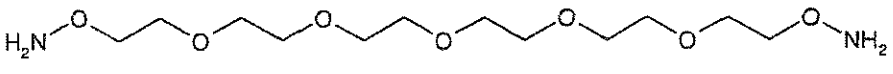
a) 3-oxapentane-1,5-dioxyamine of the formula:



b) 3,6,9-triaoxaundecane-1,11-dioxyamine of the formula:

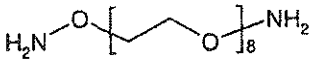


c) 3,6,9,12,15-penatoxaheptadecane-1,17-dioxyamine of the formula:



; and

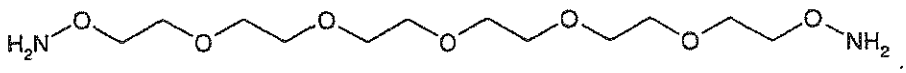
d) 3,6,9,12,15,18,21-heptaoxatricosane-1,23-dioxyamine of the formula:



[0031] In yet another embodiment, an aforementioned method is provided wherein the water soluble linker is 3,6,9-triaoxaundecane-1,11-dioxyamine of the formula:



[0032] In still another embodiment, an aforementioned method is provided wherein the water soluble linker is 3,6,9,12,15-penatoxaheptadecane-1,17-dioxyamine of the formula:



[0033] Still other methods for making the fatty acid derivatives are contemplated herein. In one embodiment, a method of preparing an aforementioned fatty acid derivative is provided comprising: a) esterifying a carboxyl group on a fatty acid to generate an ester on the fatty acid; b) activating a ω-hydroxy group on a fatty acid by introduction of a mesyl group on the fatty acid of step a); and c) coupling a water soluble linker comprising an active aminooxy group by substituting the mesyl group of step b) thereby forming a stable oxyamine-methylene bond; wherein the fatty acid derivative is water soluble.

[0034] In one embodiment, the aforementioned is provided wherein the carboxyl group is esterified by an esterifying agent selected from the group consisting of: acetyl chloride, methanol in the presence of acid, ethanol in the presence of acid, diazomethane, and methyliodide. In another embodiment, the esterifying agent is acetyl chloride.

[0035] In still another embodiment, the aforementioned is provided wherein the ω-hydroxy group is activated by an activating agent selected from the group consisting of: mesyl chloride, tosyl chloride and nosyl chloride. In one embodiment, the activating agent is mesyl chloride.

[0036] Various fatty acids are contemplated for use in the aforementioned method. In one embodiment, the aforementioned is provided wherein the fatty acid is a saturated fatty acid or unsaturated fatty acid. In another embodiment, the fatty acid is a saturated fatty acid. In yet another embodiment, the fatty acid is a branched chain fatty acid.

[0037] In still another embodiment, the aforementioned method is provided wherein the fatty acid comprises a chain length between C10 and C24, including synthetic fatty acids with odd carbon numbers. In one embodiment, an aforementioned fatty acid derivative is provided wherein the fatty acid comprises a chain length selected from the group consisting of: C10, C12, C14, C16, C18, C20, C20, C22 and C24. In another embodiment, the fatty acid has a chain length selected from the group consisting of C14, C16 and C18. In still another the fatty acid has a chain length selected from the group consisting of C13, C15 and C17.

[0038] Various water soluble polymers are also contemplated for the use in the aforementioned method. In one embodiment, the aforementioned method is provided wherein the water soluble linker comprises a water soluble polymer and at least one aminooxy group. In another embodiment, the water soluble polymer is selected from the group consisting of: polyethylene glycol (PEG), branched PEG, polysialic acid (PSA), hydroxyalkyl starch (HAS), hydroxyethyl starch (HES), carbohydrate, polysaccharides,

pullulane, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, starch, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG), polyoxazoline, polyacryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate, polyvinylpyrrolidone, polyphosphazene, polyoxazoline, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, poly(1-hydroxymethylethylene hydroxymethylformal) (PHF), and 2-methacryloyloxy-2'-ethyltrimethylammoniumphosphate (MPC). In another embodiment, the water soluble polymer is PEG.

[0039] In still another embodiment of the present disclosure, an aforementioned method is provided wherein the water soluble polymer comprises a chain length selected from the group consisting of O3, O5, O7, O9, O11, O13 and O15. In another embodiment, the water soluble linker is selected from the group consisting of:[0050]

[0040] Methods of preparing conjugated proteins are also contemplated in the present disclosure. In one embodiment, a method of preparing a conjugated therapeutic protein is provided comprising contacting an oxidized carbohydrate moiety on the therapeutic protein with an aforementioned fatty acid derivative (or a water soluble polymer as described herein) under conditions that allow conjugation; the carbohydrate moiety oxidized by incubation with a buffer comprising an oxidizing agent selected from the group consisting of sodium periodate (NaIO4), lead tetraacetate (Pb(OAc)4)and potassium perruthenate (KRuO4); wherein an oxime linkage is formed between the oxidized carbohydrate moiety and the active aminooxy group on the fatty acid derivative; and wherein the oxime linkage formation is catalyzed by a nucleophilic catalyst selected from the group consisting of o-amino benzoic acid, m-amino benzoic acid, p-amino benzoic acid, sulfanilic acid, o-aminobenzamide, o-toluidine, m-toluidine, p-toluidine, o-anisidine, m-anisidine, and p-anisidine.

[0041] In another embodiment, the aforementioned method is provided wherein the therapeutic protein is selected from the group consisting of: Factor IX (FIX), Factor VIII (FVIII), Factor VIIa (FVIIa), von Willebrand Factor (VWF), Factor FV (FV), Factor X (FX), Factor XI (FXI), Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF),ADAMTS 13 protease, IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, colony stimulating factor-1 (CSF-1), M-CSF, SCF, GM-CSF, granulocyte colony stimulating factor (G-CSF), EPO, interferon-alpha (IFN-alpha), consensus interferon, IFN-beta, IFN-gamma, IFN-omega, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-31, IL-32 alpha, IL-33, thrombopoietin (TPO),

Ang-1, Ang-2, Ang-4, Ang-Y, angiopoietin-like polypeptide 1 (ANGPTL1), angiopoietin-like polypeptide 2 (ANGPTL2), angiopoietin-like polypeptide 3 (ANGPTL3), angiopoietin-like polypeptide 4 (ANGPTL4), angiopoietin-like polypeptide 5 (ANGPTL5), angiopoietin-like polypeptide 6 (ANGPTL6), angiopoietin-like polypeptide 7 (ANGPTL7), vitronectin, vascular endothelial growth factor (VEGF), angiogenin, activin A, activin B, activin C, bone morphogenic protein-1, bone morphogenic protein-2, bone morphogenic protein-3, bone morphogenic protein-4, bone morphogenic protein-5, bone morphogenic protein-6, bone morphogenic protein-7, bone morphogenic protein-8, bone morphogenic protein-9, bone morphogenic protein-10, bone morphogenic protein-11, bone morphogenic protein-12, bone morphogenic protein-13, bone morphogenic protein-14, bone morphogenic protein-15, bone morphogenic protein receptor IA, bone morphogenic protein receptor IB, bone morphogenic protein receptor II, brain derived neurotrophic factor, cardiotrophin-1, ciliary neutrophic factor, ciliary neutrophic factor receptor, cripto, cryptic, cytokine-induced neutrophil chemotactic factor 1, cytokine-induced neutrophil, chemotactic factor 2α, cytokine-induced neutrophil chemotactic factor 2β, β endothelial cell growth factor, endothelin 1, epidermal growth factor, epigen, epiregulin, epithelial-derived neutrophil attractant, fibroblast growth factor 4, fibroblast growth factor 5, fibroblast growth factor 6, fibroblast growth factor 7, fibroblast growth factor 8, fibroblast growth factor 8b, fibroblast growth factor 8c, fibroblast growth factor 9, fibroblast growth factor 10, fibroblast growth factor 11, fibroblast growth factor 12, fibroblast growth factor 13, fibroblast growth factor 16, fibroblast growth factor 17, fibroblast growth factor 19, fibroblast growth factor 20, fibroblast growth factor 21, fibroblast growth factor acidic, fibroblast growth factor basic, glial cell line-derived neutrophic factor receptor α1, glial cell line-derived neutrophic factor receptor α2, growth related protein, growth related protein α, growth related protein β, growth related protein γ, heparin binding epidermal growth factor, hepatocyte growth factor, hepatocyte growth factor receptor, hepatoma-derived growth factor, insulin-like growth factor I, insulin-like growth factor receptor, insulin-like growth factor II, insulin-like growth factor binding protein, keratinocyte growth factor, leukemia inhibitory factor, leukemia inhibitory factor receptor α, nerve growth factor nerve growth factor receptor, neuropoietin,neurotrophin-3, neurotrophin-4, oncostatin M (OSM), placenta growth factor, placenta growth factor 2, platelet-derived endothelial cell growth factor, platelet derived growth factor, platelet derived growth factor A chain, platelet derived growth factor AA, platelet derived growth factor AB, platelet derived growth factor B chain, platelet derived growth factor BB, platelet derived growth factor receptor α, platelet derived growth factor receptor β, pre-B cell growth stimulating factor, stem cell factor (SCF),

stem cell factor receptor, TNF, TNF0, TNF1, TNF2, transforming growth factor α, transforming growth factor β, transforming growth factor β1, transforming growth factor β1.2, transforming growth factor β2, transforming growth factor β3, transforming growth factor β5, latent transforming growth factor β1, transforming growth factor β binding protein I, transforming growth factor β binding protein II, transforming growth factor β binding protein III, thymic stromal lymphopoietin (TSLP), tumor necrosis factor receptor type I, tumor necrosis factor receptor type II, urokinase-type plasminogen activator receptor, phospholipase-activating protein (PUP), insulin, lectin ricin, prolactin, chorionic gonadotropin, follicle-stimulating hormone, thyroid-stimulating hormone, tissue plasminogen activator, IgG, IgE, IgM, IgA, and IgD, α-galactosidase, β-galactosidase, DNAse, fetuin, leutinizing hormone, estrogen, insulin, albumin, lipoproteins, fetoprotein, transferrin, thrombopoietin, urokinase, integrin, thrombin, leptin, Humira (adalimumab), Prolia (denosumab), Enbrel (etanercept), a protein in Table 1, or a biologically active fragment, derivative or variant thereof.

[0042] In another embodiment, the aforementioned method is provided wherein the therapeutic protein is FVIIa. In still yet another embodiment, the aforementioned method is provided wherein the therapeutic protein is FVIII. In yet another embodiment, the aforementioned method is provided wherein the therapeutic protein is FIX.

[0043] In still another embodiment, the aforementioned method is provided wherein the oxidizing agent is sodium periodate (NaIO4). In another embodiment, the aforementioned method is provided wherein the nucleophilic catalyst is m-toluidine.

[0044] In yet another embodiment, the aforementioned method is provided further comprising purifying the conjugated therapeutic protein.

[0045] In still another embodiment, the aforementioned method is provided wherein the fatty acid derivative is prepared by a method as described herein.

[0046] Still other methods of preparing fatty acid derivatives are contemplated in the present invention. In one embodiment, a method of preparing an aforementioned fatty acid derivative is provided comprising: a) esterifying a carboxyl group on a fatty acid to generate an ester on the fatty acid; and b) coupling a water soluble linker comprising an active maleimide group to a free sulfhydryl (SH) group, thereby forming a stable thioether bond; wherein the fatty acid derivative is water soluble.

[0047] In still another embodiment, a method of preparing an aforementioned fatty acid derivative is provided comprising: a) esterifying a carboxyl group on a fatty acid to generate fatty acid ester; b) reacting the fatty acid resulting from step a) with an azide reagent thereby producing a corresponding fatty acid azide; c) hydrogenating the fatty acid azide of step b) to produce a corresponding fatty acid amine; and d) coupling a water soluble linker comprising an active NHS group to a free amine group, thereby forming a stable bond; wherein the fatty acid derivative is water soluble.

FIGURES

[0048] **Figure 1** shows the synthesis of the water soluble linker 3-oxapentane-1,5-dioxyamine.

DETAILED DESCRIPTION OF THE INVENTION

[0049] The pharmacological and immunological properties of therapeutic proteins can be improved by chemical modification and conjugation with polymeric compounds such as fatty acids and fatty acid derivatives according to the present invention.

[0050] The addition of a water soluble fatty acid derivative as described herein is one approach to improve the properties of therapeutic proteins such as the blood coagulation proteins Factor IX (FIX), Factor VIII (FVIII), Factor VIIa (FVIIa), von Willebrand Factor (VWF), Factor FV (FV), Factor X (FX), Factor XI (FXI), Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF) or ADAMTS 13 protease, as well as other known proteins or biologically/therapeutically active fragments thereof.

THERAPEUTIC PROTEINS

[0051] In certain embodiments of the invention, the aforementioned polypeptides and polynucleotides are exemplified by the following therapeutic proteins: enzymes, antigens, antibodies, receptors, blood coagulation proteins, growth factors, hormones, and ligands. In certain embodiments, the therapeutic protein is a blood coagulation protein such as Factor IX (FIX), Factor VIII (FVIII), Factor VIIa (FVIIa), von Willebrand Factor (VWF), Factor FV (FV), Factor X (FX), Factor XI (FXI), Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF) or ADAMTS 13 protease.

[0052] In certain embodiments, the therapeutic protein is immunoglobulins, cytokines such IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, colony stimulating factor-1 (CSF-1), M-CSF, SCF, GM-CSF, granulocyte colony stimulating factor (G-CSF), EPO, interferon-alpha (IFN-alpha), consensus interferon, IFN-beta, IFN-gamma, IFN-omega, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-31, IL-32 alpha, IL-33, thrombopoietin (TPO), angiopoietins, for example Ang-1, Ang-2, Ang-4, Ang-Y, the human angiopoietin-like polypeptides ANGPTL1 through 7, vitronectin, vascular endothelial growth factor (VEGF), angiogenin, activin A, activin B, activin C, bone morphogenic protein-1, bone morphogenic protein-2, bone morphogenic protein-3, bone morphogenic protein-4, bone morphogenic protein-5, bone morphogenic protein-6, bone morphogenic protein-7, bone morphogenic protein-8, bone morphogenic protein-9, bone morphogenic protein-10, bone morphogenic protein-11, bone morphogenic protein-12, bone morphogenic protein-13, bone morphogenic protein-14, bone morphogenic protein-15, bone morphogenic protein receptor IA, bone morphogenic protein receptor IB, bone morphogenic protein receptor II, brain derived neurotrophic factor, cardiotrophin-1, ciliary neutrophic factor, ciliary neutrophic factor receptor, cripto, cryptic, cytokine-induced neutrophil chemotactic factor 1, cytokine-induced neutrophil, chemotactic factor 2α, cytokine-induced neutrophil chemotactic factor 2β, β endothelial cell growth factor, endothelin 1, epidermal growth factor, epigen, epiregulin, epithelial-derived neutrophil attractant, fibroblast growth factor 4, fibroblast growth factor 5, fibroblast growth factor 6, fibroblast growth factor 7, fibroblast growth factor 8, fibroblast growth factor 8b, fibroblast growth factor 8c, fibroblast growth factor 9, fibroblast growth factor 10, fibroblast growth factor 11, fibroblast growth factor 12, fibroblast growth factor 13, fibroblast growth factor 16, fibroblast growth factor 17, fibroblast growth factor 19, fibroblast growth factor 20, fibroblast growth factor 21, fibroblast growth factor acidic, fibroblast growth factor basic, glial cell line-derived neutrophic factor receptor α1, glial cell line-derived neutrophic factor receptor α2, growth related protein, growth related protein α, growth related protein β, growth related protein γ, heparin binding epidermal growth factor, hepatocyte growth factor, hepatocyte growth factor receptor, hepatoma-derived growth factor, insulin-like growth factor I, insulin-like growth factor receptor, insulin-like growth factor II, insulin-like growth factor binding protein, keratinocyte growth factor, leukemia inhibitory factor, leukemia inhibitory factor receptor α, nerve growth factor nerve growth factor receptor, neuropoietin,neurotrophin-3, neurotrophin-4, oncostatin M (OSM), placenta growth factor, placenta growth factor 2, platelet-derived endothelial cell growth factor, platelet derived growth factor, platelet derived

growth factor A chain, platelet derived growth factor AA, platelet derived growth factor AB, platelet derived growth factor B chain, platelet derived growth factor BB, platelet derived growth factor receptor α, platelet derived growth factor receptor β, pre-B cell growth stimulating factor, stem cell factor (SCF), stem cell factor receptor, TNF, including TNF0, TNF1, TNF2, transforming growth factor α, transforming growth factor β, transforming growth factor β1, transforming growth factor β1.2, transforming growth factor β2, transforming growth factor β3, transforming growth factor β5, latent transforming growth factor β1, transforming growth factor β binding protein I, transforming growth factor β binding protein II, transforming growth factor β binding protein III, thymic stromal lymphopoietin (TSLP), tumor necrosis factor receptor type I, tumor necrosis factor receptor type II, urokinase-type plasminogen activator receptor, vascular endothelial growth factor, and chimeric proteins and biologically or immunologically active fragments thereof.

[0053] In certain embodiments, the therapeutic protein is alpha-, beta-, and gamma-interferons, colony stimulating factors including granulocyte colony stimulating factors, fibroblast growth factors, platelet derived growth factors, phospholipase-activating protein (PUP), insulin, plant proteins such as lectins and ricins, tumor necrosis factors and related alleles, soluble forms of tumor necrosis factor receptors, interleukin receptors and soluble forms of interleukin receptors, growth factors such as tissue growth factors, such as TGFαs or TGFβs and epidermal growth factors, hormones, somatomedins, pigmentary hormones, hypothalamic releasing factors, antidiuretic hormones, prolactin, chorionic gonadotropin, follicle-stimulating hormone, thyroid-stimulating hormone, tissue plasminogen activator, and immunoglobulins such as IgG, IgE, IgM, IgA, and IgD, a galactosidase, α-galactosidase, β-galactosidase, DNAse, fetuin, leutinizing hormone, estrogen, corticosteroids, insulin, albumin, lipoproteins, fetoprotein, transferrin, thrombopoietin, urokinase, DNase, integrins, thrombin, hematopoietic growth actors, leptin, glycosidases, Humira (adalimumab), Prolia (denosumab), Enbrel (etanercept), and fragments thereof, or any fusion proteins comprising any of the above mentioned proteins or fragments thereof. In addition to the aforementioned proteins, the following Table 1 provides therapeutic proteins contemplated by the present invention:

Table 1

Follicular dendritic cell secreted peptide	Angiotensin-converting enzyme	Interleukin-1 family member 6	Herstatin
Dermokine	Antithrombin-III	Prostate and testis expressed protein 2	Leucine-rich repeat-containing protein 28
Secreted frizzled-related protein 1	Apolipoprotein B-100	Group XIIA secretory phospholipase A2	LRRN4 C-terminal-like protein
Ectodysplasin-A	Apolipoprotein D	Collagen alpha-3(V) chain	Ly6/PLAUR domain-containing protein 2
Secreted frizzled-related protein 2	Apolipoprotein E	Alpha-2-macroglobulin-like protein 1	Transmembrane protein 81
Resistin	Beta-1,4-galactosyltransferase 1	Dermatopontin	Myelin protein zero-like protein 3
Osteopontin	Bone morphogenetic protein 7	Cartilage-associated protein	Protein notum homolog
Secreted frizzled-related protein 5	Complement C1q subcomponent subunit B	Desert hedgehog protein	UDP-glucuronosyltransferase 3A2
Secreted frizzled-related protein 4	C4b-binding protein alpha chain	Extracellular matrix protein 2	Protocadherin alpha-1
Secreted phosphoprotein 24	Calreticulin	Gastric intrinsic factor	Phospholipase D4
Glypican-6	Corticosteroid-binding globulin	Interleukin-33	Retinol dehydrogenase 10
Secreted frizzled-related protein 3	Carboxypeptidase A1	Bone morphogenetic protein 2	Sialic acid-binding Ig-like lectin 14
C-C motif chemokine 4	Carboxypeptidase A2	Bone morphogenetic protein 6	Transmembrane protein 161A
Melanocyte protein Pmel 17	Eotaxin	Uncharacterized protein KIAA0564	Transmembrane protein 161B
Secreted Ly-6/uPAR-related protein 1	C-C motif chemokine 13	Cerberus	Transmembrane protein 182
Beta-microseminoprotein	C-C motif chemokine 18	Carbohydrate sulfotransferase 8	Protein FAM24B
Glypican-4	C-C motif chemokine 20	Contactin-associated protein-like 3	Transmembrane protein 52
Tumor necrosis factor ligand superfamily member 15	Triggering receptor expressed on myeloid cells 2	Group XIIB secretory phospholipase A2-like protein	Major facilitator superfamily domain-containing protein 4
Resistin-like beta	C-C motif chemokine 2	Corticoliberin	UDP-glucuronosyltransferase 2A3
Tumor necrosis factor ligand superfamily member 12	Transforming growth factor-beta-induced protein ig-h3	A disintegrin and metalloproteinase with thrombospondin motifs 19	Odontogenic ameloblast-associated protein
SPARC	CD40 ligand	UPF0556 protein C19orf10	Neurosecretory protein VGF
Glypican-5	Corneodesmosin	C-X-C motif chemokine 3	Secreted phosphoprotein 2, 24kDa
Anterior gradient protein 2 homolog	Complement factor D	Cystatin-M	Protein FAM150B
Protein canopy homolog 2	Chromogranin-A	Defensin-5	Growth/differentiation factor 9
Glypican-1	Collagen alpha-1(I) chain	Defensin-6	Clusterin-like protein 1
von Willebrand factor A domain-containing protein 2	Disintegrin and metalloproteinase domain-containing protein 18	A disintegrin and metalloproteinase with thrombospondin motifs 18	Transmembrane and immunoglobulin domain-containing protein 2
WNT1-inducible-signaling pathway protein 1	Cysteine-rich secretory protein LCCL domain-containing 1	A disintegrin and metalloproteinase with thrombospondin motifs 3	C-type lectin domain-containing protein UNQ5810/PRO19627
C-C motif chemokine 1	Collagen alpha-4(IV) chain	Dickkopf-related protein 4	Epididymal-specific lipocalin-10
SPARC-related modular calcium-binding protein 2	Keratinocyte differentiation-associated protein	A disintegrin and metalloproteinase with thrombospondin motifs 5	A disintegrin and metalloproteinase with thrombospondin motifs 8
C-type lectin domain family 11 member A	Complement C4-B	Mammalian ependymin-related protein 1	Epididymal-specific lipocalin-8
Secreted Ly-6/uPAR-related protein 2	Collagen alpha-2(V) chain	Fibrillin-3	Basic proline-rich peptide P-E
Glypican-3	Complement C5	Fetuin-B	Putative uncharacterized protein C10orf99
Secreted and transmembrane protein 1	Collagen alpha-1(VII) chain	Fibroblast growth factor 6	Uncharacterized protein C17orf77
Testis-expressed sequence 264 protein	Complement component C7	Keratinocyte growth factor	Arylacetamide deacetylase-like 2

Glypican-2	Complement component C8 beta chain	Growth/differentiation factor 8	Epididymal-specific lipocalin-12
Serine protease 23	Complement component C8 gamma chain	Gastric inhibitory polypeptide	B melanoma antigen 2
39S ribosomal protein L55, mitochondrial	Collagen alpha-1(XV) chain	Glycoprotein hormone beta-5	B melanoma antigen 3
Protein NipSnap homolog 3A	Collagen alpha-1(XVI) chain	Granzyme M	Bovine seminal plasma protein homolog 1
Fibronectin	Collagen alpha-1(XVIII) chain	Gastrin-releasing peptide	Complement C1q-like protein 3
Neudesin	Collagen alpha-1(XIX) chain	Serine protease HTRA1	UPF0565 protein C2orf69
Fibroblast growth factor receptor 2	Cartilage oligomeric matrix protein	Interferon alpha-4	UPF0669 protein C6orf120
Carbonic anhydrase 6	C-reactive protein	Interferon alpha-5	Colipase-like protein C6orf127
Deleted in malignant brain tumors 1 protein	Granulocyte colony-stimulating factor	Interferon alpha-7	Uncharacterized protein C7orf69
SPARC-related modular calcium-binding protein 1	Granulocyte-macrophage colony-stimulating factor	A disintegrin and metalloproteinase with thrombospondin motifs 7	Platelet-derived growth factor receptor-like protein
Amyloid beta A4 protein	Protein CYR61	Immunoglobulin superfamily member 10	Chondroadherin-like protein
Tumor necrosis factor receptor superfamily member 6	Complement component receptor 1-like protein	Protease-associated domain-containing protein of 21 kDa	Putative uncharacterized protein UNQ6490/PRO21339
Gamma-aminobutyric acid type B receptor subunit 1	Stem cell growth factor; lymphocyte secreted C-type lectin	Abhydrolase domain-containing protein FAM108A1	Putative uncharacterized protein UNQ6493/PRO21345
Pro-neuregulin-1, membrane-bound isoform	CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase	A disintegrin and metalloproteinase with thrombospondin motifs 9	Putative uncharacterized protein UNQ5815/PRO19632
Glycoprotein hormone alpha-2	Dipeptidyl peptidase 4	Interleukin-9 receptor	Cystatin-A
Membrane metallo-endopeptidase-like 1	Dentin sialophosphoprotein	Interleukin-9	Peptidase inhibitor R3HDML
Fc receptor-like A	Endothelin-1	Inhibin beta B chain	Cystatin-9
C-C motif chemokine 4-like	Ephrin-B1	Serine protease inhibitor Kazal-type 2	DAN domain family member 5
Epithelial discoidin domain-containing receptor 1	Epidermis-specific serine protease-like protein	BMP-binding endothelial regulator protein	Insulin-like growth factor-binding protein-like 1
Mucin-1	EMILIN-1	Keratinocyte-associated protein 2	Epididymal sperm-binding protein 1
Vascular endothelial growth factor A	Endoplasmin	Laminin subunit alpha-1	Elafin
Fibulin-1	Ephrin type-A receptor 3	Leukocyte cell-derived chemotaxin-2	Protein FAM55A
Prolactin receptor	Ephrin type-B receptor 6	Gastric triacylglycerol lipase	Growth/differentiation factor 6
Proprotein convertase subtilisin/kexin type 6	Glycosyltransferase 1 domain-containing protein 1	Leucine-rich repeat and calponin homology domain-containing protein 3	Glucose-fructose oxidoreductase domain-containing protein 1
CD209 antigen	Coagulation factor X	Pancreatic lipase-related protein 2	Erythropoietin
Collagen alpha-2(XI) chain	Coagulation factor VIII	Epididymis-specific alpha-mannosidase	Glutathione peroxidase 6
Granulocyte-macrophage colony-stimulating factor receptor subunit alpha	Complement C1q tumor necrosis factor-related protein 7	Fibronectin type III domain-containing protein 7	Uncharacterized protein UNQ511/PRO1026
Elastin	Fibrillin-2	Microfibrillar-associated protein 5	Beta-defensin 128
Interleukin-15 receptor subunit alpha	Alpha-2-HS-glycoprotein	Muellerian-inhibiting factor	Interleukin-31
Midkine	Fibroblast growth factor 10	Matrix metalloproteinase-21	Interleukin-34
Integrin alpha-7	Fibrinogen alpha chain	Matrix metalloproteinase-17	Plasma kallikrein-like protein 4
Mucin-4	Fibrinogen beta chain	Matrix metalloproteinase-20	Epididymal-specific lipocalin-9
Peptidyl-glycine alpha-amidating monooxygenase	Long palate, lung and nasal epithelium carcinoma-associated protein 1	N-acetylglucosamine-1-phosphotransferase subunit gamma	cDNA FLJ60957, highly similar to Secreted frizzled-related protein 4

Apolipoprotein A-I	Gastrin	Multimerin-2	Lipase member M
Proteoglycan 4	Glycoprotein hormones alpha chain	Promotilin	CLECSF12
Tumor necrosis factor receptor superfamily member 25	N-acetylglucosamine-1-phosphotransferase subunits alpha/beta	FRAS1-related extracellular matrix protein 3	Putative inactive group IIC secretory phospholipase A2
Attractin	Granzyme A	Protein kinase C-binding protein NELL1	Serine protease MPN2
Prostate-associated microseminoprotein	Hepatocyte growth factor-like protein	Protein kinase C-binding protein NELL2	Netrin-5
Alpha-amylase 1	Insulin-like growth factor-binding protein 1	Neurotrypsin	NHL repeat-containing protein 3
Brain-derived neurotrophic factor	Insulin-like growth factor-binding protein 2	Neuroserpin	Olfactomedin-like protein 2B
C-type lectin domain family 4 member M	Insulin-like growth factor-binding protein 4	Nidogen-2	Ovochymase-2
Granulocyte colony-stimulating factor receptor	Tumor necrosis factor receptor superfamily member 10D	Abhydrolase domain-containing protein FAM108B1	Putative uncharacterized protein UNQ3029/PRO9830
Insulin-like growth factor II	Interferon alpha-1/13	Neurotrophin-4	Ovochymase-1
Carcinoembryonic antigen-related cell adhesion molecule 1	Interferon-induced helicase C domain-containing protein 1	Epididymal secretory glutathione peroxidase	Putative pregnancy-specific beta-1-glycoprotein 7
C-type lectin domain family 7 member A	Interferon alpha-2	Group 10 secretory phospholipase A2	Ovostatin homolog 2
CMRF35-like molecule 1	Interferon beta	Group IID secretory phospholipase A2	Orexigenic neuropeptide QRFP
Choline transporter-like protein 4	Interferon gamma	Lactoperoxidase	Lymphocyte antigen 6K
Pulmonary surfactant-associated protein A1	Insulin-like growth factor IB	p53 apoptosis effector related to PMP-22	Prostate and testis expressed protein 1
Spermine oxidase	Indian hedgehog protein	Placenta-specific protein 1	Putative phospholipase B-like 1
CMP-N-acetylneuraminate-beta-1,4-galactoside alpha-2,3-sialyltransferase	Neural cell adhesion molecule L1-like protein	Tuberoinfundibular peptide of 39 residues	Putative uncharacterized protein FLJ42147
Kallikrein-8	Interleukin-13	Prolargin	Otogelin
Tissue-type plasminogen activator	Interleukin-2	Secretogranin-2	Ribonuclease 8
Peroxisomal N(1)-acetyl-spermine/spermidine oxidase	Chymotrypsin-like elastase family member 2A	Endonuclease domain-containing 1 protein	Nuclear pore complex-interacting protein-like 2
Probable palmitoyltransferase ZDHHC4	Inhibin beta A chain	Semaphorin-3B	Proactivator polypeptide-like 1
Cholesteryl ester transfer protein	Pancreatic secretory trypsin inhibitor	Somatostatin	Protein spinster homolog 2
HLA class I histocompatibility antigen, A-2 alpha chain	Tumor necrosis factor receptor superfamily member 21	Dehydrogenase/reductase SDR family member 4-like 2	von Willebrand factor C domain-containing protein 2-like
Collagen alpha-1(II) chain	Inter-alpha-trypsin inhibitor heavy chain H1	Transcobalamin-1	Urotensin-2B
Pro-interleukin-16	Inter-alpha-trypsin inhibitor heavy chain H2	Trefoil factor 2	Tetraspanin-18
Leptin receptor	Inter-alpha-trypsin inhibitor heavy chain H3	Testican-1	UPF0514 membrane protein FAM159A
Decorin	Prostate-specific antigen	Serum paraoxonase/lactonase 3	Latherin
Stromal cell-derived factor 1	Kallikrein-4	Tolloid-like protein 2	Methyltransferase-like protein 7B
Tenascin	Plasma kallikrein	Trypsin-2	Protein TEX261
Disintegrin and metalloproteinase domain-containing protein 12	Calcium-activated chloride channel regulator 4	RING finger and SPRY domain-containing protein 1	Alkylated DNA repair protein alkB homolog 7
A disintegrin and metalloproteinase with thrombospondin motifs 13	Bactericidal/permeability-increasing protein-like 1	Calcium-binding and coiled-coil domain-containing protein 1	Transmembrane emp24 domain-containing protein 6
T-cell surface glycoprotein CD8 alpha chain	Leptin	Protein Wnt-2	XK-related protein 5
EGFR-coamplified and overexpressed	A disintegrin and metalloproteinase with	Ectonucleoside triphosphate	Putative V-set and immunoglobulin

WO 2012/087838 PCT/US2011/065591

protein	thrombospondin motifs 4	diphosphohydrolase 8	domain-containing protein 7
Autophagy-related protein 16-1	Hepatic triacylglycerol lipase	Protein Wnt-8b	Insulin growth factor-like family member 3
Breast cancer anti-estrogen resistance protein 3	Lymphocyte antigen 6 complex locus protein G6c	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 4	Nuclear pore complex-interacting protein-like 1
Cadherin-23	Eosinophil lysophospholipase	EMI domain-containing protein 1	Secreted phosphoprotein 1
Macrophage colony-stimulating factor 1	Lutropin subunit beta	Uncharacterized protein C6orf15	Collagen alpha-5(VI) chain
Folate receptor alpha	Microfibrillar-associated protein 1	Collectin-10	B melanoma antigen 5
Low-density lipoprotein receptor-related protein 8	Mesencephalic astrocyte-derived neurotrophic factor	Long-chain-fatty-acid--CoA ligase ACSBG2	WAP four-disulfide core domain protein 10A
E3 ubiquitin-protein ligase LRSAM1	Matrix Gla protein	Oncoprotein-induced transcript 3 protein	UPF0369 protein C6orf57
Neural cell adhesion molecule 1	72 kDa type IV collagenase	Peptidase inhibitor 15	Putative uncharacterized protein C10orf31
Neuroligin-4, X-linked	Stromelysin-1	Proline-rich acidic protein 1	Putative uncharacterized protein C11orf45
Netrin-G1	Neutrophil collagenase	Urocortin	Uncharacterized protein C12orf28
GPI transamidase component PIG-T	Mesothelin	Trypsin-X3 (EC 3.4.21.4)	Uncharacterized protein C17orf67
Kit ligand	Mucin-5AC	HHIP-like protein 2	Beta-defensin 121
Seizure 6-like protein	Mucin-6	Fractalkine	Beta-defensin 130
SLAM family member 7	Norrin	Protein Wnt-11	Histidine triad nucleotide-binding protein 2
Tumor necrosis factor	Oxytocin-neurophysin 1	Protein Wnt-7a	Apelin
Uromodulin	Beta-nerve growth factor	FCH and double SH3 domains protein 1	Placenta-specific protein 9
Tumor necrosis factor ligand superfamily member 13	Tumor necrosis factor ligand superfamily member 18	Hepatoma-derived growth factor-related protein 2	Hepatocellular carcinoma-associated protein TD26
Protein CREG1	Neurotrophin-3	Interleukin-12 subunit alpha	Persephin
EGF-like domain-containing protein 8	Platelet-derived growth factor subunit A	UPF0577 protein KIAA1324	Regulated endocrine-specific protein 18
Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1	Phosphopantothenoylcysteine decarboxylase	Complement C1q tumor necrosis factor-related protein 9	Complement C1q tumor necrosis factor-related protein 8
ADAMTS-like protein 4	Plasminogen activator inhibitor 1	Mucin-17	Bone morphogenetic protein 8A
Coagulation factor XI	Plasminogen activator inhibitor 2	Lysosomal protein NCU-G1	Protein WFDC13
Interleukin-22 receptor subunit alpha-2	Procollagen C-endopeptidase enhancer 1	Prolyl 4-hydroxylase subunit alpha-3	Protein Wnt-8a
Deformed epidermal autoregulatory factor 1 homolog	Transmembrane and ubiquitin-like domain-containing protein 2	Peptidyl-prolyl cis-trans isomerase SDCCAG10	Ig-like domain-containing protein ENSP00000270642
Prostaglandin-H2 D-isomerase	Protein disulfide-isomerase	Peptidase inhibitor 16	Abhydrolase domain-containing protein 15
Alpha-1-antitrypsin	Pigment epithelium-derived factor	Poliovirus receptor-related protein 4	Ribonuclease-like protein 9
Alpha-1-antichymotrypsin	Pepsin A	Solute carrier family 22 member 15	Uncharacterized protein C2orf66
Acyl-CoA-binding protein	Gastricsin	GPI inositol-deacylase	Uncharacterized protein C17orf99
Complement factor B	Sonic hedgehog protein	Transmembrane protein 43	Protein FAM150A
Choriogonadotropin subunit beta	Peptidoglycan recognition protein I-alpha	Angiopoietin-related protein 2	Placenta-specific 1-like protein
Versican core protein	Biglycan	Angiopoietin-related protein 6	Uncharacterized protein C18orf20
Epidermal growth factor receptor	Prolactin-inducible protein	Arylsulfatase K	Beta-defensin 110
Ecto-NOX disulfide-thiol exchanger 2	Platelet factor 4	Augurin	Neuritin-like protein
Hyaluronidase-1	Plasminogen	Brain-specific serine protease 4	Histidine-rich carboxyl terminus protein 1
Interleukin-1 receptor antagonist protein	Serum paraoxonase/arylesterase 1	DBH-like monooxygenase protein 1	C-type lectin domain family 2 member A

WO 2012/087838 PCT/US2011/065591

Interleukin-6 receptor subunit beta	Alkaline phosphatase, placental type	Uncharacterized protein C1orf56	Leucine-rich repeat-containing protein 70
Interleukin-1 receptor-like 1	Peptidyl-prolyl cis-trans isomerase B	Cerebellin-3	Serpin A13
Insulin	Bone marrow proteoglycan	Cerebellin-4	BTB/POZ domain-containing protein 17
Glycodelin	Basic salivary proline-rich protein 1	Colipase-like protein C6orf126	Uncharacterized protein C12orf53
Parathyroid hormone-related protein	Pulmonary surfactant-associated protein C	Uncharacterized protein C11orf83	C-type lectin domain family 9 member A
Nurim	Parathyroid hormone	Uncharacterized protein C16orf89	Complement C1q-like protein 4
Prolyl 4-hydroxylase subunit alpha-2	Serum amyloid P-component	Carboxypeptidase-like protein X2	CMRF35-like molecule 4
CD276 antigen	Secretogranin-1	Cystatin-9-like	Protein FAM151B
Cysteine-rich with EGF-like domain protein 1	Basement membrane-specific heparan sulfate proteoglycan core protein	Dehydrogenase/reductase SDR family member 13	Abhydrolase domain-containing protein FAM108A2/A3
CUB and sushi domain-containing protein 1	Antileukoproteinase	Beta-defensin 123	Osteocrin
Ficolin-2	Stabilin-1	Beta-defensin 132	Transmembrane protease, serine 11E2
Fc receptor-like protein 5	Extracellular superoxide dismutase [Cu-Zn]	Cytokine-like protein 1	Transmembrane protein 14E
Protein GPR89	Somatotropin	Dickkopf-related protein 2	Transmembrane protein 207
Junctional adhesion molecule A	Serpin B5	Dickkopf-like protein 1	TOMM20-like protein 1
Leucine-rich repeat-containing protein 8A	Spondin-1	Epididymal secretory protein E3-beta	Uncharacterized protein C3orf41
Multiple inositol polyphosphate phosphatase 1	Structural maintenance of chromosomes protein 3	EGF-like repeat and discoidin I-like domain-containing protein 3	Submaxillary gland androgen-regulated protein 3A
Neuropilin-1	Syntaxin-1A	Protein FAM55D	B melanoma antigen 1
Plexin-A4	Tetranectin	Fibroblast growth factor 17	Inactive carboxylesterase 4
Plexin-B1	Transforming growth factor beta-1	Fibroblast growth factor 22	Four-jointed box protein 1
Periostin	Thyroglobulin	Fibroblast growth factor-binding protein 2	Protein HSN2
Protein RIC-3	Metalloproteinase inhibitor 1	Growth/differentiation factor 3	Humanin
SLIT and NTRK-like protein 2	Metalloproteinase inhibitor 2	GLIPR1-like protein 1	Kielin/chordin-like protein
Sulfatase-modifying factor 1	Metalloproteinase inhibitor 3	Serine protease inhibitor Kazal-type 6	UPF0624 protein C6orf186
Sulfatase-modifying factor 2	Urokinase-type plasminogen activator	Interleukin-17B	Putative neurofibromin 1-like protein 4/6
Transmembrane protease, serine 6	Lactotransferrin	Interleukin-17C	Peroxidasin-like protein
Lymphotoxin-alpha	Trypsin-1	Interleukin-17D	SCO-spondin
Tumor necrosis factor receptor superfamily member 10B	Submaxillary gland androgen-regulated protein 3B	Hyaluronan and proteoglycan link protein 3	Putative uncharacterized protein UNQ9165/PRO28630
Urokinase plasminogen activator surface receptor	Tumor necrosis factor receptor superfamily member 1A	Vitelline membrane outer layer protein 1 homolog	Calcium-activated chloride channel regulator family member 3
V-set domain-containing T-cell activation inhibitor 1	Vascular endothelial growth factor receptor 1	Choriogonadotropin subunit beta variant 1	Probable serine protease UNQ9391/PRO34284
Glucagon	Vitamin D-binding protein	Lysozyme-like protein 1	Uncharacterized protein C4orf26
N-acetylmuramoyl-L-alanine amidase	Vitronectin	Matrix metalloproteinase-28	Uncharacterized protein C4orf40
Sulfhydryl oxidase 1	von Willebrand factor	Nephronectin	Uncharacterized protein C5orf55
Dehydrogenase/reductase SDR family member 4	Lymphocyte antigen 6 complex locus protein G5c	WAP four-disulfide core domain protein 12	Putative macrophage-stimulating protein MSTP9
Interleukin-18-binding protein	Zinc-alpha-2-glycoprotein	Olfactomedin-like protein 1	Uncharacterized protein C15orf61
Kin of IRRE-like protein 2	Uncharacterized protein C14orf93	Olfactomedin-like protein 2A	Chymotrypsinogen B2

WO 2012/087838 PCT/US2011/065591

Myeloid-associated differentiation marker	Retinoschisin	Serine protease 27	Beta-defensin 108A
Chordin	Alpha-1,3-mannosyltransferase ALG2	Secretoglobin family 3A member 2	Beta-defensin 111
1-acyl-sn-glycerol-3-phosphate acyltransferase gamma	C-type lectin domain family 11, member A, isoform CRA_b	A disintegrin and metalloproteinase with thrombospondin motifs 2	Putative V-set and immunoglobulin domain-containing protein 6
Advanced glycosylation end product-specific receptor	Major facilitator superfamily domain-containing protein 7	Disintegrin and metalloproteinase domain-containing protein 28	Serine protease inhibitor Kazal-type 5-like 3
NLR family CARD domain-containing protein 4	Leucine-rich repeat transmembrane neuronal protein 1	Bactericidal/permeability-increasing protein-like 2	Putative serine protease inhibitor Kazal-type 5-like 2
Pro-neuregulin-2, membrane-bound isoform	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11, mitochondrial	Acid sphingomyelinase-like phosphodiesterase 3b	Dehydrogenase/reductase SDR family member 7C
Sperm-associated antigen 11A	UPF0546 membrane protein C1orf91	Serine protease inhibitor Kazal-type 7	Beta-defensin 131
Oocyte-secreted protein 1 homolog	Carbonic anhydrase-related protein 10	Neurexophilin-4	Beta-defensin 134
Serum albumin	Cholecystokinin	Protein Wnt-9b	Beta-defensin 136
Cochlin	Codanin-1	Zymogen granule protein 16 homolog B	Beta-defensin 116
Plasma protease C1 inhibitor	Uncharacterized protein C6orf89	Semaphorin-3D	Protein FAM132A
Interleukin-7 receptor subunit alpha	Chondroitin sulfate glucuronyltransferase	Apolipoprotein L4	Protein FAM132B
Inter-alpha-trypsin inhibitor heavy chain H5	Chitinase domain-containing protein 1	Transmembrane protease, serine 11D	Beta-defensin 115
Platelet-derived growth factor D	Transmembrane protein C9orf7	Scrapie-responsive protein 1	Beta-defensin 114
Protein S100-A7	CMRF35-like molecule 9	Putative annexin A2-like protein	Serine protease inhibitor Kazal-type 9
Sialic acid-binding Ig-like lectin 10	Cytochrome P450 2S1	Bone morphogenetic protein 10	Lipase member N
Tubulointerstitial nephritis antigen-like	Crumbs protein homolog 3	Secretogranin-3	Pancreatic lipase-related protein 3
Tumor necrosis factor ligand superfamily member 13B	Dehydrogenase/reductase SDR family member 7	Complement C1q tumor necrosis factor-related protein 4	Testis, prostate and placenta-expressed protein
Long-chain-fatty-acid--CoA ligase 5	Protein ENED	Uncharacterized protein C1orf54	Neuromedin-S
Claudin-14	Complement factor H-related protein 4	Carboxypeptidase A6	Neuropeptide S
Leucine-rich repeat-containing protein 20	Leucine-rich repeat LGI family member 3	C-C motif chemokine 19	Neuronal pentraxin-like protein C16orf38
Interleukin-1 family member 7	Gliomedin	C-C motif chemokine 25	Otolin-1
Lymphocyte antigen 6 complex locus protein G5b	Glycerophosphodiester phosphodiesterase domain-containing protein 5	Chymotrypsin-like elastase family member 2B	Iron/zinc purple acid phosphatase-like protein
Acetylcholinesterase	Probable G-protein coupled receptor 113	Protein CEI	Ovostatin homolog 1
Amelogenin, X isoform	Probable G-protein coupled receptor 114	Uncharacterized protein C6orf1	Plasminogen-related protein A
Angiogenin	Glycerol-3-phosphate acyltransferase 4	Uncharacterized protein C7orf34	Polyserase-3
Anthrax toxin receptor 2	Gremlin-1	Keratinocyte-associated protein 3	Putative peptide YY-2
Annexin A2	Potassium channel subfamily K member 17	Uncharacterized protein C9orf47	Putative peptide YY-3
Apolipoprotein C-III	KDEL motif-containing protein 2	Collagen alpha-1(VIII) chain	Ribonuclease-like protein 10
Apolipoprotein L1	Layilin	Uncharacterized protein C18orf54	Ribonuclease-like protein 12
Complement C1q subcomponent subunit A	Leucine-rich repeat-containing protein 8B	Cystatin-like 1	Ribonuclease-like protein 13
Complement C1q subcomponent subunit C	Leucine-rich repeat-containing protein 8D	C2 domain-containing protein 2	Serpin A11
Calcitonin	Sialic acid-binding Ig-like lectin 6	DDRGK domain-containing protein 1	Kunitz-type protease inhibitor 4
Soluble calcium-activated nucleotidase 1	Pregnancy-specific beta-1-glycoprotein 2	Protein FAM55C	Meteorin-like protein
C-C motif chemokine 15	Ly6/PLAUR domain-containing protein 1	Collagen alpha-1(XXVI) chain	Putative testis serine protease 2

WO 2012/087838 PCT/US2011/065591

CD97 antigen (	Ly6/PLAUR domain-containing protein 5	Protein FAM19A2	Beta-defensin 112
Contactin-4	MLN64 N-terminal domain homolog	Protein FAM5B	Uncharacterized protein FLJ37543
Complement C2	Macrophage migration inhibitory factor	Fibroblast growth factor 5	Protein FAM24A
Collagen alpha-6(IV) chain	2-acylglycerol O-acyltransferase 3	Probable serine protease HTRA3	Secreted frizzled-related protein 4
Collagen alpha-2(VI) chain	Mitochondrial carrier homolog 1	Interleukin-1 family member 8	Complement C1q-like protein 2
Collagen alpha-1(XI) chain	Apolipoprotein L6	Serine protease inhibitor Kazal-type 4	Putative uncharacterized protein C17orf69
Crumbs homolog 1	Protocadherin alpha-6	Otospiralin	Putative cystatin-13
Cystatin-C	Protocadherin gamma-A12	Liver-expressed antimicrobial peptide 2	Beta-defensin 109
Neutrophil defensin 1	Voltage-gated hydrogen channel 1	Lysyl oxidase homolog 1	Beta-defensin 113
Endothelin-3	All-trans-retinol 13,14-reductase	Lysyl oxidase homolog 2	Beta-defensin 135
Low affinity immunoglobulin epsilon Fc receptor	Regulator of microtubule dynamics protein 2	Long palate, lung and nasal epithelium carcinoma-associated protein 4	Peptidase S1 domain-containing protein LOC136242
Fibroblast growth factor receptor 3	R-spondin-4	Lysozyme g-like protein 2	Growth/differentiation factor 7
Fibroblast growth factor receptor 4	Long-chain fatty acid transport protein 3	Endomucin	IgA-inducing protein homolog
Growth arrest-specific protein 6	Vesicle-trafficking protein SEC22c	Neuropeptide B	Putative lipocalin 1-like protein 1
Growth hormone receptor	Claudin-1	Kinesin-like protein KIF7	Putative serine protease 29
Bifunctional UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase	Leucine-rich repeats and immunoglobulin-like domains protein 3	Leukocyte-associated immunoglobulin-like receptor 2	Putative scavenger receptor cysteine-rich domain-containing protein LOC619207
Immunoglobulin superfamily member 8	SLAM family member 9	Calcium-dependent phospholipase A2	Secretoglobin-like protein
Interleukin-4 receptor alpha chain	Transthyretin	Proapoptotic caspase adapter protein	Putative stereocilin-like protein
Kallikrein-14	Serine/threonine-protein kinase 32B	Integrin beta-like protein 1	Insulin growth factor-like family member 2
Kallikrein-6	Platelet-derived growth factor subunit B	Tolloid-like protein 1	KIR2DL4
Laminin subunit beta-3	Noggin	Kunitz-type protease inhibitor 3	Putative zinc-alpha-2-glycoprotein-like 1
Leucyl-cystinyl aminopeptidase	Tryptase alpha-1	Protein TMEM155	Insulin growth factor-like family member 4
Mannan-binding lectin serine protease 1	Tetratricopeptide repeat protein 14	Prosalusin	Uncharacterized protein C2orf72
Mannan-binding lectin serine protease 2	XTP3-transactivated gene B protein	Protein amnionless	Replication initiation-like protein
Neutrophil gelatinase-associated lipocalin	Palmitoyltransferase ZDHHC15	Protein WFDC10B	Prostate and testis expressed protein 3
Neuropeptide Y	Zona pellucida sperm-binding protein 3	WAP four-disulfide core domain protein 8	B melanoma antigen 4
Aggrecan core protein	Leucine-rich repeat-containing protein 39	Protein Wnt-5b	Putative uncharacterized protein C1orf191
Pulmonary surfactant-associated protein B	Pancreatic triacylglycerol lipase	Protein Wnt-7b	Beta-defensin 108B-like
Poliovirus receptor-related protein 1	Transmembrane protein 139	Zona pellucida-binding protein 2	Uncharacterized protein FLJ90687
Renin	Leukemia inhibitory factor	SH3 domain-binding protein 5-like	Secreted frizzled-related protein 2
Ribonuclease pancreatic	Galectin-1	Adipocyte adhesion molecule	Basic proline-rich peptide IB-1
Semenogelin-1	C-C motif chemokine 21	Uncharacterized protein C12orf59	Fibroblast growth factor 16
Signaling lymphocytic activation molecule	CD5 antigen-like	Apolipoprotein A-I-binding protein	Serine protease inhibitor Kazal-type 8
Tissue factor pathway inhibitor	Carbohydrate sulfotransferase 9	Claudin-17	Uncharacterized protein KIAA0495
Usherin	Lipopolysaccharide-binding protein	Inactive caspase-12	Platelet basic protein-like 2
Fibroblast growth factor 23	Cysteine-rich motor neuron 1 protein	Uncharacterized protein C7orf58	Serpin E3
Interleukin-23 subunit alpha	Connective tissue growth factor	Collagen alpha-1(XXVIII) chain	CR1 receptor
Epididymal secretory protein E1	Protein eyes shut homolog	Dentin matrix protein 4	Secreted phosphoprotein 1
ADAMTS-like protein 1	Mucin-like protein 1	Uncharacterized protein C16orf48	Stress induced secreted protein 1

Chemokine-like factor	Fibroblast growth factor 19	Carboxylesterase 3	Protein Wnt
EGF-like domain-containing protein 7	Follistatin-related protein 3	Protein FAM20B	Protein Wnt (Fragment)
Tectonic-1	Hedgehog-interacting protein	GPN-loop GTPase 3	Putative serine protease LOC138652
Transmembrane protein 25	Interleukin-17 receptor B	GRAM domain-containing protein 1B	TOM1
UDP-GalNAc:beta-1,3-N-acetylgalactosaminyltransferase 1	FXYD domain-containing ion transport regulator 5	Phosphatidylinositol glycan anchor biosynthesis class U protein	Putative uncharacterized protein FLJ46089
Interleukin-15 (IL-15)	Endothelial lipase	Interleukin-27 subunit alpha	Putative uncharacterized protein C1orf134
Multiple epidermal growth factor-like domains 11	EGF-containing fibulin-like extracellular matrix protein 2	Pro-neuregulin-4, membrane-bound isoform	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 9
Mucin and cadherin-like protein	Otoraplin	Leucine-rich repeat neuronal protein 3	Uncharacterized protein C11orf44
Ribonuclease 4	Group 3 secretory phospholipase A2	NMDA receptor-regulated protein 2	Uncharacterized protein C12orf73
SH2 domain-containing protein 3C	Group XV phospholipase A2	NADH-cytochrome b5 reductase 1	Putative cystatin-9-like 2
CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase	Tumor necrosis factor ligand superfamily member 14	Parkinson disease 7 domain-containing protein 1	Putative abhydrolase domain-containing protein FAM108A5
Transmembrane protein 9	Plexin-A2	FK506-binding protein 11	Beta-defensin 133
WAP four-disulfide core domain protein 2	Papilin	C-type lectin domain family 12 member B	Fibrosin-1
Adenosine A3 receptor	Prokineticin-1	Solute carrier family 35 member F5	Probable folate receptor delta
Gamma-secretase subunit APH-1A	Ribonuclease 7	Sialic acid-binding Ig-like lectin 12	RPE-spondin
Basigin	Kunitz-type protease inhibitor 1	Protein FAM19A3	NPIP-like protein ENSP00000346774
Baculoviral IAP repeat-containing protein 7	Spondin-2	WD repeat-containing protein 82	Putative testis-specific prion protein
Calumenin	Testican-2	Adipocyte enhancer-binding protein 1	Proline-rich protein 1
Alpha-S1-casein	Inactive serine protease PAMR1	ADAMTS-like protein 3	Putative uncharacterized protein FP248
Cyclin-L1	Torsin-2A	Coiled-coil domain-containing protein 80	UPF0670 protein C8orf55
Complement factor H	Vasohibin-1	Ecto-NOX disulfide-thiol exchanger 1	Putative zinc-alpha-2-glycoprotein-like 2
Chorionic somatomammotropin hormone	Vasorin	Neuronal growth regulator 1	SPARC protein
Coxsackievirus and adenovirus receptor	Xylosyltransferase 1	Interphotoreceptor matrix proteoglycan 1	Otopetrin-1
Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	Ectonucleotide pyrophosphatase/phosphodiesterase family member 6	cDNA FLJ36603 fis, clone TRACH2015180, highly similar to Secreted frizzled-related protein 2	cDNA FLJ55667, highly similar to Secreted protein acidic and rich in cysteine
ERO1-like protein alpha	Oncostatin-M	Lipase member H	Lipase member K
Coagulation factor IX	Derlin-1	Mucin-19 (MUC-19)	C-type lectin domain family 18 member C
Low affinity immunoglobulin gamma Fc region receptor III-B	HERV-FRD_6p24.1 provirus ancestral Env polyprotein	Psoriasis susceptibility 1 candidate gene 2 protein	Putative uncharacterized protein UNQ6125/PRO20090
Ficolin-3	Prostasin	Integral membrane protein 2A	Complement C3
Fc receptor-like protein 2	Transmembrane protease, serine 11E	Vesicle transport protein SFT2B	Collagen alpha-2(IV) chain
Leucine-rich repeat transmembrane protein FLRT3	HLA class I histocompatibility antigen, Cw-16 alpha chain	von Willebrand factor A domain-containing protein 3A	Uncharacterized protein UNQ6126/PRO20091
Gelsolin	Wnt inhibitory factor 1	Protein shisa-2 homolog	Serpin-like protein HMSD
Granulysin	C-type natriuretic peptide	Signal peptidase complex subunit 3	Prostate and testis expressed protein 4
Transmembrane glycoprotein NMB	Angiopoietin-2	CD164 sialomucin-like 2 protein	Collagen alpha-1(XXII) chain
Granulins	Deoxyribonuclease gamma	Cadherin-16	Putative uncharacterized protein C13orf28

WO 2012/087838 PCT/US2011/065591

Heparanase	Carboxypeptidase A5	Cadherin-19	Cystatin-S
Ig mu chain C region	C-C motif chemokine 14	Cerebellin-2	R-spondin-1
Interleukin-1 alpha	Interleukin-5	Transmembrane protein C3orf1	C8orf2
Interleukin-31 receptor A	Interleukin-10	Sperm equatorial segment protein 1	Odorant-binding protein 2a
Junctional adhesion molecule B	C-X-C motif chemokine 2	Uncharacterized protein C6orf72	Opiorphin
Lipocalin-1	C-X-C motif chemokine 5	Uncharacterized protein C11orf24	Kidney androgen-regulated protein
Leucine-rich repeat-containing G-protein coupled receptor 6	A disintegrin and metalloproteinase with thrombospondin motifs 6	Acyl-CoA synthetase family member 2, mitochondrial	Putative uncharacterized protein UNQ5830/PRO19650/PRO19816
Latent-transforming growth factor beta-binding protein 1	Polypeptide N-acetylgalactosaminyltransferase 1	Probable UDP-sugar transporter protein SLC35A5	Putative uncharacterized protein UNQ6975/PRO21958
Matrilin-3	Fibulin-2	C-type lectin domain family 1 member A	Tachykinin-3
Myelin protein zero-like protein 1	Ficolin-1	C-type lectin domain family 3 member A	Secreted phosphoprotein 1
Neurobeachin-like protein 2	*SL cytokine*	*C-type lectin domain family 4 member E*	*Sclerostin*
Nicastrin	Follistatin	C-type lectin domain family 4 member G	ADAMTS-like protein 2
ADP-ribose pyrophosphatase, mitochondrial	FRAS1-related extracellular matrix protein 1	Probable cation-transporting ATPase 13A4	Scavenger receptor cysteine-rich domain-containing protein LOC284297
Protocadherin-15	Enamelin	UPF0480 protein C15orf24	Tryptase beta-1
Placenta growth factor	Hyaluronan and proteoglycan link protein 1	Zona pellucida sperm-binding protein 4	Tryptase delta
Protein O-linked-mannose beta-1,2-N-acetylglucosaminyltransferase 1	Leukocyte immunoglobulin-like receptor subfamily A member 3	Endoplasmic reticulum resident protein ERp27	Putative cat eye syndrome critical region protein 9
Probable hydrolase PNKD	Interleukin-17F	Transmembrane protein C16orf54	Plexin domain-containing protein 1
Pleiotrophin	Interleukin-1 receptor accessory protein	Cytochrome P450 4F12	MC51L-53L-54L homolog (Fragment)
Poliovirus receptor	Serine protease inhibitor Kazal-type 5	Cytochrome P450 4X1	COBW-like placental protein (Fragment)
Reticulon-4 receptor	Kallikrein-15	Cytochrome P450 4Z1	Cytokine receptor-like factor 2
Serum amyloid A protein	Interferon alpha-14	Protein CREG2	Beta-defensin 103
Sex hormone-binding globulin	Pregnancy-specific beta-1-glycoprotein 4	DnaJ homolog subfamily B member 9	Beta-defensin 106
SLAM family member 6	Collagenase 3	Dipeptidase 3	Hyaluronidase-3
Sarcolemmal membrane-associated protein	Matrix metalloproteinase-16	Membrane protein FAM174A	Interleukin-28 receptor alpha chain
Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	Pituitary adenylate cyclase-activating polypeptide	Thioredoxin domain-containing protein 15	Glycosyltransferase 54 domain-containing protein
Thyroxine-binding globulin	Prokineticin-2	Protein FAM19A4	Chordin-like protein 1
Transmembrane and coiled-coil domain-containing protein 1	Latent-transforming growth factor beta-binding protein 3	Adenosine monophosphate-protein transferase FICD	Putative uncharacterized protein UNQ9370/PRO34162
Transmembrane protease, serine 3	Somatoliberin	Prenylcysteine oxidase-like	Netrin receptor UNC5B
Tumor necrosis factor receptor superfamily member 10C	Thrombospondin type-1 domain-containing protein 1	Phytanoyl-CoA hydroxylase-interacting protein-like	Fibroblast growth factor receptor FGFR-1 secreted form protein (Fragment)
Tumor necrosis factor receptor superfamily member 11B	Angiogenic factor with G patch and FHA domains 1	FXYD domain-containing ion transport regulator 4	Uncharacterized protein ENSP00000244321
Serotransferrin	TGF-beta receptor type III	Growth/differentiation factor 11	ECE2
Tryptase beta-2	Thyrotropin subunit beta	Cerebral dopamine neurotrophic factor	EPA6
Protein YIPF5	Uncharacterized protein C19orf36	GPN-loop GTPase 2	Putative soluble interleukin 18 receptor 1

WO 2012/087838 PCT/US2011/065591

Vesicle-associated membrane protein-associated protein B/C	Complement C1q tumor necrosis factor-related protein 2	Growth hormone-inducible transmembrane protein	Putative abhydrolase domain-containing protein FAM108A6
cDNA, FLJ96669, highly similar to Homo sapiens secreted protein, acidic, cysteine-rich (osteonectin)(SPARC), mRNA	Ectonucleotide pyrophosphatase/phosphodiesterase family member 5	Glycerophosphodiester phosphodiesterase domain-containing protein 2	Putative V-set and immunoglobulin domain-containing-like protein ENSP00000303034
cDNA FLJ77519, highly similar to Homo sapiens secreted frizzled related protein mRNA	Polypeptide N-acetylgalactosaminyltransferase-like protein 2	WAP, kazal, immunoglobulin, kunitz and NTR domain-containing protein 1	B cell maturation antigen transcript variant 4 (Tumor necrosis factor receptor superfamily member 17)
T-cell differentiation antigen CD6	Slit homolog 1 protein	KDEL motif-containing protein 1	UPF0672 protein C3orf58
Pikachurin	Growth hormone variant	Adipophilin	Methylthioribose-1-phosphate isomerase
Fibrinogen-like protein 1	Angiopoietin-related protein 3	Lactase-like protein	17-beta hydroxysteroid dehydrogenase 13
Interleukin-32	Angiopoietin-related protein 7	Chondromodulin-1	Aminopeptidase B
Matrilin-4	Ecto-ADP-ribosyltransferase 5	Collagen alpha-6(VI) chain	Dermcidin
Sperm-associated antigen 11B	Carbonic anhydrase-related protein 11	Leucine-rich repeat-containing protein 33	Meteorin
Coagulation factor XII	Probable ribonuclease 11	MANSC domain-containing protein 1	Methyltransferase-like protein 7A
Hepcidin	Probable carboxypeptidase X1	Lipocalin-15	NL3
Klotho	Protein FAM3D	Arylsulfatase I	N-acetyltransferase 15
Serglycin	C-X-C motif chemokine 14	Mesoderm development candidate 2	Ephrin-A4
Tomoregulin-2	Beta-defensin 127	Dickkopf-related protein 1	Protein Plunc
Chordin-like protein 2	Beta-defensin 129	Podocan	Kallikrein-11
Tumor necrosis factor receptor superfamily member 6B	Cysteine-rich secretory protein LCCL domain-containing 2	Fibronectin type III domain-containing protein 1	WNT1 induced secreted protein 1 splice variant x (Fragment)
UPF0414 transmembrane protein C20orf30	Fibroblast growth factor 21	Neurotrimin	Interleukin-1 family member 10
C-type lectin domain family 4 member C	Plasma alpha-L-fucosidase	Olfactory receptor 10W1	PLA2G2D
UPF0317 protein C14orf159, mitochondrial	Gastrokine-1	Protein PARM-1	Proteoglycan 3
Netrin-G2	Gastrokine-2	PDZ domain-containing protein 2	Insulin-like peptide INSL5
Metalloreductase STEAP2	Glutathione peroxidase 7	Proepiregulin	Olfactomedin-like protein 3
Sushi domain-containing protein 4	HHIP-like protein 1	Polycystic kidney disease protein 1-like 1	Extracellular glycoprotein lacritin
Protein YIF1B	Interferon kappa	WLPL514	Retinol dehydrogenase 13
Apolipoprotein M	Apolipoprotein C-I	Matrix metalloproteinase-26	Neutrophil defensin 3
C4b-binding protein beta chain	Procollagen C-endopeptidase enhancer 2	RELT-like protein 2	GLGQ5807
T-cell surface glycoprotein CD8 beta chain	Left-right determination factor 1	Solute carrier family 35 member E3	TUFT1
C-C motif chemokine 3-like 1	Leucine-rich repeat LGI family member 4	Zinc transporter ZIP9	DRLV8200
Fibroblast growth factor 8	BRCA1-A complex subunit Abraxas	Noelin-2	IDLW5808
Sialomucin core protein 24	Leucine zipper protein 2	Seizure 6-like protein 2	UBAP2
Programmed cell death 1 ligand 2	Neurexophilin-3	Semaphorin-3A	C1q/TNF-related protein 8
Secreted and transmembrane 1	Osteomodulin	Semaphorin-4C	KIR2DL4 (Fragment)
Complement C1q tumor necrosis factor-related protein 6	Kazal-type serine protease inhibitor domain-containing protein 1	Abhydrolase domain-containing protein 14A	Chemokine-like factor super family 2 transcript variant 2
EGF-like module-containing mucin-like hormone receptor-like 3	Sperm acrosome membrane-associated protein 3	Ankyrin repeat domain-containing protein 36	Keratinocytes associated transmembrane protein 1

WO 2012/087838 PCT/US2011/065591

Noelin-3	Secretoglobin family 3A member 1	Protein shisa-4	GKGM353
Odorant-binding protein 2b	Tsukushin	Neuromedin-U	MATL2963
Urotensin-2	Claudin-2 (SP82)	Nodal homolog	NINP6167
Vitrin	Complement factor H-related protein 2	Synaptogyrin-2	POM121-like
WNT1-inducible-signaling pathway protein 3	Immunoglobulin superfamily containing leucine-rich repeat protein	Brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 2	RTFV9368 (SLE-dependent upregulation 1)
cDNA FLJ75759, highly similar to Homo sapiens follistatin-like 3 (secreted glycoprotein) (FSTL3), mRNA	Leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein 1	Coiled-coil domain-containing protein 104	Leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein 4
Angiotensin-converting enzyme 2	Kin of IRRE-like protein 3	Transmembrane 4 L6 family member 20	KCNQ2
Adiponectin	Hematopoietic cell signal transducer	Transmembrane protein 107	ELCV5929
Angiopoietin-related protein 4	Follitropin subunit beta	Transmembrane protein 143	KVVM3106
Apolipoprotein A-V	Melanoma inhibitory activity protein 3	Transmembrane protein 178	ISPF6484
Asporin	Leucine-rich repeat-containing protein 4	Transmembrane protein 205	LKHP9428
Bactericidal permeability-increasing protein	Zinc transporter 5	Transmembrane protein 41A	VNFT9373
CUB domain-containing protein 1	Leucine-rich repeat neuronal protein 1	Transmembrane protein 50A	ACAH3104
Cartilage intermediate layer protein 1	Apical endosomal glycoprotein	Transmembrane protein 50B	RVLA1944
Beta-Ala-His dipeptidase	Serum amyloid A-4 protein	Interleukin-28B	Wpep3002
Collagen alpha-1(V) chain	Probetacellulin	Neuronal pentraxin-2	ZDHHC11
Collagen alpha-1(XXV) chain	Beta-1,4-galactosyltransferase 7	Collectrin	AGLW2560
Estradiol 17-beta-dehydrogenase 11	3-hydroxybutyrate dehydrogenase type 2	Transmembrane protein 92	TSSP3028
DnaJ homolog subfamily C member 10	C1GALT1-specific chaperone 1	Transmembrane protein 95	RFVG5814
EGF-like domain-containing protein 6	Beta-casein	Transmembrane protein 9B	SHSS3124
Coagulation factor XIII A chain	Kappa-casein	Probable carboxypeptidase PM20D1	MMP19
Glucose-6-phosphate isomerase	Transmembrane protein C2orf18	Tetraspanin-12	GSQS6193
Appetite-regulating hormone	Carboxypeptidase N catalytic chain	Tetraspanin-13	VGPW2523
Interleukin-12 subunit beta	CD320 antigen	Tetraspanin-15	LMNE6487
Interleukin-22	Chondroitin sulfate synthase 1	UPF0513 transmembrane protein	ALLA2487
Intelectin-1	Chondroitin sulfate synthase 2	Mitochondrial uncoupling protein 4	GALI1870
Leucine-rich glioma-inactivated protein 1	CMRF35-like molecule 7	Polyserase-2	FRSS1829
Lymphocyte antigen 96	Protein canopy homolog 3	Probable palmitoyltransferase ZDHHC24	MRSS6228
Matrilysin	Short-chain dehydrogenase/reductase 3	Zona pellucida sperm-binding protein 1	GRPR5811
Mucin-20	Delta-like protein 4	Zona pellucida sperm-binding protein 2	AVLL5809
Proprotein convertase subtilisin/kexin type 9	Delta and Notch-like epidermal growth factor-related receptor	Conserved oligomeric Golgi complex subunit 7	CR1 C3b/C4b receptor SCR9 (or 16) C-term. exon SCR = short consensus repeat
Peptidoglycan recognition protein	Dolichol kinase	Adiponectin receptor protein 2	PIKR2786
Interferon-induced 17 kDa protein	Endothelin-converting enzyme-like 1	Inhibin beta C chain	S100 calcium binding protein A7-like 3
Protein Wnt-4	Integral membrane protein 2B	Brorin	GTWW5826 (LP5085 protein)
Allograft inflammatory factor 1-like	Insulin-like growth factor-binding protein 5	Semaphorin-3C	KTIS8219 (HCG2020043)
Armadillo repeat-containing X-linked protein 3	Endothelial cell-selective adhesion molecule	Heparan sulfate glucosamine 3-O-sulfotransferase 2	Hyaluronan and proteoglycan link protein 4

WO 2012/087838 PCT/US2011/065591

Chondroitin sulfate N-acetylgalactosaminyltransferase 1	Signal peptide, CUB and EGF-like domain-containing protein 1	Leptin receptor overlapping transcript-like 1	Micronovel
Chitotriosidase-1	Complement factor H-related protein 3	SPARC-like protein 1	SAMK3000
Claudin domain-containing protein 1	Prorelaxin H1	Fibulin-7	VFLL3057
Erlin-2	Follistatin-related protein 1	Protein HEG homolog 1	CVWG5837
Glycosyltransferase 8 domain-containing protein 1	Globoside alpha-1,3-N-acetylgalactosaminyltransferase 1	Fibrinogen C domain-containing protein 1	VGSA5840
Golgi membrane protein 1	Gamma-glutamyl hydrolase	Phospholipase A1 member A	GHPS3125
Probable G-protein coupled receptor 125	Cadherin-24	Basic salivary proline-rich protein 2	GRTR3118
Interleukin-20 receptor alpha chain	Glycerol-3-phosphate acyltransferase 3	Spermatogenesis-associated protein 6	PAMP6501
Galectin-7	G-protein coupled receptor 56	Sushi repeat-containing protein SRPX2	LTLL9335
NKG2D ligand 4	Hyaluronan-binding protein 2	Twisted gastrulation protein homolog 1	VCEW9374
L-amino-acid oxidase	Proheparin-binding EGF-like growth factor	Torsin-1B	AHPA9419
Prolyl 3-hydroxylase 1	Histidine-rich glycoprotein	Protein Wnt-5a	MDHV1887
GPI ethanolamine phosphate transferase 2	Carbohydrate sulfotransferase 14	Acrosin-binding protein	HSAL5836
GPI ethanolamine phosphate transferase 3	Interleukin-20 receptor beta chain	C-type lectin domain family 18 member B	LHLC1946
Calcium-binding mitochondrial carrier protein SCaMC-2 (Small calcium-binding mitochondrial carrier protein 2)	Ectonucleotide pyrophosphatase/phosphodiesterase family member 3	Lysosomal-associated transmembrane protein 4A	Long palate, lung and nasal epithelium carcinoma-associated protein 3 (Ligand-binding protein RYA3)
Pulmonary surfactant-associated protein A2	Insulin-like growth factor-binding protein 7	Semaphorin-3E	LPPA601
Splicing factor, arginine/serine-rich 16	Kallistatin	Ameloblastin	PINK1
Alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 6	Fibronectin type III domain-containing protein 3B	Major facilitator superfamily domain-containing protein 5	SERH2790
Single Ig IL-1-related receptor	Leukemia inhibitory factor receptor	Angiopoietin-1	FLFF9364
Tectonic-3	Lin-7 homolog B	Angiopoietin-4	APELIN
Tumor necrosis factor ligand superfamily member 11	Thioredoxin-related transmembrane protein 1	Multiple epidermal growth factor-like domains 9	GLSH6409
Tumor necrosis factor receptor superfamily member 19	Disintegrin and metalloproteinase domain-containing protein 32	Acid sphingomyelinase-like phosphodiesterase 3a	SFVP2550
Palmitoyltransferase ZDHHC9	Ly6/PLAUR domain-containing protein 3	ADAMTS-like protein 5	RRLF9220
Fibulin-5	C-type lectin domain family 14 member A	Spexin	PTML5838
Protein Z-dependent protease inhibitor	Protein cornichon homolog	Putative trypsin-6	VLGN1945
Alpha-2-macroglobulin	Protein FAM151A	Proto-oncogene protein Wnt-1	AVPC1948
Agouti-related protein	FK506-binding protein 14	Bone morphogenetic protein 3b	AWQG2491
Pancreatic alpha-amylase	Neuropilin and tolloid-like protein 2	Bone morphogenetic protein 5	PSVL6168
Natriuretic peptides B	Protocadherin beta-13	Bone morphogenetic protein 8B	LCII3035
Atrial natriuretic factor	Prenylcysteine oxidase 1	Protein FAM26D	PPRR6495
Neutral ceramidase	Peflin	C1q-related factor	RLSC6348
Beta-2-microglobulin	Peptidyl-prolyl cis-trans isomerase-like 1	WAP four-disulfide core domain protein 1	CSRP2BP
Bone morphogenetic protein 4	Prostate stem cell antigen	Cerebellin-1	GLLV3061
Biotinidase	Protein patched homolog 2	Carboxypeptidase O	GWSI6489

WO 2012/087838 PCT/US2011/065591

Scavenger receptor cysteine-rich type 1 protein M130	Chitobiosyldiphosphodolichol beta-mannosyltransferase	Myelin protein zero-like protein 2 (Epithelial V-like antigen 1)	cDNA FLJ53955, highly similar to Secreted frizzled-related protein 4
Carboxypeptidase B2	Protein sel-1 homolog 1	Serine protease 1-like protein 1	PPIF
Carboxypeptidase Z	ProSAAS	Coiled-coil domain-containing protein 70	VSSW1971
C-C motif chemokine 5	Sialic acid-binding Ig-like lectin 9	C-C motif chemokine 28	KLIA6249
C-C motif chemokine 7	SLIT and NTRK-like protein 1	Uncharacterized protein C4orf29	ALLW1950
C-C motif chemokine 8	Statherin	CUB domain-containing protein 2	GVEI466
CD59 glycoprotein	Testisin	Trem-like transcript 4 protein	ESFI5812
Complement factor I	Transmembrane channel-like protein 5	Uncharacterized protein C6orf58	GNNC2999
Clusterin	Transmembrane protease, serine 4	Chondroadherin	AAGG6488
Collagen alpha-2(I) chain	Metastasis-suppressor KiSS-1	Cartilage intermediate layer protein 2	HHSL751
Collagen alpha-1(III) chain	Islet amyloid polypeptide	Uncharacterized protein C10orf25	Beta-defensin 108B
Collagen alpha-1(IV) chain	Trem-like transcript 2 protein	Isthmin-1	Beta-defensin 118
Collagen alpha-3(IV) chain	Thioredoxin domain-containing protein 12	Cystatin-8	Beta-defensin 124
Collagen alpha-5(IV) chain	Vascular endothelial growth factor B	Cardiotrophin-1 (CT-1)	Beta-defensin 125
Collagen alpha-3(VI) chain	Vascular endothelial growth factor C	Chymotrypsinogen B	Beta-defensin 126
Complement component C6	Reticulocalbin-3	C-X-C motif chemokine 9	Deoxyribonuclease-1-like 2
Collagen alpha-1(IX) chain	Fibrillin-1	C-X-C motif chemokine 13	Stanniocalcin-2
Collagen alpha-1(X) chain	Protein FAM3A	EMILIN-3	Endothelial cell-specific molecule 1
Collagen alpha-1(XVII) chain	Protein G7c	Secretagogin	Carboxylesterase 7
Collagen alpha-1(XXI) chain	Neuropilin and tolloid-like protein 1	Epididymal secretory protein E3-alpha	Protein NOV homolog
Coatomer subunit alpha	Pregnancy-specific beta-1-glycoprotein 11	Epiphycan	UPF0528 protein FAM172A
Complement receptor type 1	Serpin B4	Protein FAM5C	Interleukin-27 subunit beta
Cystatin-SN	ADAM DEC1	Fibroblast growth factor 20	Protein FAM3C
Deoxyribonuclease-1	ADP-dependent glucokinase	Fibroblast growth factor-binding protein 3	Stromal cell-derived factor 2-like protein 1
Extracellular matrix protein 1	Alpha-amylase 2B	Transmembrane protein 204	Butyrophilin subfamily 1 member A1
Low affinity immunoglobulin gamma Fc region receptor III-A	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 3	Phosphatidylethanolamine-binding protein 4	Keratinocyte-associated transmembrane protein 2
Alpha-fetoprotein	Calcitonin gene-related peptide 2	Coagulation factor V	Immunoglobulin alpha Fc receptor
Heparin-binding growth factor 2	Carboxypeptidase E	Coagulation factor VII	EMILIN-2
Fibrinogen gamma chain	Cardiotrophin-like cytokine factor 1	Pro-MCH	Ephrin type-A receptor 10
Growth/differentiation factor 5	Collagen alpha-2(VIII) chain	Folate receptor gamma	Exostosin-like 2
Glial cell line-derived neurotrophic factor	Crumbs homolog 2	Mucin-7	Follistatin-related protein 4
Insulin-like growth factor-binding protein 3	Dentin matrix acidic phosphoprotein 1	Galanin-like peptide	Follistatin-related protein 5
Insulin-like growth factor IA	Down syndrome cell adhesion molecule	Hemicentin-1	Transmembrane protein 66
Ig gamma-1 chain C region	Immunoglobulin superfamily member 1	Interleukin-6	Growth/differentiation factor 2
Ig gamma-2 chain C region	Interleukin-4	Embryonic growth/differentiation factor 1	GDNF family receptor alpha-4
Ig gamma-3 chain C region	Interleukin-6 receptor subunit alpha	Interleukin-8	Ig gamma-4 chain C region
Insulin-like 3	Interleukin-24	Gremlin-2	Lymphocyte antigen 86
Inter-alpha-trypsin inhibitor heavy chain	Ladinin-1	Stromelysin-2	Inhibin beta E chain
UPF0378 protein KIAA0100	Lipase member I	Probable G-protein coupled receptor 171	GRAM domain-containing protein 1C

WO 2012/087838 PCT/US2011/065591

Kininogen-1	Pancreatic lipase-related protein 1	Pappalysin-2	Interferon alpha-10
Laminin subunit alpha-2	Leucine-rich alpha-2-glycoprotein	Microfibril-associated glycoprotein 4	Interferon alpha-16
Laminin subunit alpha-4	Matrix-remodeling-associated protein 5	Neuromedin-B	Interferon alpha-6
Laminin subunit beta-1	Netrin-4	Mimecan	Immunoglobulin superfamily member 21
Protein-lysine 6-oxidase	Hepatocyte growth factor receptor	Matrix metalloproteinase-19	Agrin
Multimerin-1	C-C motif chemokine 22	Interleukin-11	Prolactin
Vasopressin-neurophysin 2-copeptin	Nyctalopin	Interleukin-17A	Kelch-like protein 11
Nidogen-1	Osteocalcin	Interleukin-18	Protein Wnt-16
Phospholipase A2,	Basic salivary proline-rich protein 3	Interleukin-26	Properdin
Perforin-1	Pregnancy-specific beta-1-glycoprotein 10	Interleukin-28A	Kallikrein-13
Phosphatidylinositol-glycan-specific phospholipase D	Leucine-rich repeat transmembrane protein FLRT2	Transmembrane emp24 domain-containing protein 3	1-acyl-sn-glycerol-3-phosphate acyltransferase delta
Fibrocystin	R-spondin-3	Interleukin-29	Kallikrein-9
Phospholipid transfer protein	Sialoadhesin	Insulin-like peptide INSL6	Vitamin K-dependent protein S
Prostatic acid phosphatase	Trypsin-3	Protein Wnt-2b	Butyrophilin-like protein 8
Vitamin K-dependent protein Z	Dipeptidase 2	Pregnancy-specific beta-1-glycoprotein 1	Laminin subunit beta-4
Salivary acidic proline-rich phosphoprotein 1/2	Collagen and calcium-binding EGF domain-containing protein 1	Sperm acrosome membrane-associated protein 4	Lymphatic vessel endothelial hyaluronic acid receptor 1
Pregnancy zone protein	Germ cell-specific gene 1-like protein	Laminin subunit gamma-3	Cystatin-SA
Prorelaxin H2	Leucine-rich repeat-containing protein 31	Lysyl oxidase homolog 3	Transmembrane protein 59
Semaphorin-4D	Apolipoprotein O	Neurotensin/neuromedin N	Apolipoprotein(a)-like protein 2
Slit homolog 2 protein	Dystroglycan	MAM domain-containing protein 2	Lysozyme-like protein 2
Alpha-tectorin	Neutrophil defensin 4	Microfibrillar-associated protein 2	Lysozyme-like protein 4
Tenascin-X	Amphoterin-induced protein 3	Melanoma inhibitory activity protein 2	Reelin
Trefoil factor 3	Gamma-secretase subunit APH-1B	Matrix metalloproteinase-24	Retinol-binding protein 4
Transferrin receptor protein 1	Apolipoprotein C-IV	Matrix metalloproteinase-25	Carbonic anhydrase 14
Protransforming growth factor alpha	Arylsulfatase G	Netrin-1	Tubulointerstitial nephritis antigen
Transforming growth factor beta-2	Glia-activating factor	Netrin-3	Neuropeptide W
Tumor necrosis factor ligand superfamily member 6	Caspase recruitment domain-containing protein 18	Alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1	Alpha-1,3-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase B
Tumor necrosis factor receptor superfamily member 1B	Heparan sulfate glucosamine 3-O-sulfotransferase 3A1	Alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3	Transmembrane emp24 domain-containing protein 5
Tumor necrosis factor receptor superfamily member 5	Thyrotropin-releasing hormone-degrading ectoenzyme	Melanoma-derived growth regulatory protein	Complement C1q tumor necrosis factor-related protein 3
Thrombopoietin	Guanylin	FMRFamide-related peptides	Podocan-like protein 1
VIP peptides	Choline transporter-like protein 3	Otoconin-90	Pregnancy-specific beta-1-glycoprotein 5
Acidic mammalian chitinase	*17-beta-hydroxysteroid dehydrogenase* 14	*Neurturin*	*Keratocan*
Cysteine-rich secretory protein 2	Immunoglobulin lambda-like polypeptide 1	Neurexophilin-1	Group IIE secretory phospholipase A2
Haptoglobin-related protein	DnaJ homolog subfamily B member 14	Neurexophilin-2	Left-right determination factor 2
C-C motif chemokine 26	F-box only protein 8	Platelet factor 4 variant	NKG2D ligand 2
Collectin-11	Fibroleukin	Nociceptin	Macrophage metalloelastase

WO 2012/087838 PCT/US2011/065591

Cysteine-rich with EGF-like domain protein 2	Methionine-R-sulfoxide reductase B3, mitochondrial	V-set and transmembrane domain-containing protein 1	Triggering receptor expressed on myeloid cells 1
C-X-C motif chemokine 16	Leucine-rich repeat LGI family member 2	Proline-rich protein 4	Cytokine receptor-like factor 1
Fibroblast growth factor-binding protein 1	Vesicle transport protein GOT1B	Prolactin-releasing peptide	Secretin
Interleukin-1 family member 5	Integral membrane protein GPR177	Sérine protease 33	Stromal cell-derived factor 2
Interleukin-1 family member 9	Probable G-protein coupled receptor 78	Pregnancy-specific beta-1-glycoprotein 8	Lysozyme-like protein 6
Kallikrein-5	HEPACAM family member 2	Retbindin	Serpin A9
Matrilin-2	Interleukin-27 receptor subunit alpha	FMRFamide-related peptides	Sclerostin domain-containing protein 1
Cell surface glycoprotein CD200 receptor 1	Proenkephalin-A	Ribonuclease K6	Lysocardiolipin acyltransferase 1
Lysophosphatidic acid phosphatase type 6	Integrin alpha-10	Ribonuclease T2	Plasma glutamate carboxypeptidase
Nucleotide exchange factor SIL1	KTEL motif-containing protein 1	Repetin	Slit homolog 3 protein
Thrombospondin type-1 domain-containing protein 4	Leukocyte immunoglobulin-like receptor subfamily A member 5	Complement C1r subcomponent-like protein	C3 and PZP-like alpha-2-macroglobulin domain-containing protein 8
WNT1-inducible-signaling pathway protein 2	Leucine-rich repeat and fibronectin type-III domain-containing protein 3	Uncharacterized glycosyltransferase AER61	Retinoic acid receptor responder protein 2
Bromodomain-containing protein 9	Uteroglobin	Semaphorin-3G	Cartilage acidic protein 1
CD99 antigen-like protein 2	Netrin-G1 ligand	Secretoglobin family 1C member 1	Stanniocalcin-1
Uncharacterized protein C1orf159	Pannexin-1	Secretoglobin family 1D member 1	Beta-tectorin
Carbohydrate sulfotransferase 12	Protocadherin-12	Secretoglobin family 1D member 2	Post-GPI attachment to proteins factor 3
Probable serine carboxypeptidase CPVL	Protocadherin alpha-10	Serpin A12	Germ cell-specific gene 1 protein
Mucin-3A	Protocadherin beta-10	Serpin I2	Interleukin-21 receptor
CUB and zona pellucida-like domain-containing protein 1	Osteopetrosis-associated transmembrane protein 1	von Willebrand factor C and EGF domain-containing protein	V-set and immunoglobulin domain-containing protein 4
Polypeptide N-acetylgalactosaminyltransferase 14	Beta-galactoside alpha-2,6-sialyltransferase 1	A disintegrin and metalloproteinase with thrombospondin motifs 15	Scavenger receptor cysteine-rich domain-containing group B protein
Galectin-9	GPI transamidase component PIG-S	Sodium channel subunit beta-2	Prothyroliberin
Leucine-rich repeat-containing protein 17	Proline-rich transmembrane protein 3	Metalloproteinase inhibitor 4	Semaphorin-4A
Leucine-rich repeat neuronal protein 2	Sulfhydryl oxidase 2	T-cell immunomodulatory protein	
Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 3	A disintegrin and metalloproteinase with thrombospondin motifs 16	A disintegrin and metalloproteinase with thrombospondin motifs 10	Tumor necrosis factor receptor superfamily member 27
Tuftelin	SH2 domain-containing protein 3A	Thymic stromal lymphopoietin	Toll-like receptor 7
Brain mitochondrial carrier protein	SHC-transforming protein 4	Transmembrane protein 130	
Signal peptide, CUB and EGF-like domain-containing protein 3	Disintegrin and metalloproteinase domain-containing protein 23	Unique cartilage matrix-associated protein	Thioredoxin domain-containing protein 16
14-3-3 protein sigma	Transducin beta-like protein 2	Urocortin-2	Alpha-2-antiplasmin
Alpha-1-acid glycoprotein 1	Tudor domain-containing protein 10	Urocortin-3 (	WAP four-disulfide core domain protein 3
Alpha-1-acid glycoprotein 2	Transmembrane 9 superfamily member 3	Protein AMBP	Protein WFDC9
von Willebrand factor A domain-containing protein 1	Von Willebrand factor D and EGF domain-containing protein	Complement C1q tumor necrosis factor-related protein 9-like	A disintegrin and metalloproteinase with thrombospondin motifs 14
Disintegrin and metalloproteinase domain-containing protein 9	A disintegrin and metalloproteinase with thrombospondin motifs 17	Growth inhibition and differentiation-related protein 88	Adipocyte plasma membrane-associated protein

WO 2012/087838 PCT/US2011/065591

Angiotensinogen	Transmembrane channel-like protein 2	Protein Wnt-10a	Peroxidasin homolog
Apolipoprotein A-II (Apo-AII) (ApoA-II)	Pregnancy-specific beta-1-glycoprotein 3	Protein Wnt-3a	Progressive ankylosis protein homolog
Apolipoprotein A-IV (Apo-AIV) (ApoA-IV)	Tenomodulin	Proto-oncogene protein Wnt-3	Chitinase-3-like protein 1
Apolipoprotein C-II (Apo-CII) (ApoC-II)	Tetraspanin-6	Protein Wnt-6	UPF0672 protein CXorf36
Beta-2-glycoprotein 1	Thioredoxin domain-containing protein 5	Protein Wnt-9a	Arylsulfatase J
Apoptosis-related protein 3	Vascular endothelial growth factor D	Cytokine SCM-1 beta	Cortistatin
Beta-secretase 2	Pregnancy-specific beta-1-glycoprotein 9	Zymogen granule membrane protein 16	Ceruloplasmin
Histo-blood group ABO system transferase	Semaphorin-3F	Zona pellucida-binding protein 1	Angiopoietin-related protein 5
Cathepsin L2	Acid phosphatase-like protein 2	Anterior gradient protein 3 homolog	Coiled-coil domain-containing protein 126
C-C motif chemokine 3	Apolipoprotein O-like	Amelotin	CD177 antigen
C-type lectin domain family 1 member B	Beta-defensin 119	Uncharacterized protein C5orf46	Protein canopy homolog 4
Calcium-activated chloride channel regulator 1	A disintegrin and metalloproteinase with thrombospondin motifs 12	Uncharacterized aarF domain-containing protein kinase 1	Fibronectin type-III domain-containing protein C4orf31
Chymase	Protein FAM131A	Draxin	Protein FAM180A
Collagen alpha-1(VI) chain	Protein FAM3B	Fibroblast growth factor 18	Platelet basic protein
Complement component C8 alpha chain	Beta-galactosidase-1-like protein	C-X-C motif chemokine 11	Interferon epsilon
Complement component C9	Lysozyme g-like protein 1	Ly6/PLAUR domain-containing protein 6	Intelectin-2
Glucose-fructose oxidoreductase domain-containing protein 2	Inter-alpha-trypsin inhibitor heavy chain H5-like protein	Chymotrypsin-like elastase family member 1	Alpha-1,3-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase A
DnaJ homolog subfamily B member 11	Sperm acrosome-associated protein 5	Erythropoietin receptor	Matrix extracellular phosphoglycoprotein
Ectonucleotide pyrophosphatase/phosphodiesterase family member 7	Leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein 2	MAM domain-containing glycosylphosphatidylinositol anchor protein 2	cDNA FLJ77863, highly similar to Homo sapiens secreted and transmembrane 1 (SECTM1), mRNA
Endoplasmic reticulum aminopeptidase 1	Surfactant-associated protein 2	Matrix metalloproteinase-27	Epididymal-specific lipocalin-6
Receptor tyrosine-protein kinase erbB-3	Adiponectin receptor protein 1	Inactive serine protease 35	Afamin
Endoplasmic reticulum resident protein ERp44	Multiple epidermal growth factor-like domains 6	Coiled-coil domain-containing protein 134	Probable cation-transporting ATPase 13A5
IgGFc-binding protein	Neuroendocrine protein 7B2	Suprabasin	Glutathione peroxidase 3
Complement factor H-related protein 1	Alpha-1B-glycoprotein	Secretoglobin family 1D member 4	Claudin-18
Polypeptide N-acetylgalactosaminyltransferase 2	WAP, kazal, immunoglobulin, kunitz and NTR domain-containing protein 2	V-set and transmembrane domain-containing protein 2A	Putative killer cell immunoglobulin-like receptor like protein KIR3DP1
Hemopexin	Arylacetamide deacetylase-like 1	ADM	Secretory phospholipase A2 receptor
Hepatocyte growth factor activator	Histatin-3	Uncharacterized protein C2orf82	Haptoglobin
Major histocompatibility complex class I-related gene protein	Pro-neuregulin-3, membrane-bound isoform	Insulin growth factor-like family member 1	Carcinoembryonic antigen-related cell adhesion molecule 20
Insulin-like growth factor-binding protein 6	Agouti-signaling protein	Cadherin-like protein 29	Bone morphogenetic protein 3
Ig delta chain C region	Claudin-8	Bone morphogenetic protein 15	Bone marrow stromal antigen 2
Interleukin-1 beta	UPF0454 protein C12orf49	Plasma serine protease inhibitor	Cytochrome P450 20A1
Low-density lipoprotein receptor-related protein 10	von Willebrand factor A domain-containing protein 5B1	Carcinoembryonic antigen-related cell adhesion molecule 21	Bactericidal/permeability-increasing protein-like 3
Junctional adhesion molecule C	Cadherin-6	Alpha-lactalbumin	Protein dpy-19 homolog 2

WO 2012/087838 PCT/US2011/065591

Uncharacterized protein KIAA0319	Cathelicidin antimicrobial peptide	Sister chromatid cohesion protein DCC1	Group IIF secretory phospholipase A2
Laminin subunit alpha-5	Laminin subunit gamma-1	Galectin-3-binding protein	Carboxypeptidase B
Fibronectin type III domain-containing protein 4	Dehydrogenase/reductase SDR family member 7B	Dynein heavy chain domain-containing protein 1	Glycosyltransferase 8 domain-containing protein 2
Lipoprotein lipase	C-C motif chemokine 16	C-C motif chemokine 17	Protein FAM19A1
Interstitial collagenase	C-C motif chemokine 24	Fatty acyl-CoA reductase 1	GDNF family receptor alpha-like
Matrix metalloproteinase-9	HEAT repeat-containing protein C7orf27	Fin bud initiation factor homolog	Probable glutathione peroxidase 8
Mucin-16	Collagen alpha-2(IX) chain	Polymeric immunoglobulin receptor	Cystatin-D
Mucin-2	Collagen alpha-3(IX) chain	Prion-like protein doppel	Cystatin-F
Mucin-5B	Colipase	C-X-C motif chemokine 6	Platelet-activating factor acetylhydrolase
Myocilin	Collagen alpha-1(XXVII) chain	C-X-C motif chemokine 10	Pappalysin-1
Oxidized low-density lipoprotein receptor 1	Carboxypeptidase N subunit 2	Beta-defensin 1	Solute carrier family 22 member 12
Prostate tumor overexpressed gene 1 protein	Leucine-rich repeat transmembrane neuronal protein 4	Hyaluronan and proteoglycan link protein 2	Chorionic somatomammotropin hormone-like 1
Receptor-interacting serine/threonine-protein kinase 2	Collagen triple helix repeat-containing protein 1	Disintegrin and metalloproteinase domain-containing protein 30	Regulator of microtubule dynamics protein 3
Equilibrative nucleoside transporter 3	Endothelin-2	Suppressor of fused homolog	Retinol dehydrogenase 14
Selenoprotein P	Fibromodulin	Folate receptor beta	Galanin
Pulmonary surfactant-associated protein D	Fc receptor-like B	Extracellular sulfatase Sulf-2	Transcobalamin-2
Stimulated by retinoic acid gene 6 protein homolog	Zinc finger RAD18 domain-containing protein C1orf124	Tumor necrosis factor receptor superfamily member 14	Catechol-O-methyltransferase domain-containing protein 1
Trefoil factor 1	Growth/differentiation factor 15	Artemin	Tripeptidyl-peptidase 1
Tissue factor pathway inhibitor 2	Glia-derived nexin	Collagen alpha-1(XII) chain	Trem-like transcript 1 protein
Prothrombin	Progonadoliberin-1	Collagen alpha-1(XIV) chain	Guanylate cyclase activator 2B
Toll-like receptor 9	Granzyme K	Beta-defensin 2	Inducible T-cell costimulator
Intercellular adhesion molecule 4	Interferon alpha-17	Interleukin-21	
Interleukin-19	Interferon alpha-21	Interleukin-3	
Isthmin-2	Interferon alpha-8	Interleukin-7	Notch homolog 2 N-terminal-like protein
Kin of IRRE-like protein 1	Interferon omega-1	Inhibin alpha chain	Laminin subunit beta-2
Kallikrein-10	Early placenta insulin-like peptide	Laminin subunit alpha-3	Neuropilin-2
Latent-transforming growth factor beta-binding protein 4	EGF, latrophilin and seven transmembrane domain-containing protein 1	Dehydrogenase/reductase SDR family member on chromosome X	EGF-containing fibulin-like extracellular matrix protein 1
Paired immunoglobulin-like type 2 receptor alpha	Fibronectin type 3 and ankyrin repeat domains protein 1	FXYD domain-containing ion transport regulator 6	Receptor-type tyrosine-protein phosphatase kappa
Regenerating islet-derived protein 3 alpha	Lysyl oxidase homolog 4	Serine incorporator 2	Regenerating islet-derived protein 4
E3 ubiquitin-protein ligase RNF5	Lumican	Stromelysin-3	Tachykinin-4
Protachykinin-1	Adropin	Secreted phosphoprotein 1	Matrix metalloproteinase-23
Secreted frizzled-related protein 1, isoform CRA_a	Leucine-rich repeat transmembrane protein FLRT1	Serine beta-lactamase-like protein LACTB, mitochondrial	Complement C1q tumor necrosis factor-related protein 5
Plasminogen-related protein B	Nucleobindin-2	Galectin-3	Opticin
Probable palmitoyltransferase ZDHHC16	Phospholipase A2	Pancreatic prohormone	Pre-small/secreted glycoprotein

Angiopoietin-related protein 1	Proenkephalin-B	Pregnancy-specific beta-1-glycoprotein 6	Pentraxin-related protein PTX3
UPF0510 protein C19orf63	Peptidoglycan recognition protein I-beta	Dickkopf-related protein 3	Carboxylesterase 8
Scavenger receptor cysteine-rich type 1 protein M160	Immunoglobulin superfamily containing leucine-rich repeat protein 2	Dehydrogenase/reductase SDR family member 11	Thioredoxin-related transmembrane protein 4
ER degradation-enhancing alpha-mannosidase-like 2	V-set and immunoglobulin domain-containing protein 2	Regenerating islet-derived protein 3 gamma	Major facilitator superfamily domain-containing protein 2
Beta-galactosidase-1-like protein 2	Peptide YY	RING finger protein 43	Kallikrein-12
Interleukin-17 receptor E	Retinol-binding protein 3	Semenogelin-2	Brevican core protein
Interleukin-20	Atherin	Mucin-15	Porimin
Interleukin-25	Translocation protein SEC63 homolog	Bone sialoprotein 2	Torsin-1A
PDZ domain-containing protein 11	Transforming growth factor beta-3	Lymphotactin	C-C motif chemokine 23
Relaxin-3	Protein Wnt-10b	Growth-regulated alpha protein	Testican-3
Retinoid-inducible serine carboxypeptidase	Renalase	R-spondin-2	Basic salivary proline-rich protein 4
Short palate, lung and nasal epithelium carcinoma-associated protein 2	Proprotein convertase subtilisin/kexin type 4	Transmembrane and coiled-coil domain-containing protein 3	Tumor necrosis factor receptor superfamily member 18
WAP four-disulfide core domain protein 5	Carboxypeptidase A4	VEGF co-regulated chemokine 1	Brother of CDO
Platelet-derived growth factor C	Olfactomedin-4	ADM2	Beta-1,4-galactosyltransferase 4
Disintegrin and metalloproteinase domain-containing protein 33	Insulin-like growth factor-binding protein complex acid labile chain	Hydroxysteroid 11-beta-dehydrogenase 1-like protein	Dehydrogenase/reductase SDR family member 9
BSD domain-containing protein 1	Amelogenin, Y isoform	Delta-like protein 1	Eppin
Cell adhesion molecule 3	Arylsulfatase F	Ephrin-A1	Otoancorin
CDC45-related protein	Choriogonadotropin subunit beta variant 2	Fibroblast growth factor receptor-like 1	Tenascin-R
Chondrolectin	Beta-defensin 104	GDNF family receptor alpha-3	Growth factor
Diacylglycerol O-acyltransferase 2	Beta-defensin 105	Platelet receptor Gi24	Protein TSPEAR
3-keto-steroid reductase	Beta-defensin 107	Progonadoliberin-2	Hephaestin
Interleukin-17 receptor C	Protein WFDC11	Kallikrein-7	Butyrophilin-like protein 3
Interleukin-17 receptor D	WAP four-disulfide core domain protein 6	Apolipoprotein F	Butyrophilin-like protein 9
Integrator complex subunit 1	Epigen	Protein CASC4	Laminin subunit gamma-2
Junctional adhesion molecule-like	Protein FAM19A5	VIP36-like protein	Protein LMBR1L
E3 ubiquitin-protein ligase LNX	Claudin-6	Magnesium transporter protein 1	Mucin-21
Leucine-rich repeat transmembrane neuronal protein 3	*Carcinoembryonic antigen-related cell* adhesion molecule 19	*Amiloride-sensitive amine oxidase* [copper-containing]	Endoplasmic reticulum mannosyl-oligosaccharide 1,2-alpha-mannosidase
Methionine adenosyltransferase 2 subunit beta	A disintegrin and metalloproteinase with thrombospondin motifs 1	DNA damage-regulated autophagy modulator protein 2	Pancreatic secretory granule membrane major glycoprotein GP2
Podocalyxin-like protein 2	Protein COQ10 A, mitochondrial	Transmembrane protein C17orf87	Semaphorin-4B
Prominin-2	Uncharacterized protein C19orf41	Complement factor H-related protein 5	Semaphorin-5B
Plexin domain-containing protein 2	Uncharacterized protein C21orf63	FK506-binding protein 7	Epsilon-sarcoglycan
Roundabout homolog 4	Protein delta homolog 2	Serine incorporator 1	Guanylate-binding protein 5
Lactosylceramide alpha-2,3-sialyltransferase	*Cocaine- and amphetamine-regulated* transcript protein	*Transmembrane and ubiquitin-like* domain-containing protein 1	Ectonucleoside triphosphate diphosphohydrolase 6
SID1 transmembrane family member 2	Lipoma HMGIC fusion partner-like 1 protein	Protein ERGIC-53-like	Serpin B3

WO 2012/087838 PCT/US2011/065591

Sushi domain-containing protein 1	Leucine-rich repeat-containing protein 18	Toll-like receptor 10	Protein RMD5 homolog B
Serine/threonine-protein kinase TAO2	Leucine-rich repeat-containing protein 25	Toll-like receptor 8	Scavenger receptor class A member 5
Transmembrane protease, serine 2	Leucine-rich repeat-containing protein 3B	Selenoprotein T	Semaphorin-6B
UDP-glucuronic acid decarboxylase 1	Leucine-rich repeat-containing protein 3	Sialic acid-binding Ig-like lectin 11	Transmembrane protein 108
Uncharacterized protein C10orf58	Ly6/PLAUR domain-containing protein 4	Sorting nexin-24	Sushi domain-containing protein 3
Thioredoxin-related transmembrane protein 2	Vitamin K epoxide reductase complex subunit 1	Complement C1q tumor necrosis factor-related protein 1	Latent-transforming growth factor beta-binding protein 2
CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase	A disintegrin and metalloproteinase with thrombospondin motifs 20	Putative uncharacterized protein UNQ6494/PRO21346	Putative uncharacterized protein UNQ6190/PRO20217
Putative uncharacterized protein ENSP00000380674	Putative uncharacterized protein ENSP00000381830	Secreted and transmembrane 1 precusor variant	Secreted and transmembrane 1 precusor variant
Transmembrane protein 119	Cat eye syndrome critical region protein 1	C-type lectin domain family 18 member A	Collagen alpha-1(XX) chain
Transmembrane protein 98	Testis-expressed protein 101	Cysteine-rich secretory protein 3	Netrin receptor UNC5D
Pre-B lymphocyte protein 3	Xylosyltransferase 2	Complement C4-A	Mucin-13
Putative uncharacterized protein C14orf144	Protein FAM20A	Putative uncharacterized protein PRO2829	ATP-dependent metalloprotease YME1L1
Membrane-bound transcription factor site-1 protease	Transmembrane and immunoglobulin domain-containing protein 1	Calcium-activated chloride channel regulator 2	Proprotein convertase subtilisin/kexin type 5
Ficolin (Collagen/fibrinogen domain containing) 3 (Hakata antigen) (NL3) (Ficolin (Collagen/fibrinogen domain containing) 3 (Hakata antigen), isoform CRA_b)	Putative killer cell immunoglobulin-like receptor-like protein KIR3DX1 (Leukocyte receptor cluster member 12)	Neuroblastoma suppressor of tumorigenicity 1	

WO 2012/087838 PCT/US2011/065591

[0054] The therapeutic proteins provided herein should not be considered to be exclusive. Rather, as is apparent from the disclosure provided herein, the methods of the invention are applicable to any protein wherein attachment of a water soluble fatty acid derivative is desired according to the invention. For example, therapeutic proteins are described in US 2007/0026485, incorporated herein by reference in its entirety.

Blood coagulation proteins

[0055] In one aspect, the starting material of the present invention is a blood coagulation protein, which can be derived from human plasma, or produced by recombinant engineering techniques, as described in patents US Patent No. 4,757,006; US Patent No. 5,733,873; US Patent No. 5,198,349; US Patent No. 5,250,421; US Patent No. 5,919,766; and EP 306 968.

[0056] Therapeutic polypeptides such as blood coagulation proteins including Factor IX (FIX), Factor VIII (FVIII), Factor VIIa (FVIIa), von Willebrand Factor (VWF), Factor FV (FV), Factor X (FX), Factor XI (FXI), Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF) and ADAMTS 13 protease are rapidly degraded by proteolytic enzymes and neutralized by antibodies. This reduces their half-life and circulation time, thereby limiting their therapeutic effectiveness. Relatively high doses and frequent administration are necessary to reach and sustain the desired therapeutic or prophylactic effect of these coagulation proteins. As a consequence, adequate dose regulation is difficult to obtain and the need of frequent intravenous administrations imposes restrictions on the patient's way of living.

[0057] As described herein, blood coagulation proteins including, but not limited to, Factor IX (FIX), Factor VIII (FVIII), Factor VIIa (FVIIa), von Willebrand Factor (VWF), Factor FV (FV), Factor X (FX), Factor XI, Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF) and ADAMTS 13 protease are contemplated by the invention. As used herein, the term "blood coagulation protein" refers to any Factor IX (FIX), Factor VIII (FVIII), Factor VIIa (FVIIa), von Willebrand Factor (VWF), Factor FV (FV), Factor X (FX), Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF) and ADAMTS 13 protease which exhibits biological activity that is associated with that particular native blood coagulation protein.

[0058] The blood coagulation cascade is divided into three distinct segments: the intrinsic, extrinsic, and common pathways (Schenone et al., Curr Opin Hematol. 2004;11:272-7). The cascade involves a series of serine protease enzymes (zymogens) and protein cofactors. When required, an inactive zymogen precursor is converted into the active form, which consequently converts the next enzyme in the cascade.

[0059] The intrinsic pathway requires the clotting factors VIII, IX, X, XI, and XII. Initiation of the intrinsic pathway occurs when prekallikrein, high-molecular-weight kininogen, factor XI (FXI) and factor XII (FXII) are exposed to a negatively charged surface. Also required are calcium ions and phospholipids secreted from platelets.

[0060] The extrinsic pathway is initiated when the vascular lumen of blood vessels is damaged. The membrane glycoprotein tissue factor is exposed and then binds to circulating factor VII (FVII) and to small preexisting amounts of its activated form FVIIa. This binding facilitates full conversion of FVII to FVIIa and subsequently, in the presence of calcium and phospholipids, the conversion of factor IX (FIX) to factor IXa (FIXa) and factor X (FX) to factor Xa (FXa). The association of FVIIa with tissue factor enhances the proteolytic activity by bringing the binding sites of FVII for the substrate (FIX and FX) into closer proximity and by inducing a conformational change, which enhances the enzymatic activity of FVIIa.

[0061] The activation of FX is the common point of the two pathways. Along with phospholipid and calcium, factors Va (FVa) and Xa convert prothrombin to thrombin (prothrombinase complex), which then cleaves fibrinogen to form fibrin monomers. The monomers polymerize to form fibrin strands. Factor XIIIa (FXIIIa) covalently bonds these strands to one another to form a rigid mesh.

[0062] Conversion of FVII to FVIIa is also catalyzed by a number of proteases, including thrombin, FIXa, FXa, factor XIa (FXIa), and factor XIIa (FXIIa). For inhibition of the early phase of the cascade, tissue factor pathway inhibitor targets FVIIa/tissue factor/FXa product complex.

Factor VIIa

[0063] FVII (also known as stable factor or proconvertin) is a vitamin K–dependent serine protease glycoprotein with a pivotal role in hemostasis and coagulation (Eigenbrot, Curr Protein Pept Sci. 2002;3:287-99).

[0064] FVII is synthesized in the liver and secreted as a single-chain glycoprotein of 48 kD. FVII shares with all vitamin K–dependent serine protease glycoproteins a similar protein domain structure consisting of an amino-terminal gamma-carboxyglutamic acid (Gla) domain with 9-12 residues responsible for the interaction of the protein with lipid membranes, a carboxy-terminal serine protease domain (catalytic domain), and two epidermal growth factor–like domains containing a calcium ion binding site that mediates interaction with tissue factor. Gamma-glutamyl carboxylase catalyzes carboxylation of Gla residues in the amino-terminal portion of the molecule. The carboxylase is dependent on a reduced form of vitamin K for its action, which is oxidized to the epoxide form. Vitamin K epoxide reductase is required to convert the epoxide form of vitamin K back to the reduced form.

[0065] The major proportion of FVII circulates in plasma in zymogen form, and activation of this form results in cleavage of the peptide bond between arginine 152 and isoleucine 153. The resulting activated FVIIa consists of a NH2-derived light chain (20 kD) and a COOH terminal–derived heavy chain (30 kD) linked via a single disulfide bond (Cys 135 to Cys 262). The light chain contains the membrane-binding Gla domain, while the heavy chain contains the catalytic domain.

[0066] The plasma concentration of FVII determined by genetic and environmental factors is about 0.5 mg/mL (Pinotti et al., Blood. 2000;95:3423-8). Different FVII genotypes can result in several-fold differences in mean FVII levels. Plasma FVII levels are elevated during pregnancy in healthy females and also increase with age and are higher in females and in persons with hypertriglyceridemia. FVII has the shortest half-life of all procoagulant factors (3-6 h). The mean plasma concentration of FVIIa is 3.6 ng/mL in healthy individuals and the circulating half-life of FVIIa is relatively long (2.5 h) compared with other coagulation factors.

[0067] Hereditary FVII deficiency is a rare autosomal recessive bleeding disorder with a prevalence estimated to be 1 case per 500,000 persons in the general population (Acharya et al., J Thromb Haemost. 2004;2248-56). Acquired FVII deficiency from inhibitors is also very rare. Cases have also been reported with the deficiency occurring in association with drugs such as cephalosporins, penicillins, and oral anticoagulants. Furthermore, acquired FVII deficiency has been reported to occur spontaneously or with other conditions, such as myeloma, sepsis, aplastic anemia, with interleukin-2 and antithymocyte globulin therapy.

[0068] Reference polynucleotide and polypeptide sequences include, e.g., GenBank Accession Nos. J02933 for the genomic sequence, M13232 for the cDNA (Hagen et al. PNAS 1986; 83: 2412-6), and P08709 for the polypeptide sequence (references incorporated herein in their entireties). A variety of polymorphisms of FVII have been described, for example see Sabater-Lleal et al. (Hum Genet. 2006; 118:741-51) (reference incorporated herein in its entirety).

Factor IX

[0069] FIX is a vitamin K-dependent plasma protein that participates in the intrinsic pathway of blood coagulation by converting FX to its active form in the presence of calcium ions, phospholipids and FVIIIa. The predominant catalytic capability of FIX is as a serine protease with specificity for a particular arginine-isoleucine bond within FX. Activation of FIX occurs by FXIa which causes excision of the activation peptide from FIX to produce an activated FIX molecule comprising two chains held by one or more disulphide bonds. Defects in FIX are the cause of recessive X-linked hemophilia B.

[0070] Hemophilia A and B are inherited diseases characterized by deficiencies in FVIII and FIX polypeptides, respectively. The underlying cause of the deficiencies is frequently the result of mutations in FVIII and FIX genes, both of which are located on the X chromosome. Traditional therapy for hemophilias often involves intravenous administration of pooled plasma or semi-purified coagulation proteins from normal individuals. These preparations can be contaminated by pathogenic agents or viruses, such as infectious prions, HIV, parvovirus, hepatitis A, and hepatitis C. Hence, there is an urgent need for therapeutic agents that do not require the use of human serum.

[0071] The level of the decrease in FIX activity is directly proportional to the severity of hemophilia B. The current treatment of hemophilia B consists of the replacement of the missing protein by plasma-derived or recombinant FIX (so-called FIX substitution or replacement treatment or therapy).

[0072] Polynucleotide and polypeptide sequences of FIX can be found for example in the UniProtKB/Swiss-Prot Accession No. P00740, and US Pat. No. 6,531,298.

Factor VIII

[0073] Coagulation factor VIII (FVIII) circulates in plasma at a very low concentration and is bound non-covalently to von Willebrand factor (VWF). During hemostasis, FVIII is separated from VWF and acts as a cofactor for activated factor IX (FIXa)-mediated FX activation by enhancing the rate of activation in the presence of calcium and phospholipids or cellular membranes.

[0074] FVIII is synthesized as a single-chain precursor of approximately 270-330 kD with the domain structure A1-A2-B-A3-C1-C2. When purified from plasma (e.g., "plasma-derived" or "plasmatic"), FVIII is composed of a heavy chain (A1-A2-B) and a light chain (A3-C1-C2). The molecular mass of the light chain is 80 kD whereas, due to proteolysis within the B domain, the heavy chain is in the range of 90-220 kD.

[0075] FVIII is also synthesized as a recombinant protein for therapeutic use in bleeding disorders. Various in vitro assays have been devised to determine the potential efficacy of recombinant FVIII (rFVIII) as a therapeutic medicine. These assays mimic the in vivo effects of endogenous FVIII. In vitro thrombin treatment of FVIII results in a rapid increase and subsequent decrease in its procoagulant activity, as measured by in vitro assays. This activation and inactivation coincides with specific limited proteolysis both in the heavy and the light chains, which alter the availability of different binding epitopes in FVIII, e.g. allowing FVIII to dissociate from VWF and bind to a phospholipid surface or altering the binding ability to certain monoclonal antibodies.

[0076] The lack or dysfunction of FVIII is associated with the most frequent bleeding disorder, hemophilia A. The treatment of choice for the management of hemophilia A is replacement therapy with plasma derived or rFVIII concentrates. Patients with severe haemophilia A with FVIII levels below 1%, are generally on prophylactic therapy with the aim of keeping FVIII above 1% between doses. Taking into account the average half-lives of the various FVIII products in the circulation, this result can usually be achieved by giving FVIII two to three times a week.

[0077] Reference polynucleotide and polypeptide sequences include, e.g., *UniProtKB/Swiss-Prot P00451 (FA8_HUMAN); Gitschier J et al., Characterization of the* human Factor VIII gene, Nature, 312(5992): 326-30 (1984); Vehar GH et al., Structure of human Factor VIII, Nature, 312(5992):337-42 (1984); Thompson AR. Structure and Function of the Factor VIII gene and protein, Semin Thromb Hemost, 2003:29;11-29 (2002).

Von Willebrand Factor

[0078] Von Willebrand factor (VWF) is a glycoprotein circulating in plasma as a series of multimers ranging in size from about 500 to 20,000 kD. Multimeric forms of VWF are composed of 250 kD polypeptide subunits linked together by disulfide bonds. VWF mediates initial platelet adhesion to the sub-endothelium of the damaged vessel wall. Only the larger multimers exhibit hemostatic activity. It is assumed that endothelial cells secrete large polymeric forms of VWF and those forms of VWF which have a low molecular weight (low molecular weight VWF) arise from proteolytic cleavage. The multimers having large molecular masses are stored in the Weibel-Pallade bodies of endothelial cells and liberated upon stimulation.

[0079] VWF is synthesized by endothelial cells and megakaryocytes as prepro-VWF that consists to a large extent of repeated domains. Upon cleavage of the signal peptide, pro-VWF dimerizes through disulfide linkages at its C-terminal region. The dimers serve as protomers for multimerization, which is governed by disulfide linkages between the free end termini. The assembly to multimers is followed by the proteolytic removal of the propeptide sequence (Leyte et al., Biochem. J. 274 (1991), 257-261).

[0080] The primary translation product predicted from the cloned cDNA of VWF is a 2813-residue precursor polypeptide (prepro-VWF). The prepro-VWF consists of a 22 amino acid signal peptide and a 741 amino acid propeptide, with the mature VWF comprising 2050 amino acids (Ruggeri Z.A., and Ware, J., FASEB J., 308-316 (1993).

[0081] Defects in VWF are causal to von Willebrand disease (VWD), which is characterized by a more or less pronounced bleeding phenotype. VWD type 3 is the most severe form in which VWF is completely missing, and VWD type 1 relates to a quantitative loss of VWF and its phenotype can be very mild. VWD type 2 relates to qualitative defects of VWF and can be as severe as VWD type 3. VWD type 2 has many sub forms, some being associated with the loss or the decrease of high molecular weight multimers. Von Willebrand disease type 2a (VWD-2A) is characterized by a loss of both intermediate and large multimers. VWD-2B is characterized by a loss of highest-molecular-weight multimers. Other diseases and disorders related to VWF are known in the art.

[0082] The polynucleotide and amino acid sequences of prepro-VWF are available at GenBank Accession Nos. NM_000552 and NP_000543, respectively.

[0083] Other blood coagulation proteins according to the present invention are described in the art, e.g. Mann KG, Thromb Haemost, 1999;82:165-74.

A. **Polypeptides**

[0084] In one aspect, the starting material of the present invention is a protein or polypeptide. As described herein, the term therapeutic protein refers to any therapeutic protein molecule which exhibits biological activity that is associated with the therapeutic protein. In one embodiment of the invention, the therapeutic protein molecule is a full-length protein.

[0085] Therapeutic protein molecules contemplated include full-length proteins, precursors of full length proteins, biologically active subunits or fragments of full-length proteins, as well as biologically active derivatives and variants of any of these forms of therapeutic proteins. Thus, therapeutic protein include those that (1) have an amino acid sequence that has greater than about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% or greater amino acid sequence identity, over a region of at least about 25, about 50, about 100, about 200, about 300, about 400, or more amino acids, to a polypeptide encoded by a referenced nucleic acid or an amino acid sequence described herein; and/or (2) specifically bind to antibodies, e.g., polyclonal or monoclonal antibodies, generated against an immunogen comprising a referenced amino acid sequence as described herein, an immunogenic fragment thereof, and/or a conservatively modified variant thereof.

[0086] According to the present invention, the term "recombinant therapeutic protein" includes any therapeutic protein obtained via recombinant DNA technology. In certain embodiments, the term encompasses proteins as described herein.

[0087] As used herein, "endogenous therapeutic protein" includes a therapeutic protein which originates from the mammal intended to receive treatment. The term also includes therapeutic protein transcribed from a transgene or any other foreign DNA present in said mammal. As used herein, "exogenous therapeutic protein" includes a blood coagulation protein which does not originate from the mammal intended to receive treatment.

[0088] As used herein, "plasma-derived blood coagulation protein" or "plasmatic" includes all forms of the protein found in blood obtained from a mammal having the property participating in the coagulation pathway.

[0089] As used herein "biologically active derivative" or "biologically active variant" includes any derivative or variant of a molecule having substantially the same functional and/or biological properties of said molecule, such as binding properties, and/or the same structural basis, such as a peptidic backbone or a basic polymeric unit.

[0090] An "analog," "variant" or "derivative" is a compound substantially similar in structure and having the same biological activity, albeit in certain instances to a differing degree, to a naturally-occurring molecule. For example, a polypeptide variant refers to a polypeptide sharing substantially similar structure and having the same biological activity as a reference polypeptide. Variants or analogs differ in the composition of their amino acid sequences compared to the naturally-occurring polypeptide from which the analog is derived, based on one or more mutations involving (i) deletion of one or more amino acid residues at one or more termini of the polypeptide and/or one or more internal regions of the naturally-occurring polypeptide sequence (e.g., fragments), (ii) insertion or addition of one or more amino acids at one or more termini (typically an "addition" or "fusion") of the polypeptide and/or one or more internal regions (typically an "insertion") of the naturally-occurring polypeptide sequence or (iii) substitution of one or more amino acids for other amino acids in the naturally-occurring polypeptide sequence. By way of example, a "derivative" refers to a polypeptide sharing the same or substantially similar structure as a reference polypeptide that has been modified, e.g., chemically.

[0091] In various embodiments, analogs, variants or derivatives are designed to allow, for example, conjugation of another molecule to the protein analog, variant or derivative, thereby forming a conjugated protein according to the present invention.

[0092] Variant or analog polypeptides include insertion variants, wherein one or more amino acid residues are added to a therapeutic protein amino acid sequence of the invention. Insertions may be located at either or both termini of the protein, and/or may be positioned within internal regions of the therapeutic protein amino acid sequence. Insertion variants, with additional residues at either or both termini, include for example, fusion proteins and proteins including amino acid tags or other amino acid labels. In one aspect, the blood coagulation protein molecule optionally contains an N-terminal Met, especially when the molecule is expressed recombinantly in a bacterial cell such as E. coli.

[0093] In deletion variants, one or more amino acid residues in a therapeutic protein polypeptide as described herein are removed. Deletions can be effected at one or both termini of the therapeutic protein polypeptide, and/or with removal of one or more residues within the therapeutic protein amino acid sequence. Deletion variants, therefore, include fragments of a therapeutic protein polypeptide sequence.

[0094] In substitution variants, one or more amino acid residues of a therapeutic protein polypeptide are removed and replaced with alternative residues. In one aspect, the substitutions are conservative in nature and conservative substitutions of this type are well known in the art. Alternatively, the invention embraces substitutions that are also non-conservative. Exemplary conservative substitutions are described in Lehninger, [Biochemistry, 2nd Edition; Worth Publishers, Inc., New York (1975), pp.71-77] and are set out immediately below.

CONSERVATIVE SUBSTITUTIONS

SIDE CHAIN CHARACTERISTIC	AMINO ACID
Non-polar (hydrophobic):	
A. Aliphatic	A L I V P
B. Aromatic	F W
C. Sulfur-containing	M
D. Borderline	G
Uncharged-polar:	
A. Hydroxyl	S T Y
B. Amides	N Q
C. Sulfhydryl	C
D. Borderline	G
Positively charged (basic)	K R H
Negatively charged (acidic)	D E

[0095] Alternatively, exemplary conservative substitutions are set out immediately below.

CONSERVATIVE SUBSTITUTIONS II

ORIGINAL RESIDUE	EXEMPLARY SUBSTITUTION
Ala (A)	Val, Leu, Ile
Arg (R)	Lys, Gln, Asn
Asn (N)	Gln, His, Lys, Arg
Asp (D)	Glu
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
His (H)	Asn, Gln, Lys, Arg
Ile (I)	Leu, Val, Met, Ala, Phe,
Leu (L)	Ile, Val, Met, Ala, Phe
Lys (K)	Arg, Gln, Asn
Met (M)	Leu, Phe, Ile
Phe (F)	Leu, Val, Ile, Ala
Pro (P)	Gly
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp, Phe, Thr, Ser
Val (V)	Ile, Leu, Met, Phe, Ala

B. Polynucleotides

[0096] Nucleic acids encoding a therapeutic protein of the invention include, for example and without limitation, genes, pre-mRNAs, mRNAs, cDNAs, polymorphic variants, alleles, synthetic and naturally-occurring mutants.

[0097] Polynucleotides encoding a therapeutic protein of the invention also include, without limitation, those that (1) specifically hybridize under stringent hybridization conditions to a nucleic acid encoding a referenced amino acid sequence as described herein, and conservatively modified variants thereof; (2) have a nucleic acid sequence that has greater than about 95%, about 96%, about 97%, about 98%, about 99%, or higher nucleotide sequence identity, over a region of at least about 25, about 50, about 100, about 150, about

200, about 250, about 500, about 1000, or more nucleotides (up to the full length sequence of 1218 nucleotides of the mature protein), to a reference nucleic acid sequence as described herein. Exemplary "stringent hybridization" conditions include hybridization at 42oC in 50% formamide, 5X SSC, 20 mM Na•PO4, pH 6.8; and washing in 1X SSC at 55oC for 30 minutes. It is understood that variation in these exemplary conditions can be made based on the length and GC nucleotide content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining appropriate hybridization conditions. See Sambrook et al., Molecular Cloning: A Laboratory Manual (Second ed., Cold Spring Harbor Laboratory Press, 1989) §§ 9.47-9.51.

[0098] A "naturally-occurring" polynucleotide or polypeptide sequence is typically from a mammal including, but not limited to, primate, e.g., human; rodent, e.g., rat, mouse, hamster; cow, pig, horse, sheep, or any mammal. The nucleic acids and proteins of the invention can be recombinant molecules (e.g., heterologous and encoding the wild type sequence or a variant thereof, or non-naturally occurring).

C. Production of therapeutic proteins

[0099] Production of a therapeutic protein includes any method known in the art for (i) the production of recombinant DNA by genetic engineering, (ii) introducing recombinant DNA into prokaryotic or eukaryotic cells by, for example and without limitation, transfection, electroporation or microinjection, (iii) cultivating said transformed cells, (iv) expressing therapeutic protein, e.g. constitutively or upon induction, and (v) isolating said blood coagulation protein, e.g. from the culture medium or by harvesting the transformed cells, in order to obtain purified therapeutic protein.

[00100] In other aspects, the therapeutic protein is produced by expression in a suitable prokaryotic or eukaryotic host system characterized by producing a pharmacologically acceptable blood coagulation protein molecule. Examples of eukaryotic cells are mammalian cells, such as CHO, COS, HEK 293, BHK, SK-Hep, and HepG2.

[00101] A wide variety of vectors are used for the preparation of the therapeutic protein and are selected from eukaryotic and prokaryotic expression vectors. Examples of vectors for prokaryotic expression include plasmids such as, and without limitation, pRSET, pET, and pBAD, wherein the promoters used in prokaryotic expression vectors include one or more of, and without limitation, lac, trc, trp, recA, or araBAD. Examples of vectors for eukaryotic expression include: (i) for expression in yeast, vectors such as, and without limitation, pAO,

pPIC, pYES, or pMET, using promoters such as, and without limitation, AOX1, GAP, GAL1, or AUG1; (ii) for expression in insect cells, vectors such as and without limitation, pMT, pAc5, pIB, pMIB, or pBAC, using promoters such as and without limitation PH, p10, MT, Ac5, OpIE2, gp64, or polh, and (iii) for expression in mammalian cells, vectors such as and without limitation pSVL, pCMV, pRc/RSV, pcDNA3, or pBPV, and vectors derived from, in one aspect, viral systems such as and without limitation vaccinia virus, adeno-associated viruses, herpes viruses, or retroviruses, using promoters such as and without limitation CMV, SV40, EF-1, UbC, RSV, ADV, BPV, and β-actin.

[00102] In various embodiments of the invention, therapeutic proteins are modified by conjugating water soluble fatty acid derivatives or water soluble linkers to one or more carbohydrates on the therapeutic protein. Thus, in one embodiment, the therapeutic protein is a glycoprotein and is purified from a host cell that allows glycosylation (i.e., the protein is glycosylated in vivo and subsequently purified as a glycoprotein.). In various embodiments, the therapeutic protein is or is not a glycoprotein and is glycosylated in vitro following purification from a host cell. In vitro glycosylation methods are well known in the art (See, e.g., Meynial-Salles I and Combes D, Journal of Biotechnology 1996, 46:1-14;/ Solá RJ and Griebenow K, BioDrugs 2010, 24:9-21). Of course, one of skill in the art could (1) purify the therapeutic protein; (2) modify the therapeutic protein to allow for in vitro, optionally site-specific, glycosylation (e.g., amino acid deletions/insertion/substitutions); and (3) glycosylate the modified protein in vitro according to procedures known in the art.

D. Administration

[00103] In one embodiment a conjugated therapeutic protein of the present invention may be administered by injection, such as intravenous, intramuscular, or intraperitoneal injection.

[00104] To administer compositions comprising a conjugated therapeutic protein of the present invention to human or test animals, in one aspect, the compositions comprise one or more pharmaceutically acceptable carriers. The terms "pharmaceutically" or "pharmacologically acceptable" refer to molecular entities and compositions that are stable, inhibit protein degradation such as aggregation and cleavage products, and in addition do not produce allergic, or other adverse reactions when administered using routes well-known in the art, as described below. "Pharmaceutically acceptable carriers" include any and all clinically useful solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like, including those agents disclosed above.

[00105] As used herein, "effective amount" includes a dose suitable for treating a disease or disorder or ameliorating a symptom of a disease or disorder. In one embodiment, "effective amount" includes a dose suitable for treating a mammal having a bleeding disorder as described herein.

[00106] The compositions may be administered orally, topically, transdermally, parenterally, by inhalation spray, vaginally, rectally, or by intracranial injection. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intracisternal injection, or infusion techniques. Administration by intravenous, intradermal, intramuscular, intramammary, intraperitoneal, intrathecal, retrobulbar, intrapulmonary injection and or surgical implantation at a particular site is contemplated as well. Generally, compositions are essentially free of pyrogens, as well as other impurities that could be harmful to the recipient.

[00107] Single or multiple administrations of the compositions can be carried out with the dose levels and pattern being selected by the treating physician. For the prevention or treatment of disease, the appropriate dosage will depend on the type of disease to be treated, as described above, the severity and course of the disease, whether drug is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the drug, and the discretion of the attending physician.

[00108] The present invention also relates to a pharmaceutical composition comprising an effective amount of a conjugated therapeutic protein as defined herein. The pharmaceutical composition may further comprise a pharmaceutically acceptable carrier, diluent, salt, buffer, or excipient. The pharmaceutical composition can be used for treating the above-defined bleeding disorders. The pharmaceutical composition of the invention may be a solution or a lyophilized product. Solutions of the pharmaceutical composition may be subjected to any suitable lyophilization process. As an additional aspect, the invention includes kits which comprise a composition of the invention packaged in a manner which facilitates its use for administration to subjects. In one embodiment, such a kit includes a compound or composition described herein (e.g., a composition comprising a conjugated therapeutic protein), packaged in a container such as a sealed bottle or vessel, with a label affixed to the container or included in the package that describes use of the compound or composition in practicing the method. In one embodiment, the kit contains a first container having a composition comprising a conjugated therapeutic protein and a second container having a physiologically acceptable reconstitution solution for the composition in the first container.

In one aspect, the compound or composition is packaged in a unit dosage form. The kit may further include a device suitable for administering the composition according to a specific route of administration. Preferably, the kit contains a label that describes use of the therapeutic protein or peptide composition.

FATTY ACIDS, FATTY ACID DERIVATIVES, AND PROTEIN-FATTY ACID DERIVATIVE CONJUGATES

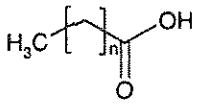
[00109] In one aspect, a therapeutic protein derivative (i.e., a conjugated therapeutic protein) molecule provided herein is bound to a water-soluble fatty acid derivative. As used herein, a "water soluble fatty acid derivative" comprises a fatty acid (i.e., a carboxylic acid) conjugated to a water soluble linker (e.g., an aminooxy linker) as described herein. Such fatty acid derivatives, according to the invention, are stable (i.e., are not released from the protein), water soluble, and capable of binding to human serum albumin.

A. Fatty acids

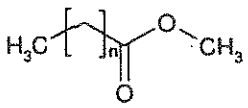
[00110] Fatty acids (i.e., FA or FAs) include, but not are limited to, saturated fatty acids, unsaturated fatty acids, branched chain fatty acids (Mukheriji et al., Prog Lipid Res 2003;42:359-76) and derivatives thereof that are capable of binding human serum albumin according to the present invention.

[00111] By way of example, fatty acids have the following general structure:

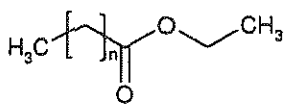
Saturated FA: general structure



Saturated FA methyl ester: general structure



Saturated FA ethyl ester: general structure



[00112] Fatty acids, according to various embodiments of the present invention, also comprise various alternative structures (e.g. methyl- or ethyl esters) or other structures such as containing terminal groups in ω-position (e.g. hydroxyl, amino, thio and carboxyl groups).

[00113] In one embodiment, the fatty acid is a naturally-occuring fatty acid. In various embodiments, the fatty acid is a short chain fatty acid (e.g., less than six carbons), a medium chain fatty acid (e.g., 6-12 carbons), long chain fatty acids (e.g., longer than 12 carbons), or a very long chain fatty acid (e.g., longer than 22 carbons). In another embodiment, the fatty acid has between 4 and 28 carbons. In one embodiment, the fatty acid is in the cis configuration. In still another embodiment, the fatty acid is in the trans configuration.

[00114] In one embodiment, the fatty acid is a saturated fatty acid between 12 and 20 carbons in length. Such fatty acids are known in the art, e.g., C12 (Dodecanoic acid, Lauric acid), C14 (Tetradecanoic acid, Myristic acid), C16 (Hexadecanoic acid, Palmitic acid), C18 (Octadecanoic acid, Stearic acid) and C20 (Eicosanoic acid, Arachidic acid). Examples of unsaturated fatty acids are Myristoleic acid (C14:1), Palmitoleic acid (C16:1), Oleic acid (C18:1), Linoleic acid (C18:2) and Arachidonic acid (C20:4). Most of the fatty acids are commercially available and can be prepared by different chemical methods (Recent Developments in the Synthesis of Fatty Acid Derivatives, Editors: Knothe G and Derksen JTB, AOCS Press 1999, ISBN 1-893997-00-6.)

[00115] In various embodiments of the present invention, the fatty acid comprises 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, or 50 carbons.

B. Fatty acid derivatives

[00116] The present invention provides the preparation of a novel class of activated fatty acid derivatives (FA derivatives) which can bind human serum albumin. The FA derivatives contain a water soluble spacer or linker, which allows the handling and manipulation of FA derivatives in aqueous solution (i.e., the fatty acid derivatives according to the present invention are water soluble, unlike the corresponding fatty acids from which they are derived). In one embodiment, the FA derivatives contain an active aminooxy group, which allows the coupling of the FA derivative to an oxidized carbohydrate moiety (predominantly N-glycans) of therapeutic proteins to form stable oxime linkages. As used herein, a "stable" linkage means that a covalent bond is formed which is "non-releasable" or non-hydrolyzable.

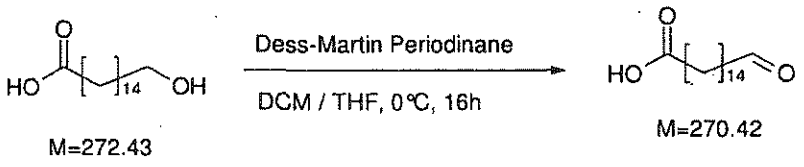
[00117] The chemical modification via carbohydrates might be the preferred option for coagulation proteins like FVIII, FIX and FVIIa to form conjugates with high residual activity.

[00118] By way of example and without limitation, the following strategy represents one embodiment according to the present invention to prepare a water soluble FA derivative containing an active aminooxy group.

[00119] Strategy 1:

[00120] The ω-hydroxy group of a FA derivative (e.g. 16-hydroxyhexadecanoic acid) is subjected to oxidation with Dess Martin periodinane to generate an aldehyde group. In the next step a diaminooxy linker containing a water soluble PEG chain (e.g. 3,6,9-triaoxaundecane-1,11-dioxyamine) is coupled to this aldehyde group to form a stable oxime linkage. The following schematic represents one example according to Strategy 1:

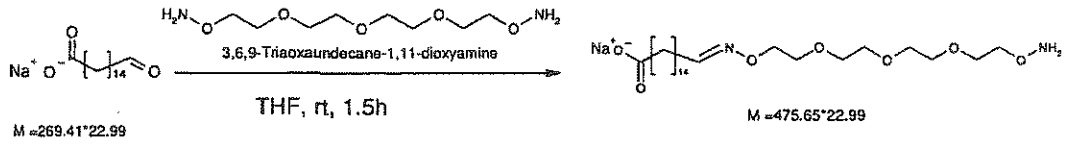
[00121] Step 1: 16-hydroxystearic acid was selectively oxidized with Dess-Martin reagent to yield 16-oxostearic acid. The crude product was purified by chromatography using silica gel as separating agent.



16-hydroxyhexadecanoic acid

16-oxohexadecanoic acid

[00122] Step 2: The 16-oxo moiety of 16-oxostearic acid sodium salt was reacted with 3,6,9-triaoxaundecane-1,11-dioxyamine. The crude product was purified by chromatography using silica gel as separating agent.



16-oxohexadecanoic acid sodium salt

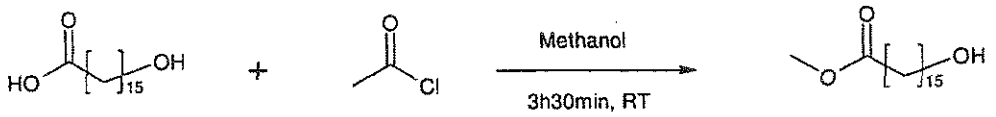
16-(2-(2-(2-(2-Aminoxyethoxy)-ethoxy)-ethoxy)-ethoxyimino)-hexadecanoic acid sodium salt

[00123] Alternatively, another embodiment, the following strategy is employed to prepare a water soluble FA derivatives containing an active aminooxy group.

[00124] Strategy 2:

[00125] The carboxyl group of a ω-hydroxy fatty acid (e.g. 16-hydroxyhexadecanoic acid) is esterified with acetyl chloride. The ω-hydroxy group of this methyl ester derivative is activated with mesyl chloride to introduce a better leaving group. Then the mesyl group is reacted with a diaminooxy linker containing a water soluble PEG chain (e.g. 3,6,9-triaoxaundecane-1,11-dioxyamine) to form a stable aminooxy-methylene bond. Optionally the methyl ester can be hydrolyzed in alkaline solution to generate a free carboxyl group. The following schematic represents one example according to Strategy 2:

[00126] Step 1: The carboxylic acid moiety of 16-hydroxyhexadecanoic acid was protected by forming the methyl ester using acetyl chloride as the methylation reagent.



M= 272.43

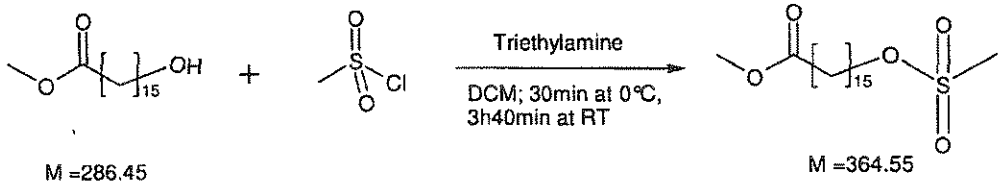
16-Hydroxyhexa-decanoic acid

Acetyl chloride

M= 286.46

16-Hydroxyhexadecanoic acid methyl ester

[00127] Step 2: The 16-hydroxy moiety of 16-hydroxyhexadecanoic acid methyl ester was activated by substituting the hydroxyl group with mesyl, which is the better leaving group.



M =286.45

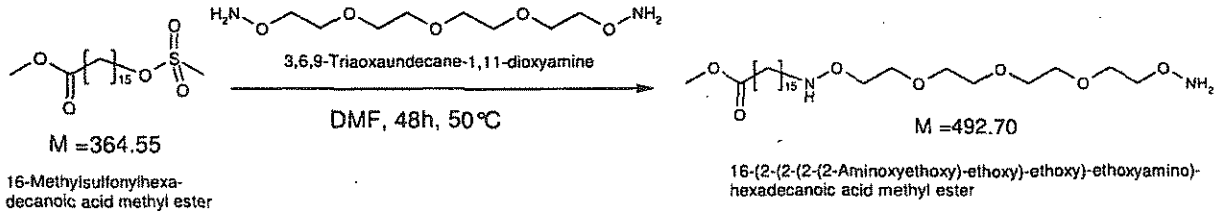
16-Hydroxyhexadeca-noic acid methyl ester

Mesyl chloride

M =364.55

16-Methylsulfonylhexadecanoic acid methyl ester

[00128] Step 3: The 16-mesyl moiety of 16-methylsulfonylhexadecanoic acid methyl ester was substituted by one aminooxy moiety of the bifunctional 3,6,9-triaoxaundecane-1,11-dioxyamine. The crude product is purified by chromatography using silica gel as separating agent.



[00129] Additional strategies are also contemplated by the present invention. The present invention, for example, is not restricted to aminooxy chemistry for coupling to the aldehyde groups of oxidized carbohydrate residues. As described herein, the use of other chemistries including, but not limited to hydrazides for coupling to aldehyde groups, NHS esters for coupling to amino groups and maleimides for coupling to free SH-groups of therapeutic proteins are also contemplated.

[00130] By way of example, a fatty acid methyl ester prepared as described above is reacted with a commercially-available MAL-PEG-COOH (mal-PEG(12)-COOH / IRIS Biotech GmbH, Marktredwitz, Germany) as described herein. By way of still another example, a fatty acid ester with a reactive amino group is reacted with a commercially available NHS-PEG-NHS (NHS-dPEG(4)-NHS / IRIS Biotech GmbH, Marktredwitz, Germany) as described herein.

[00131] Water soluble linkers include, but are not limited to, water soluble polymers (e.g. PEG). The linker can consist of any chemical structure containing one or more functional groups, which increase its water solubility. These functional groups could have the ability to form a negative or positive charge, thereby making the linker water soluble. In one embodiment this functional group includes, but is not limited to a sulfo or carboxyl group. In addition any polar functional group can be used, which makes the linker more water soluble. Examples for this are hydroxyl, amino, amido, maleimido, aminooxy and hydrazide groups as well as N-hydroxy succinimide (NHS) esters and sulfo NHS esters.

[00132] In various embodiments, the water soluble polymer includes, but is not limited to, polyethylene glycol (PEG), branched PEG, polysialic acid (PSA), hydroxyalkyl starch (HAS), hydroxyethyl starch (HES), carbohydrate, polysaccharides, pullulane, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, starch, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG), polyoxazoline, polyacryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate,

polyvinylpyrrolidone, polyphosphazene, polyoxazoline, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, poly(1-hydroxymethylethylene hydroxymethylformal) (PHF), 2-methacryloyloxy-2'-ethyltrimethylammoniumphosphate (MPC).

[00133] In one embodiment, the water soluble polymer is PEG. In various embodiments of the invention, the water soluble polymer comprises a chain length of between approximately 3 to 25 oxygens. For example, the water soluble polymer comprises 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, or 25 oxygens, in various embodiments according the present invention.

C. *Protein-fatty acid derivative conjugates*

[00134] Aminooxy linker systems (i.e., wherein a water soluble fatty acid derivative comprises an aminooxy linker) are contemplated by the present invention. For example, in one embodiment of the invention, the reaction of hydroxylamine or hydroxylamine derivatives with aldehydes (e.g., on a carbohydrate moiety following oxidation by sodium periodate) to form an oxime group is applied to the preparation of protein conjugates. For example, a glycoprotein (e.g., a therapeutic protein according to the present invention) is first oxidized with an oxidizing agent such as sodium periodate (NaIO4) (Rothfus JA et Smith *EL., J Biol Chem 1963, 238, 1402-10*; and Van Lenten L and Ashwell G., *J Biol Chem 1971*, 246, 1889-94). The periodate oxidation of glycoproteins is based on the classical Malaprade reaction described in 1928, the oxidation of vicinal diols with periodate to form an active aldehyde group (Malaprade L., Analytical application, Bull Soc Chim France, 1928, 43, 683-96). Additional examples for such an oxidizing agent are lead tetraacetate (Pb(OAc)4), manganese acetate (MnO(Ac)3), cobalt acetate (Co(OAc)2), thallium acetate (TlOAc), cerium sulfate (Ce(SO4)2) (US 4,367,309) or potassium perruthenate (KRuO4) (Marko et al., J Am Chem Soc 1997,119, 12661-2). By "oxidizing agent" a mild oxidizing compound which is capable of oxidizing vicinal diols in carbohydrates, thereby generating active aldehyde groups under physiological reaction conditions is meant.

[00135] The second step is the coupling of the polymer (e.g., fatty acid derivative) containing an aminooxy group to the oxidized carbohydrate moiety to form an oxime linkage. In one embodiment of the invention, this step can be carried out in the presence of catalytic amounts of the nucleophilic catalyst aniline or aniline derivatives (Dirksen A et Dawson PE, Bioconjugate Chem. 2008; Zeng Y et al., Nature Methods 2009;6:207-9). The aniline

catalysis dramatically accelerates the oxime ligation allowing the use of very low concentrations of the reagents. In another embodiment of the invention the oxime linkage is stabilized by reduction with NaCNBH3 to form an alkoxyamine linkage. Additional catalysts are described below. In another embodiment, this step is carried out in the presence of m-toluidine.

[00136] In one embodiment of the invention, the reaction steps to conjugate a water soluble linker (e.g., fatty acid derivative) to a protein are carried out separately and sequentially (i.e., starting materials (e.g., therapeutic protein, water soluble linker, etc), reagents (e.g., oxidizing agents, aniline, etc) and reaction products (e.g., oxidized carbohydrate on a therapeutic protein, activated aminooxy water soluble linker, etc) are separated between individual reaction steps). In another embodiment, the starting materials and reagents necessary to complete a conjugation reaction according to the present invention is carried out in a single vessel. In one embodiment the native protein is mixed with the aminooxy- polymer reagent. Subsequently the oxidizing reagent is added and the conjugation reaction is performed.

[00137] Additional information on aminooxy technology can be found in the following references, each of which is incorporated in their entireties: EP 1681303A1 (HASylated erythropoietin); WO 2005/014024 (conjugates of a polymer and a protein linked by an oxime linking group); WO96/40662 (aminooxy-containing linker compounds and their application in conjugates); WO 2008/025856 (Modified proteins); Peri F et al., Tetrahedron 1998, 54, 12269-78; Kubler-Kielb J and Pozsgay V., J Org Chem 2005, 70, 6887-90; Lees A et al., Vaccine 2006, 24(6), 716-29; and Heredia KL et al., Macromolecules 2007, 40(14), 4772-9.

[00138] The coupling of the water soluble linker can be carried out by direct coupling to the protein, e.g., via a free sulfhydryl group or free amino group on the protein) or via a linker molecule described herein. The conjugation is in one aspect performed by direct coupling (or coupling via linker systems) of the water soluble linker to a therapeutic protein under formation of stable bonds.

[00139] Thus, in various embodiments of the invention, the fatty acid derivatives described herein are designed to allow conjugation to a therapeutic protein. For example, the fatty acid derivatives are designed to include various terminal reactive groups, as described herein.

[00140] In certain aspects, therapeutic proteins are conjugated to a water soluble fatty acid derivatives by any of a variety of chemical methods (Roberts JM et al., Advan Drug Delivery Rev 2002;54:459-76). For example, in one embodiment a therapeutic protein is modified by the conjugation of fatty acid derivatives to free amino groups of the protein using N-hydroxysuccinimide (NHS) esters. In another embodiment the water soluble fatty acid derivative is coupled to free SH groups using maleimide chemistry. In another embodiment the water soluble fatty acid derivative is coupled by use of hydrazide or aminooxy chemistry to the carbohydrate moieties of the therapeutic protein after prior oxidation.

[00141] In one embodiment of the invention, a therapeutic protein is modified via lysine residues by use of water soluble fatty acid derivatives containing an active N-hydroxysuccinimide (NHS) ester. This derivative reacts with the lysine residues of the therapeutic protein under mild conditions by forming a stable amide bond.

[00142] In another embodiment of the invention, linkage through a peptide bond between a carboxyl group on one of either the protein or fatty acid derivative and an amine group of the protein or fatty acid derivative, or an ester linkage between a carboxyl group of the protein or fatty acid derivative and a hydroxyl group of the therapeutic protein or fatty acid derivative, is contemplated. Another linkage by which the therapeutic protein is covalently bonded to the fatty acid derivative is via a Schiff base, e.g., between a free amino group on the protein being reacted with an aldehyde group formed at a terminal end of a fatty acid. The generated Schiff base is in one aspect stabilized by specific reduction with NaCNBH3 to form a secondary amine. An alternative approach is the generation of terminal free amino groups in the fatty acid derivative by reductive amination with NH4Cl after prior oxidation. Bifunctional reagents can be used for linking two amino or two hydroxyl groups. For example, a fatty acid derivative containing an amino group is coupled to amino groups of the protein with reagents like BS3 (Bis(sulfosuccinimidyl)suberate / Pierce, Rockford, IL). In addition heterobifunctional cross linking reagents like Sulfo-EMCS (N-ε-Maleimidocaproyloxy) sulfosuccinimide ester / Pierce) are used for instance to link amine and thiol groups.

[00143] In another approach, a fatty acid derivative with an active hydrazide group is prepared and coupled to the carbohydrate moiety of the protein after prior oxidation and generation of aldehyde functions.

[00144] As described above, a free amine group of the therapeutic protein reacts with the 1-carboxyl group of a fatty acid derivative to form a peptidyl bond or an ester linkage is formed between the 1-carboxylic acid group and a hydroxyl or other suitable active group on a protein.

[00145] In various embodiments, the therapeutic protein is linked to or associated with the fatty acid derivative in stoichiometric amounts (e.g., 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:7, 1:8, 1:9, or 1:10, etc.). In various embodiments, 1-6, 7-12 or 13-20 fatty acid derivatives are linked to the protein. In still other embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more fatty acid derivatives are linked to the protein.

[00146] In various embodiments, the therapeutic protein is modified to introduce glycosylation sites (i.e., sites other than the native glycosylation sites). Such modification may be accomplished using standard molecular biological techniques known in the art. Moreover, the therapeutic protein, prior to conjugation to a water soluble linker via one or more carbohydrate moieties, may be glycosylated in vivo or in vitro. These glycosylated sites can serve as targets for conjugation of the proteins with water soluble linkers (US Patent Application No. 20090028822, US Patent Application No. 2009/0093399, US Patent Application No. 2009/0081188, US Patent Application No. 2007/0254836, US Patent Application No. 2006/0111279, and DeFrees S. et al., Glycobiology, 2006, 16, 9, 833-43).

[00147] In one embodiment, the conjugated protein retains the full functional activity of native therapeutic protein products, and provides an extended half-life in vivo, as compared to native therapeutic protein products. In another embodiment, the conjugated protein retains at least 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44. 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56,57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, or 150 percent (%) biological activity relative to native protein.

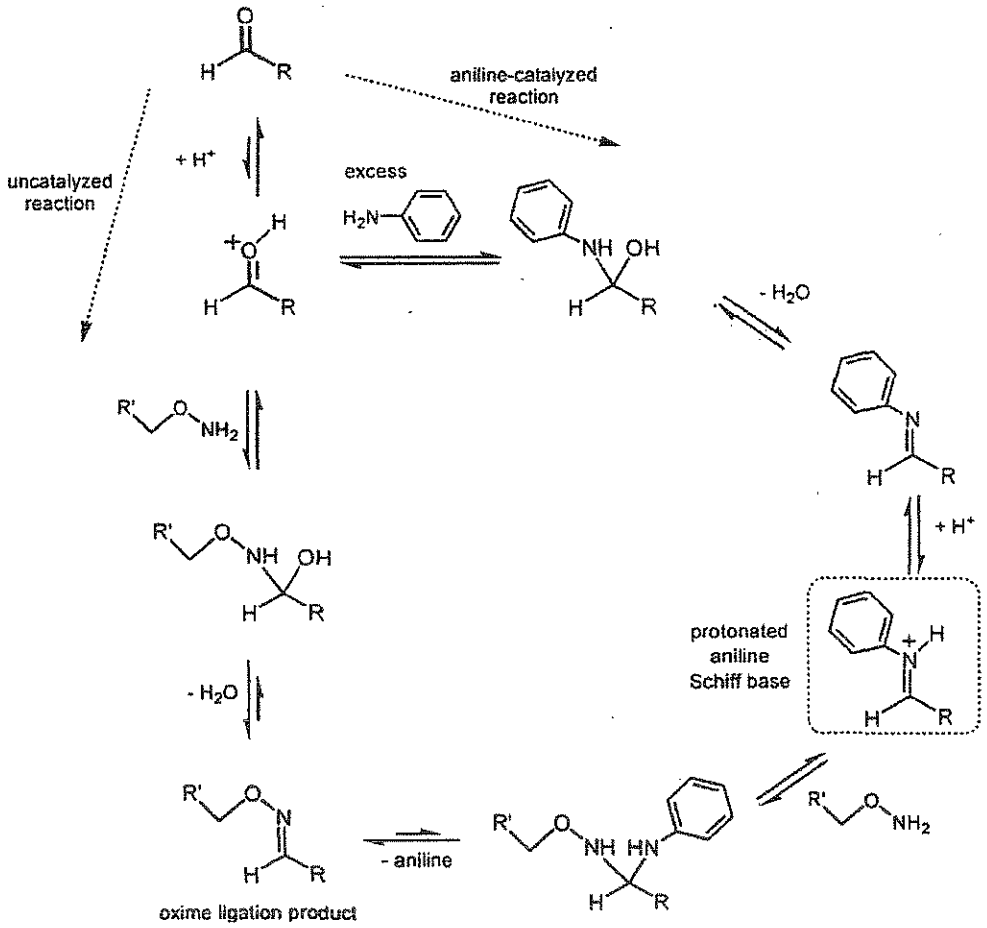
[00148] In a related aspect, the biological activities of the conjugated protein and native blood coagulation protein are determined by the ratios of chromogenic activity to blood coagulation factor antigen value (blood coagulation factor:Chr: blood coagulation factor:Ag).

[00149] In still another embodiment of the invention, the half-life of the conjugated protein is decreased or increased 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, or 10-fold relative to the in vivo half-life of native therapeutic protein.

NUCLEOPHILIC CATALYSTS

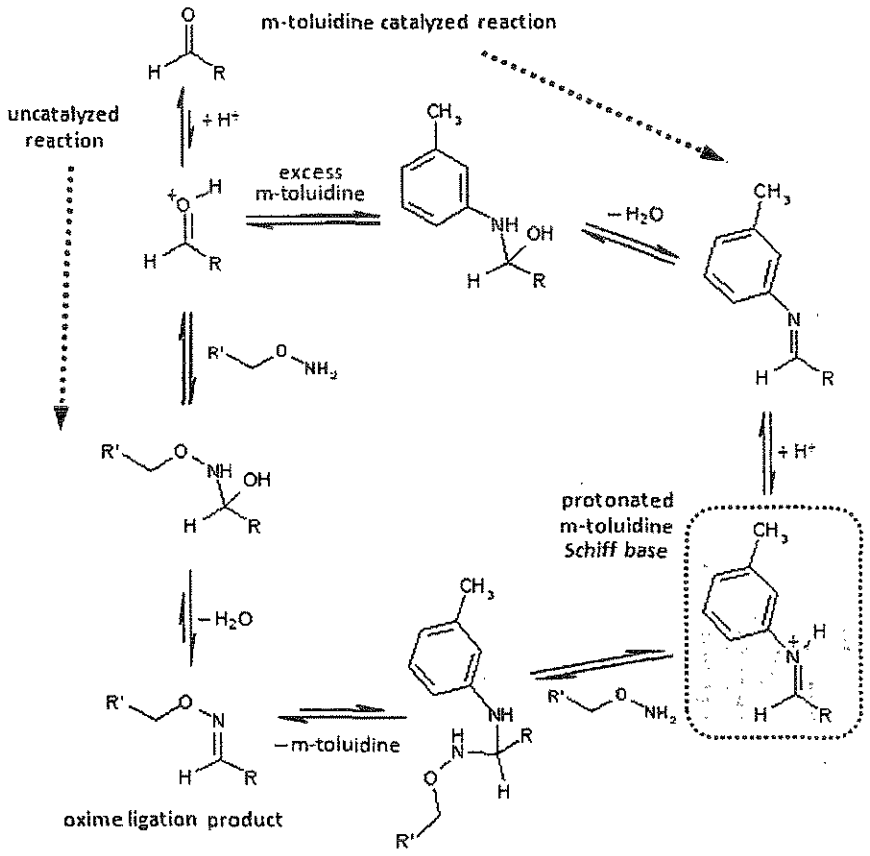
[00150] As described herein, the conjugation of water soluble fatty acid derivatives to therapeutic proteins can be catalyzed by aniline. Aniline strongly catalyzes aqueous reactions of aldehydes and ketones with amines to form stable imines such as hydrazones and oximes. The following diagram compares an uncatalyzed versus the aniline-catalyzed oxime ligation reaction (adapted from Kohler JJ, ChemBioChem 2009;10:2147-50).

:



[00151] However, considering the numerous health risks associated with aniline, alternative catalysts are desirable. The present invention provides aniline derivatives as alternative oxime ligation catalysts. Such aniline derivatives include, but are not limited to, o-amino benzoic acid, m-amino benzoic acid, p-amino benzoic acid, sulfanilic acid, o-

aminobenzamide, o-toluidine, m-toluidine, p-toluidine, o-anisidine, m-anisidine, and p-anisidine. The following diagram compares an uncatalyzed versus the m-toluidine-catalyzed oxime ligation reaction (PCT/US2011/45873):



adapted from Kohler JJ, ChemBioChem 2009;10:2147-50

[00152] In one embodiment of the invention, m-toluidine (aka meta-toluidine, m-methylaniline, 3-methylaniline, or 3-amino-1-methylbenzene) is used to catalyze the conjugation reactions described herein. M-toluidine and aniline have similar physical properties and essentially the same pKa value (m-toluidine:pKa 4.73, aniline:pKa 4.63).

[00153] The nucleophilic catalysts of the invention are useful for oxime ligation (e.g, using aminooxy linkage) or hydrazone formation (e.g., using hydrazide chemistry). In various embodiments of the invention, the nucleophilic catalyst is provided in the conjugation reaction at a concentration of of 0.1, 0.2, 0.3, 0.5, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 11, 12, 13, 14, 15, 16, 17, 18,

19, 20, 25, 30, 35, 40, 45, or 50 mM. In one embodiment, the nucleophilic catalyst is provided between 1 to 10 mM. In various embodiments of the invention, the pH range of conjugation reaction is between 4.0 and 7.0, between 4.5 and 7.0, between 5.0 and 6.5, between 5.0 and 6.5. In various embodiments, the pH of the conjugation reaction is pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5. In one embodiment, the pH is between 5.5 to 6.5.

PURIFICATION OF CONJUGATED PROTEINS

[00154] In various embodiments, purification of a protein that has been incubated with an oxidizing agent and/or a therapeutic protein that has been conjugated with a water soluble fatty acid derivative according to the invention, is desired. Numerous purification techniques are known in the art and include, without limitation, chromatographic methods, filtration methods, and precipitation methods (See, e.g., Guide to Protein Purification, Meth. Enzymology Vol 463 (edited by Burgess RR and Deutscher MP), 2nd edition, Academic Press 2009).

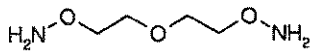
[00155] The following examples are not intended to be limiting but only exemplary of specific embodiments of the invention.

EXAMPLES

Example 1

Preparation of the homobifunctional linker $NH_2[OCH_2CH_2]_2ONH_2$

[00156] The homobifunctional linker $NH_2[OCH_2CH_2]_2ONH_2$

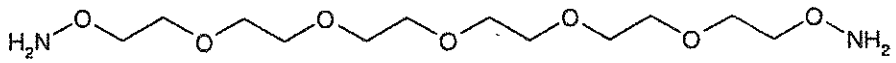


[00157] 3oxapentane-1,5-dioxyamine containing two active aminooxy groups was synthesized according to Boturyn et al. (Tetrahedron 1997;53:5485-92) in a two step organic reaction employing a modified Gabriel-Synthesis of primary amines (Figure 1). In the first step one molecule of 2,2-dichlorodiethylether was reacted with two molecules of endo-N-hydroxy-5-norbornene-2,3-dicarboximide in *DMF*. The desired homobifunctional product was prepared from the resulting intermediate by hydrazinolysis in ethanol.

Example 2

Preparation of the homobifunctional linker $NH_2[OCH_2CH_2]_6ONH_2$

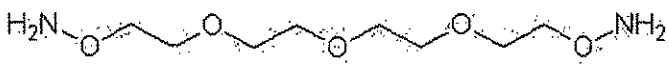
[00158] The homobifunctional linker $NH_2[OCH_2CH_2]_6ONH_2$



[00159] 3,6,9,12,15-penatoxa-heptadecane-1,17-dioxyamine containing two active aminooxy groups was synthesized according to Boturyn et al. (Tetrahedron 1997;53:5485-92) in a two step organic reaction employing a modified Gabriel-Synthesis of primary amines. In the first step one molecule of hexaethylene glycol dichloride was reacted with two molecules of endo-N-hydroxy-5-norbornene-2,3-dicarboximide in DMF. The desired homobifunctional product was prepared from the resulting intermediate by hydrazinolysis in ethanol.

Example 3

Preparation of the homobifunctional linker $NH_2[OCH_2CH_2]_4ONH_2$



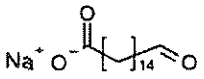
[00160] The homobifunctional linker $NH_2[OCH_2CH_2]_4ONH_2$ (3,6,9-Triaoxaundecane-1,11-dioxyamine) containing two active aminooxy groups was synthesized according to Boturyn et al. (Tetrahedron 1997;53:5485-92) in a two step organic reaction employing a modified Gabriel-Synthesis of primary amines: In the first step one molecule bis-(2-(2-chloroethoxy)-ethyl)-ether was reacted with two molecules endo-N-hydroxy-5-norbornene-2,3-dicarboximide in DMF. The final homobifunctional product was prepared from the resulting intermediate by hydrazinolysis in ethanol.

Example 4

Preparation of 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyimino)-hexadecanoic acid sodium salt

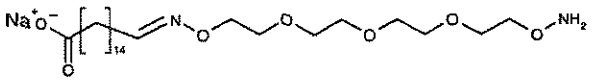
[00161] The fatty acid–aminooxy linker 16-(2-(2-(2-(2-Aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyimino)-hexadecanoic acid sodium salt was synthesized according to Halligan and Nair (Arkivoc 2006 (ii) 101-106) and Hubbs and Heathcock (J Am Chem Soc, 2003;125:12836-43) in a two step reaction.

[00162] Intermediate 1: 16-oxohexadecanoic acid sodium salt



[00163] To a cooled solution (0°C) of 16-hydroxyhexadecanoic acid (800 mg, 2.9 mmol) in dichloromethane (10ml) and tetrahydrofurane (20ml) Dess-Martin periodinane (1636 mg, 3.7 mmol) was added at 0°C. The mixture was stirred for 3.5hrs at 0°C and 2.5hrs at room temperature under Ar-atmosphere. Then a 15%-solution of sodium thiosulfate in saturated sodium bicarbonate solution was added, the mixture was stirred at room temperature for 1.5hrs. Intermediate 1 was extracted with diethylether, after drying over sodium sulfate the organic layer was evaporated to dryness and purified by column chromatography using silica gel as separating agent and a solvent mixture of toluene / ethylacetate. Yield: 34% (white solid).

[00164] Product: 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyimino)-hexadecanoic acid sodium salt



[00165] To a solution of 3,6,9-triaoxaundecane-1,11-dioxyamine (146mg, 0.65mmol) in anhydrous tetrahydrofurane (3ml) Intermediate 1 (19.1 mg, 0.07 mmol) was added. The mixture was stirred for 1.5 hrs at room temperature under Ar-atmosphere. Then the mixture was evaporated to dryness and purified by column chromatography using silica gel as separating agent and a solvent mixture of dichloromethane / methanol / Huenig's base. Yield: 71% (white solid).

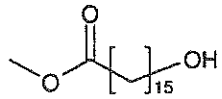
[00166] Mass spectrometry (ESI): m/z = 477,3529 for $[M+2H]^+$

Example 5

Preparation of 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexadecanoic acid methyl ester

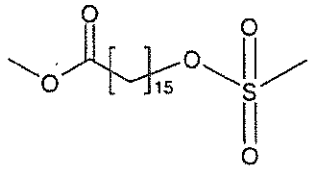
[00167] The fatty acid methyl ester–aminooxy linker 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexadecanoic acid methyl ester was synthesized according to Hang et al. (J Am Chem Soc 2007;129:2744-5) in a three step reaction.

[00168] Intermediate 1: 16-hydroxyhexadecanoic acid methyl ester



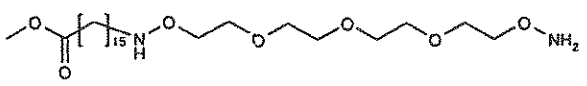
[00169] To a cooled (0°C) solution of 16-hydroxyhexadecanoic acid (3000 mg, 10.79mmol) in anhydrous methanol (27 ml) acetyl chloride (3.837 ml, 53.96 mmol) was added dropwise at 0°C within 2min, then the mixture was stirred at room temperature for 3.5hrs under Ar-atmosphere. Subsequently the mixture was evaporated to dryness, the residue was dissolved in dichloromethane (100 ml), washed with saturated sodium bicarbonate solution (2x50ml) and Brine solution (1x50 ml). After drying over sodium sulfate the collected organic layer was evaporated to dryness and vacuum dried at room temperature. Yield: 92% (white solid).

[00170] Intermediate 2: 16-methylsulfonylhexadecanoic acid methyl ester



[00171] To a cooled solution (0°C) of intermediate1 (2807 mg, 9.80 mmol) in dichloromethane (40ml) triethylamine (1.502 ml, 10.78mmol) was added, then a precooled solution (0°C) of mesyl chloride (0.834ml, 10.78 mmol) in dichloromethane (5ml) was added dropwise within 10min at 0°C. The mixture was stirred for 30min at 0°C and for 2.75hrs at room temperature. Then the mixture was diluted with dichloromethane (150 ml), washed with water (1x100 ml), saturated sodium bicarbonate solution (2x100 ml) and Brine solution (1x100 ml). After drying over sodium sulfate the collected organic layer was evaporated to dryness and vacuum dried at room temperature. Yield: 95% (white pale yellow solid).

[00172] Product: 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexadecanoic acid methyl ester



[00173] To a solution of 3,6,9-triaoxaundecane-1,11-dioxyamine (517 mg, 2.30 mmol) in anhydrous N,N-dimethylformamide (22 ml) a solution of intermediate 2 (70 mg, 0.19mmol) in anhydrous N,N-dimethylformamide (7ml) was added dropwise for 1hr at room temperature under Ar-atmosphere; the mixture was stirred for 2 days at different temperatures (room temperature, 50°C, 80°C) to complete the reaction. Then the mixture was evaporated to dryness, the crude product was purified by column chromatography using silica gel as separating agent and a solvent mixture of dichloromethane / methanol. Yield: 31% (colorless partially solidified white oil).

[00174] Mass spectrometry (ESI): m/z = 493,3824 for $[M+H]^+$

Example 6

Coupling of 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexa-decanoic acid methyl ester to the carbohydrate moiety of coagulation Factor VIII

[00175] FA-rFVIII is prepared by using a two-step procedure. In the first step rFVIII is oxidized with $NaIO_4$ and purified by anion exchange chromatography (AEC). Subsequently the oxidized rFVIII is modified with the FA-aminoxy reagent.

[00176] rFVIII (45mg starting material) is oxidized with $NaIO_4$ (final concentration 200 µM). After an incubation time of 30 minutes (22°C), the oxidation reaction is stopped by adding an 1 M aqueous L-cysteine solution (final concentration: 10 mM). The oxidized rFVIII is purified by anion exchange chromatography on EMD TMAE(M). Then 25 µl of a 10% (w/v) solution of 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexa-decanoic acid methyl ester (prepared according to Example 3) in DMSO is added to the eluate (protein concentration 2 mg/ml) and the coupling reaction is performed for 18 hours at 4°C. Then the FA-rFVIII conjugate is further purified by HIC on Phenyl Sepharose 4 FF. Finally the eluate is concentrated by UF/DF using a 30kD membrane (MILLIPORE).

Example 7

Coupling of 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexa-decanoic acid methyl ester to the carbohydrate moiety of coagulation Factor IX

[00177] 10 mg rFIX is dissolved in reaction buffer (20 mM L-histidine, 5 mM $CaCl_2$, 150 mM NaCl, pH 6.0) to give a final concentration of 2.5 mg/ml. To this solution a 5mM aqueous $NaIO_4$ solution was added to get a final concentration of 100µM. The reaction mixture was incubated for 1h at 4°C under gentle stirring in the dark. Then the mixture is

loaded onto a pre-equilibrated PD-10 desalting colums for removal of excess $NaIO_4$. To this mixture 8 µl of of a 10% (w/v) solution of 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexa-decanoic acid methyl ester (prepared according to Example 3) in DMSO is added and the coupling reaction is carried out at pH 6.0 for 18 h at 4°C. Then the conjugate is further purified by IEX on Q-Sepharose FF. Finally the eluate is concentrated by UF/DF using Vivaspin devices.

Example 8

Coupling of 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexa-decanoic acid methyl ester to the carbohydrate moiety of coagulation Factor VIIa

[00178] 10 mg rFVIIa is dissolved in reaction buffer ((50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to give a final concentration of 2.0 mg/ml. To this solution a 5 mM aqueous $NaIO_4$ solution is added to get a final concentration of 50 µM. The reaction mixture is incubated for 1h at 4°C under gentle stirring in the dark. Then the mixture is loaded onto a pre-equilibrated PD-10 desalting colums for removal of excess $NaIO_4$. To this mixture 8 µl of of a 10% (w/v) solution of 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexa-decanoic acid methyl ester (prepared according to Example 3) in DMSO is added and the coupling reaction is carried out at pH 6.0 for 18 h at 4°C. Then the conjugate is further purified by IEX on Q-Sepharose FF. Finally the eluate is concentrated by UF/DF using Vivaspin devices.

Example 9

Coupling of 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexa-decanoic acid methyl ester to the carbohydrate moiety of coagulation Factor VIII

[00179] 10 mg rFVIII are disssolved in Hepes-buffer (50 mM Hepes, 150 mM NaCl, 5 mM calcium chloride, pH 6.0) to give a protein concentration of 2 mg/ml. Then 10 µl of a 10% (w/v) solution of 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexa-decanoic acid methyl ester (prepared according to Example 3) in DMSO is added to the FVIII solution. Subsequently an aqueous solution of the nucleophilic catalyst m-toluidine (50 mM) is prepared and added to the mixture within 15 minutes to get a final concentration of 10 mM. Finally a 40 mM aqueous sodium periodate solution is added to give a concentration of 400 µM. The reaction mixture is incubated for 120 minutes in the dark at a temperature of 22 °C under gentle stirring. Then the reaction is stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation

for 60 min. Subsequently the reaction mixture is loaded onto an IEX column filled with Q-Sepharose FF (1.6 x 8 cm). The column is washed with 20 column volumes equilibration buffer (20 mM Hepes, 5 mM $CaCl_2$, pH 7.4) and the FA-rFVIII conjugate is eluted with buffer B (20 mM Hepes, 5. mM $CaCl_2$, 0.5 M NaCl, pH 7.4). Finally the product is subjected to UF/DF with Vivaspin devices using Hepes buffer, 7.4 (20 mM Hepes, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4) as diafiltration buffer.

Example 10

Coupling of 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexa-decanoic acid methyl ester to the carbohydrate moiety of coagulation Factor IX

[00180] 7 mg rFIX are dissolved in His – buffer (20 mM His, 150 mM NaCl, 5 mM $CaCl_2$, pH 6.0) to give a protein concentration of 2 mg/ml. Then 7 µl of a 10% (w/v) solution of 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexa-decanoic acid methyl ester (prepared according to Example 3) in DMSO is added to the FIX solution. Then an aqueous solution of the nucleophilic catalyst m-toluidine (50 mM) is prepared and added to the mixture within 15 minutes to get a final concentration of 10 mM. Finally a 40 mM aqueous sodium periodate solution is added to give a concentration of 250 µM. The reaction mixture is incubated for 120 minutes in the dark at a temperature of 22 °C under gentle stirring. Subsequently the reaction is quenched by the addition of L-cysteine (final concentration: 5 mM) for 30 min at room temperature.

[00181] The FA-rFIX conjugate is purified by anion exchange chromatography. The reaction mixture is diluted with 10 ml buffer A (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5) and loaded onto a 5ml HiTrap Q FF column (GE Healthcare, Fairfield, CT) equilibrated with buffer A. The column is washed with 5 CV using the same buffer. Then the column is eluted with buffer B (50 mM Hepes, 1 M NaCl, 5 mM $CaCl_2$, pH 7.5). The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose. The final diafiltration step is performed against 20 mM Hepes buffer, pH 7.2 containing 150 mM NaCl and 5 mM $CaCl_2$.

Example 11

Coupling of 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexa-decanoic acid methyl ester to the carbohydrate moiety of coagulation Factor VIIa

[00182] 10 mg rFVIIa are dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0). Then 10 µl of a 10% (w/v) solution of 16-(2-(2-

(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexa-decanoic acid methyl ester (prepared according to Example 3) *in DMSO is added to the FVIIa solution. Subsequently* an aqueous solution of the nucleophilic catalyst m-toluidine (50 mM) is prepared and added to the mixture within 15 minutes to get a final concentration of 10 mM. Finally a 40 mM aqueous sodium periodate solution is added to give a concentration of 250 µM. The reaction mixture is incubated for 120 minutes in the dark at a temperature of 22 °C under gentle stirring. Subsequently the reaction is quenched by the addition of L-cysteine (final concentration: 5 mM) for 30 min at room temperature. The FA-rFVIIa conjugate is purified by anion exchange chromatography. The reaction mixture is diluted with 10 ml buffer A (50 mM Hepes, pH 7.4) and loaded onto a 5 ml HiTrap Q FF column (GE Healthcare, Fairfield, CT) equilibrated with buffer A. The column is washed with 5 CV using the same buffer. Then the column is eluted with buffer B (50 mM Hepes, 1 M NaCl, 5 mM $CaCl_2$, pH 7.4). The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose. The final diafiltration step is performed against 20 mM Hepes buffer, pH 7.2 containing 150 mM NaCl and 5 mM $CaCl_2$.

Example 12

Coupling of a FA derivative containing an active aminooxy group to an oxidized carbohydrate moiety in the presence of a nucleophilic catalyst

[00183] The coupling of a FA derivative containing an active aminooxy group to an oxidized therapeutic protein (such as the proteins set out in Table 1 herein) is also provided.

[00184] For coupling of a FA derivative containing an aminooxy group, the carbohydrate moieties (predominantly N-glycans) of a therapeutic protein (e.g., EPO, G-CSF, or insulin; *see Table 1; concentration: 0.5- 3 mg/ml) are first oxidized with* $NaIO_4$ *(concentration: 200* µM). Then the reaction is stopped by addition of L-cysteine (final concentration: 5 mM) and the reagents are separated by UF/DF. After diafiltration the FA aminooxy reagent (i.e., the FA derivative) is added using a 25 M excess and the coupling reaction is performed at pH 6.0 for 1 hour at room temperature under gentle stirring in the presence of the nucleophilic *catalyst m-toluidine (concentration: 10 mM). Subsequently the reaction mixture is loaded* onto an ion exchange (IEX) column. The column is washed with > 5 CV washing buffer and the conjugate is eluted with a linear NaCl gradient. Finally the conjugate containing fractions are subjected to UF/DF.

Example 13

Coupling of a FA derivative containing an active aminooxy group to an oxidized carbohydrate moiety in the presence of a nucleophilic catalyst

[00185] For coupling of a FA derivative containing an aminooxy group to the carbohydrate moiety of a therapeutic protein the protein (e.g., EPO, G-CSF, or insulin; see Table 1; concentration: 0.5- 3 mg/ml) is incubated at pH 6.0 with $NaIO_4$ (concentration: 300 μM) for 1 hour at room temperature in the presence of the nucleophilic catalyst m-toluidine (concentration: 10 mM). Then the reaction is stopped by addition of L-cysteine (final concentration: 5 mM) and the reaction mixture is loaded onto an ion exchange (IEX) column. The column is washed with > 5 CV washing buffer and the conjugate is eluted with a linear NaCl gradient. Finally the conjugate containing fractions are subjected to UF/DF.

Example 14

Coupling of a FA derivative containing an active aminooxy group to an oxidized blood coagulation protein in the presence of a nucleophilic catalyst

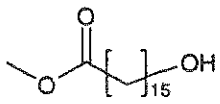
[00186] The coupling of a FA derivative containing an active aminooxy group to an oxidized blood coagulation protein (such as FIX, FVIII and FVIIa as described in the above examples) may be carried out in the presence of a nucleophilic catalyst such as m-toluidine in a concentration range of 2-20 mM.

Example 15

Preparation of a water soluble MAL fatty acid linker

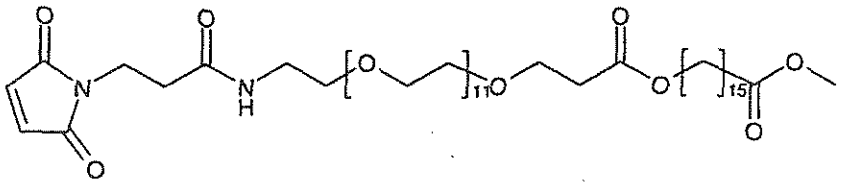
[00187] A fatty acid linker containing a water soluble PEG chain in ω-position and an active MAL group is prepared in a two-step synthesis:

[00188] Step 1: Preparation of 16-hydroxyhexadecanoic acid methyl ester:



[00189] Commercially available 16-hydroxyhexadecanoic acid is esterified with acetyl chloride in methanol for 5hrs at room temperature according to Example 3 to give the corresponding methyl ester (Hang et al., Chemical probes for the rapid detection of fatty-acylated proteins in mammalian cells, JACS 2007;129:2744-5).

[00190] Step 2: Preparation of the MAL fatty acid linker:



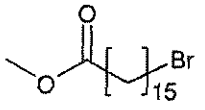
[00191] 16-hydroxyhexadecanoic acid methyl ester is reacted with commercially available MAL-PEG-COOH (mal-Peg(12)-COOH / IRIS Biotech GmbH, Marktredwitz, Germany) employing a Mitsunobu reaction in THF at room temperature over night (Toyokuni et al., Synthesis of a New Heterobifunctional Linker, N-[4-(Aminooxy)butyl]-maleimide, for Facile Access to a Thiol-Reactive 18F-Labeling Agent, Bioconjugate Chem 2003;14:1253-9).

Example 16

Preparation of a water soluble NHS fatty acid linker

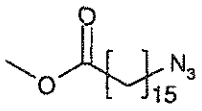
[00192] A fatty acid linker containing a water soluble PEG chain in ω-position and a terminal active NHS ester is prepared by use of a four-step synthesis:

[00193] Step 1: Preparation of 16-bromohexadecanoic acid methyl ester



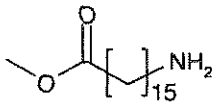
[00194] Commercially available 16-bromohexadecanoic acid is esterified with methanol and catalytic amount of concentrated sulfuric acid over night at reflux temperature to give the corresponding methyl ester (Zinic et al., Positionally Isomeric Organic Gelators: Structure–Gelation Study, Racemic versus Enantiomeric Gelators, and Solvation Effects, Chem Eur J 2010;16:3066-82).

[00195] Step 2: Preparation of 16-azidohexadecanoic acid methyl ester



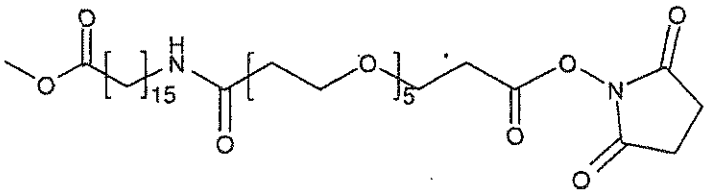
[00196] 16-bromohexadecanoic acid methyl ester is reacted with sodium azide in acetonitrile for four days at reflux temperature to give the corresponding azide (Zinic et al., Positionally Isomeric Organic Gelators: Structure–Gelation Study, Racemic versus Enantiomeric Gelators, and Solvation Effects, Chem. Eur. J. 2010;16:3066-82).

[00197] Step 3: Preparation of 16-aminohexadecanoic acid methyl ester



[00198] 16-azidohexadecanoic acid methyl ester is catalytically hydrogenated with palladium / activated charcoal in methanol at 3bar for three hours to give the corresponding amine (Zinic et al., Positionally Isomeric Organic Gelators: Structure–Gelation Study, Racemic versus Enantiomeric Gelators, and Solvation Effects, Chem. Eur. J. 2010;16:3066-82).

[00199] Step 4: Preparation of the NHS fatty acid linker



[00200] 16-aminohexadecanoic acid methyl ester is reacted with commercially available NHS-PEG-NHS (NHS-dPEG(4)-NHS / IRIS Biotech GmbH, Marktredwitz, Germany) in 1,4-dioxane at room temperature for three days to give the NHS fatty acid linker (Cline et al., The Aminolysis of N-Hydroxysuccinimide Esters. A Structure-Reactivity Study, JACS 1987;109:3087-91).

Example 17

Analytical characterization of protein FA conjugates

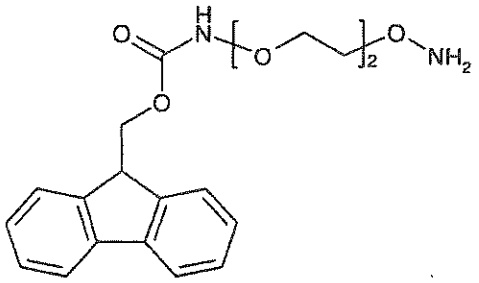
[00201] The albumin binding properties of the FA-rFVIII, rFIX or FVIIa samples prepared according to the examples herein are verified in vitro by use of Enzyme Linked Immunosorbent Assay (ELISA) systems. A 96 well plate is coated with a polyclonal antibody directed against human serum albumin (HSA). The next step is the blocking of the ELISA plate with a PBS-gelatine buffer. Then HSA is bound to the antibody followed by binding of the FA-protein sample, which is diluted to different concentrations. Finally the HSA-protein sample is detected by a peroxidase labeled anti-VIII, anti-FIX or anti FVIIa antibody. Peroxidase activity is detected by using tetramethyl-benzidine (TMB) as substrate. The developed color intensity is measured with an ELISA reader at 450 nm. The binding of the FA-protein sample to HSA is evaluated by plotting the different sample concentrations on the x-axis and their corresponding absorbance values on the y-axis.

Example 18

Preparation of a water soluble PEGylated fatty acid linker containing an aminooxy group

[00202] A fatty acid linker containing a water soluble PEG chain with an aminooxy moiety at the ω-position connected to the fatty acid carboxyl group is prepared in a three step synthesis using 3-oxapentane-1,5-dioxyamine as described in Example 1.

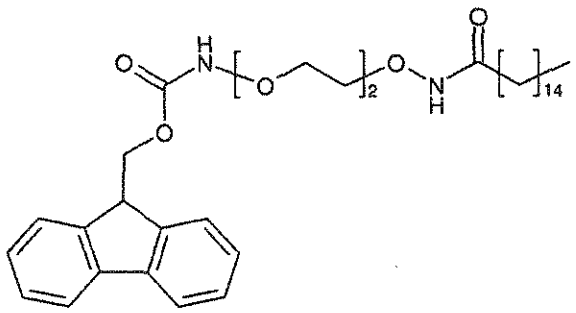
[00203] Step 1: Preparation of N-(9-fluorenylmethoxycarbonyl)-3-oxapentane-1,5-dioxyamine



[00204] 3-oxapentane-1,5-dioxyamine was reacted with 9-fluorenylmethylchloroformate in 1,4-dioxane at ambient temperature for 1 hour. The solution was evaporated under reduced pressure and the crude product was purified employing silica gel chromatography with dichloromethane / methanol 20/1 (v/v) as the solvent mixture to give pure mono-FMOC

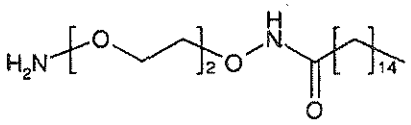
protected dioxyamine (Boturyn et al., Synthesis of Fluorescent Probes for the Detection of Abasic Sites in DNA, Tetrahedron 1997; Vol.53, No.15, 5485-92).

[00205] Step 2: Preparation of hexadecanoic acid (2-(2-(N-(9-fluorenylmethoxycarbonyl) aminooxy) ethoxy) ethoxy) amide



[00206] N-(9-fluorenylmethoxycarbonyl)-3-oxapentane-1,5-dioxyamine is reacted with commercially available palmitic acid N-hydroxysuccinimide ester in THF at ambient temperature over night to give the mono-FMOC protected aminooxy-fatty acid conjugate (Jong et al., Synthesis of ceramides using N-hydroxysuccinimide esters, Journal of Lipid Research 1972; Vol.13, 819-22).

[00207] Step 3: Preparation of hexadecanoic acid (2-(2-aminooxyethoxy) ethoxy) amide



[00208] Mono-FMOC protected aminooxy-fatty acid conjugate is reacted with Piperidine in dichloromethane at ambient temperature to give the deprotected aminooxy-fatty acid linker as the final product (Boturyn et al., Synthesis of Fluorescent Probes for the Detection of Abasic Sites in *DNA*, Tetrahedron 1997; Vol.53, No.15, 5485-92).

Example 19

Coupling of a MAL fatty acid linker with A1PI

[00209] A fatty acid linker containing a MAL group is prepared as described in Example 15 by reaction of 16-hydroxyhexadecanoic acid methyl ester with commercially available MAL-PEG-COOH (*mal-Peg(12)-COOH / IRIS Biotech GmbH, Marktredwitz, Germany*) employing a Mitsunobu reaction. This linker is coupled to the free SH-group of A1PI.

[00210] 10 mg of purified A1PI (concentration: 1 mg/ml) is dissolved in reaction buffer (20 mM phosphate, 5 mM EDTA, pH 7.0) and 10 µL of a 5% (w/v) solution of the MAL fatty acid linker in DMSO is added to the A1PI solution. The modification reaction is performed for 2 hours at room temperature followed by a quenching step using L-cysteine (final concentration: 10 mM). After the addition of L-cysteine the reaction mixture is incubated under gentle shaking for an additional hour at the same temperature. The modified A1PI is diluted with equilibration buffer (25 mM phosphate, pH 6.5) to correct the solutions conductivity to < 4.5 mS/cm and loaded onto a pre-packed HiTrap Q FF (GE-Healthcare) with a column volume (CV) of 5 ml. Then the column is equilibrated with 10 CV equilibration buffer (flow rate: 2 ml/min). Finally the PEG-A1PI is eluted with a linear gradient with elution buffer (25 mM Na_2HPO_4. 1 M NaCl, pH 6.5).

Example 20

Coupling of a NHS fatty acid linker with coagulation factor VIII

[00211] A fatty acid linker containing a NHS group is prepared as described in Example 16 by reaction of 16-hydroxyhexadecanoic acid methyl ester with commercially available NHS-PEG-NHS (NHS-dPEG(4)-NHS (IRIS Biotech GmbH, Marktredwitz, Germany). This linker is coupled to the free amino groups of lysine residues of coagulation factor VIII.

[00212] 10 mg rFVIII is disssolved in Hepes-buffer (50 mM Hepes, 150 mM NaCl, 5 mM calcium chloride, pH 6.0) to give a protein concentration of 2 mg/ml. Then 10 µl of a 10% (w/v) solution of NHS fatty acid linker in DMSO is added to the FVIII solution. The reaction mixture is incubated for 120 minutes in the dark at a temperature of 22 °C under gentle stirring. Then the reaction is stopped by the addition of an aqueous glycine solution (1 M) to give a final concentration of 20 mM in the reaction mixture. The mixture is incubated for 15 min at room temperature under gentle stirring and subsequently loaded onto an IEX column filled with Q-Sepharose FF (1.6 x 8 cm). The column is washed with 20 column volumes equilibration buffer (20 mM Hepes, 5 mM $CaCl_2$, pH 7.4) and the FA-rFVIII conjugate is eluted with buffer B (20 mM Hepes, 5 mM $CaCl_2$, 0.5 M NaCl, pH 7.4). Finally the product is subjected to UF/DF with Vivaspin devices using Hepes buffer, 7.4 (20 mM Hepes, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4) as diafiltration buffer.

Claims

1. A water soluble fatty acid derivative comprising a fatty acid or fatty acid ester attached to a water soluble linker, said fatty acid derivative stably attached to a therapeutic protein, wherein the water soluble linker comprises a water soluble polymer, at least one first functional group attached to the therapeutic protein, wherein the first functional group is an aminooxy group, and a second functional group attached to the fatty acid or fatty acid ester, wherein the second functional group is an aminooxy group.

2. The fatty acid derivative according to claim 1 that binds human serum albumin (HSA) in vitro or in vivo,

has increased half-life relative to a native therapeutic protein, and

wherein the fatty acid is a saturated fatty acid or unsaturated fatty acid.

3. The fatty acid derivative of claim 2 wherein the fatty acid is a branched chain fatty acid.

4. The fatty acid derivative according to any of claims 1-3 wherein the fatty acid comprises a chain length selected from the group consisting of C10, C12, C14, C16, C18, C20, C22, and C24.

5. The fatty acid derivative according to any of claims 1-4 wherein the fatty acid is attached to the water soluble linker at a group on the fatty acid selected from the group consisting of: terminal carboxyl group and ω-group,

wherein the ω-group is selected from the group consisting of: hydroxyl, amino, thio, and carboxyl.

6. The fatty acid derivative according to claim 1 or 2 wherein the fatty acid is 16-hydroxyhexadecanoic acid or the fatty acid ester is 16-hydroxyhexadecanoic acid methyl ester.

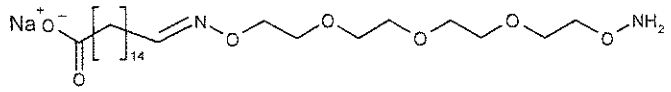
7. The fatty acid derivative according to claim 1 or 2 wherein the fatty acid ester is selected from the group consisting of: methyl ester and ethyl ester.

8. The fatty acid derivative according to any one of claims 1-3 wherein the fatty acid derivative is stably attached to the therapeutic protein by an oxime linkage,

wherein the oxime linkage is formed between an aminooxy group on the water soluble linker and an aldehyde group of an oxidized carbohydrate on the therapeutic protein.

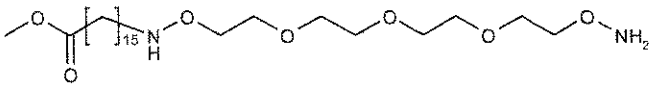
9. The fatty acid derivative according to any one of claims 1-3 wherein the fatty acid derivative is selected from the group consisting of:

a) 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyimino)-hexadecanoic acid sodium salt of the formula:



; and

b) 16-(2-(2-(2-(2-aminooxyethoxy)-ethoxy)-ethoxy)-ethoxyamino)-hexadecanoic acid methyl ester,



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10. A method of preparing a fatty acid derivative according to claim 1 comprising:

a) oxidizing an ω-hydroxy group on a fatty acid to generate an aldehyde group on the fatty acid; and

b) coupling a water soluble linker comprising an active aminooxy group to the aldehyde group to form a stable oxime linkage;

wherein said fatty acid derivative is water soluble;

wherein the ω-hydroxy group is oxidized by an oxidation reagent selected from the group consisting of: Dess Martin periodinane reagent, Tempo reagent, oxalyl chloride/DMSO, tetrapropylammoniumperruthenate (TPAP) and crome VI reagents (Collins reagent, pyridinium chloro chromate (PCC) and pyridinium dichromate);

wherein the fatty acid is a saturated fatty acid or unsaturated fatty acid.

11. A method of preparing a fatty acid derivative according to claim 1 comprising:

a) esterifying a carboxyl group on a fatty acid to generate an ester on the fatty acid;

b) activating an ω-hydroxy group on a fatty acid to generate a mesyl group on the fatty acid of step a); and

c) coupling a water soluble linker comprising an active aminooxy group by substituting the mesyl group of step b) thereby forming a stable oxyimine-methylene bond;

wherein said fatty acid derivative is water soluble;

wherein the carboxyl group is esterified by an esterifying agent selected from the group consisting of: acetyl chloride, methanol in the presence of acid, ethanol in the presence of acid, diazomethane, and methyliodide;

wherein the ω-hydroxy group is activated by an activating agent selected from the group consisting of: mesyl chloride, tosyl chloride and nosyl chloride; and

wherein the fatty acid is a saturated fatty acid or unsaturated fatty acid.

12. The method of claim 10 or 11 wherein the fatty acid is a branched chain fatty acid.

13. The method according to claim 10 or 11 wherein the fatty acid comprises a chain length selected from the group consisting of C10, C12, C14, C16, C18, C20, C22, and C24, optionally

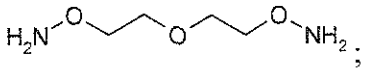
wherein the fatty acid is 16-hydroxyhexadecanoic acid.

14. The fatty acid derivative according to claim 1 or the method according to claim 10 or 11 wherein the water soluble polymer is selected from the group consisting of: polyethylene glycol (PEG), branched PEG, polysialic acid (PSA), hydroxyalkyl starch (HAS), hydroxyethyl starch (HES), carbohydrate, polysaccharides, pullulane, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, starch, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG), polyoxazoline, polyacryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate, polyvinylpyrrolidone, polyphosphazene, polyoxazoline, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, poly(1-hydroxymethylethylene hydroxymethylformal) (PHF), and 2-methacryloyloxy-2'-ethyltrimethylammoniumphosphate (MPC).

15. The fatty acid derivative or method according to claim 14 wherein the water soluble polymer is PEG and comprises a chain length selected from the group consisting of O3, O5, O7, O9, O11, O13 and O15.

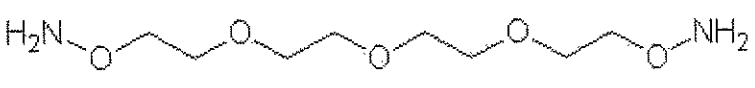
16. The fatty acid derivative or method of claim 15 wherein the water soluble linker is selected from the group consisting of:

a) 3-oxapentane-1,5-dioxyamine of the formula:



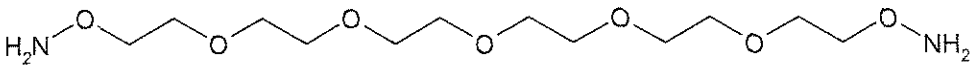
;

b) 3,6,9-triaoxaundecane-1,11-dioxyamine of the formula:



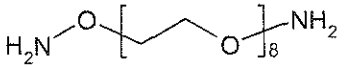
;

c) 3,6,9,12,15-penatoxaheptadecane-1,17-dioxyamine of the formula:



; and

d) 3,6,9,12,15,18,21-heptaoxatricosane-1,23-dioxyamine of the formula:



17. A method of preparing a conjugated therapeutic protein comprising contacting an oxidized carbohydrate moiety on the therapeutic protein with a fatty acid derivative according to claim 1 under conditions that allow conjugation;

said carbohydrate moiety oxidized by incubation with a buffer comprising an oxidizing agent selected from the group consisting of sodium periodate (NaIO4), lead tetraacetate (Pb(OAc)4) and potassium perruthenate (KRuO4);

wherein an oxime linkage is formed between the oxidized carbohydrate moiety and the active aminooxy group on the fatty acid derivative;

and wherein said oxime linkage formation is catalyzed by a nucleophilic catalyst selected from the group consisting of aniline, o-amino benzoic acid, m-amino benzoic acid, p-amino benzoic acid, sulfanilic acid, o-aminobenzamide, o-toluidine, m-toluidine, p-toluidine, o-anisidine, m-anisidine, and p-ânisidine.

18. The fatty acid derivative according to any of claims 1 to 9 or the method according to claim 17 wherein the therapeutic protein is selected from the group consisting of: Factor IX (FIX), Factor VIII (FVIII), Factor VIIa (FVIIa), von Willebrand Factor (VWF), Factor FV (FV), Factor X (FX), Factor XI (FXI), Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF),ADAMTS 13 protease, IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, colony stimulating factor-1 (CSF-1), M-CSF, SCF, GM-CSF, granulocyte colony stimulating factor (G-CSF), EPO, interferon-alpha (IFN-alpha), consensus interferon, IFN-beta, IFN-gamma, IFN-omega, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-31, IL-32 alpha, IL-33, thrombopoietin (TPO), Ang-1, Ang-2, Ang-4, Ang-Y, angiopoietin-like polypeptide 1 (ANGPTL1), angiopoietin-like polypeptide 2 (ANGPTL2), angiopoietin-like polypeptide 3 (ANGPTL3), angiopoietin-like polypeptide 4 (ANGPTL4), angiopoietin-like polypeptide 5 (ANGPTL5), angiopoietin-like polypeptide 6 (ANGPTL6), angiopoietin-like polypeptide 7 (ANGPTL7), vitronectin, vascular endothelial growth factor (VEGF), angiogenin, activin A, activin B, activin C, bone morphogenic protein-1, bone morphogenic protein-2, bone morphogenic protein-3, bone morphogenic protein-4, bone morphogenic protein-5, bone morphogenic protein-6, bone morphogenic protein-7, bone morphogenic protein-8, bone morphogenic protein-9, bone morphogenic protein-10, bone morphogenic protein-11, bone morphogenic protein-12, bone morphogenic protein-13, bone morphogenic protein-14, bone morphogenic protein-15, bone morphogenic protein receptor IA, bone morphogenic protein receptor IB, bone morphogenic protein receptor II, brain derived neurotrophic factor, cardiotrophin-1, ciliary neutrophic factor, ciliary neutrophic factor receptor, cripto, cryptic, cytokine-induced neutrophil chemotactic factor 1, cytokine-induced neutrophil, chemotactic factor 2α, cytokine-induced neutrophil chemotactic factor 2β, β endothelial cell growth factor, endothelin 1, epidermal growth factor, epigen, epiregulin, epithelial-derived neutrophil attractant, fibroblast growth factor 4, fibroblast growth factor 5, fibroblast growth factor 6, fibroblast growth factor 7, fibroblast growth factor 8, fibroblast growth factor 8b, fibroblast growth factor 8c, fibroblast growth factor 9, fibroblast growth factor 10, fibroblast growth factor 11, fibroblast growth factor 12, fibroblast growth factor 13, fibroblast growth factor 16, fibroblast growth factor 17, fibroblast growth factor 19, fibroblast growth factor 20, fibroblast growth factor 21, fibroblast growth factor acidic, fibroblast growth factor basic, glial cell line-derived neutrophic factor receptor α1, glial cell line-derived neutrophic factor receptor α2, growth related protein,

growth related protein α, growth related protein β, growth related protein γ, heparin binding epidermal growth factor, hepatocyte growth factor, hepatocyte growth factor receptor, hepatoma-derived growth factor, insulin-like growth factor I, insulin-like growth factor receptor, insulin-like growth factor II, insulin-like growth factor binding protein, keratinocyte growth factor, leukemia inhibitory factor, leukemia inhibitory factor receptor α, nerve growth factor nerve growth factor receptor, neuropoietin,neurotrophin-3, neurotrophin-4, oncostatin M (OSM), placenta growth factor, placenta growth factor 2, platelet-derived endothelial cell growth factor, platelet derived growth factor, platelet derived growth factor A chain, platelet derived growth factor AA, platelet derived growth factor AB, platelet derived growth factor B chain, platelet derived growth factor BB, platelet derived growth factor receptor α, platelet derived growth factor receptor β, pre-B cell growth stimulating factor, stem cell factor (SCF), stem cell factor receptor, TNF, TNF0, TNF1, TNF2, transforming growth factor α, transforming growth factor β, transforming growth factor β1, transforming growth factor β1.2, transforming growth factor β2, transforming growth factor β3, transforming growth factor β5, latent transforming growth factor β1, transforming growth factor β binding protein I, transforming growth factor β binding protein II, transforming growth factor β binding protein III, thymic stromal lymphopoietin (TSLP), tumor necrosis factor receptor type I, tumor necrosis factor receptor type II, urokinase-type plasminogen activator receptor, phospholipase-activating protein (PUP), insulin, lectin ricin, prolactin, chorionic gonadotropin, follicle-stimulating hormone, thyroid-stimulating hormone, tissue plasminogen activator, IgG, IgE, IgM, IgA, and IgD, α-galactosidase, β-galactosidase, DNAse, fetuin, leutinizing hormone, estrogen, insulin, albumin, lipoproteins, fetoprotein, transferrin, thrombopoietin, urokinase, integrin, thrombin, leptin, Humira (adalimumab), Prolia (denosumab), Enbrel (etanercept), a protein in Table 1, or a biologically active fragment, derivative or variant thereof.