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 ~~OF THE INVENTION~~

[0010] The present ~~invention~~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 ~~invention~~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 ($NaIO_4$), lead tetraacetate ($Pb(OAc)_4$) and potassium perruthenate ($KRuO_4$);

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 ~~invention~~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 ~~invention~~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 ~~invention~~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

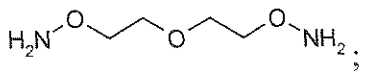
protein has the ability to impart a negative or positive charge, thereby making the linker water soluble. In still another embodiment, the functional group is selected from the group consisting of a sulfo group, carboxyl group, hydroxyl group, amino group, amido group, maleimido group, aminooxy group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

[0017] Numerous water soluble polymers are contemplated in the present ~~invention~~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.

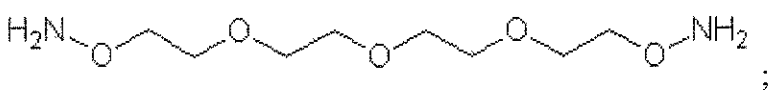
[0018] In one embodiment, a fatty acid derivative is provided wherein the water soluble linker is selected from the group consisting of:

3-oxapentane-1,5-dioxyamine



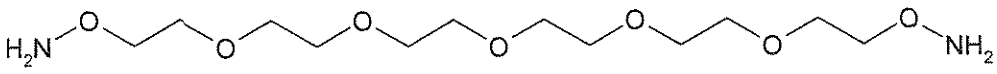
;

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



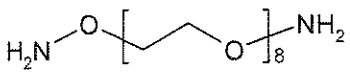
;

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



; and

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

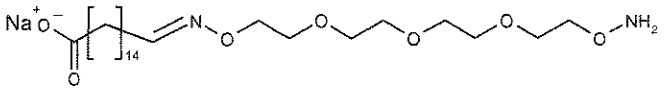


[0019] In another embodiment of the ~~invention~~present disclosure, a fatty acid derivative is provided wherein the fatty acid derivative is stably attached to the therapeutic protein by an oxime linkage. In another embodiment, the oxime linkage is formed between an oxime group on the water soluble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

[0020] In yet another embodiment of the ~~invention~~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.

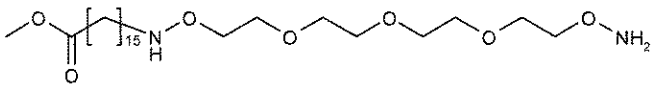
[0021] In one embodiment of the ~~invention~~present disclosure, a fatty acid derivative is provided wherein the fatty acid derivative is selected from the group consisting of:

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* ~~invention~~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

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 ~~invention~~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 ~~invention~~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.

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 ~~invention~~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 ~~invention~~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),