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.

[0008] Recently, an improved method was described comprising mild periodate oxidation of sialic acids to generate aldehydes followed by reaction with an aminooxy group containing reagent in the presence of catalytic amounts of aniline (Dirksen A et Dawson PE, Bioconjugate Chem. 2008;19,2543-8; and 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 reagent.

[0009] Notwithstanding the methods available of conjugating water soluble polymers to therapeutic proteins, there remains a need to develop materials and methods for conjugating water soluble polymers to proteins that improves the protein's pharmacodynamic and/or pharmacokinetic properties while minimizing the costs associated with the various reagents.

SUMMARY ~~OF THE INVENTION~~

[0010] The present ~~invention~~disclosure provides materials and methods for conjugating polymers to proteins that improves the protein's pharmacodynamic and/or pharmacokinetic properties while minimizing the costs associated with the various reagents.

[0010a] In one aspect, there is provided a modified blood coagulation protein comprising:

(a) a blood coagulation protein selected from the group consisting of

(i) a blood coagulation protein with Factor IX (FIX) biological activity; and

(ii) a blood coagulation protein with Factor VIII (FVIII) biological activity; and

(b) at least one polysialic acid (PSA) containing an active aminooxy group bound to said blood coagulation protein of (a) at one or more oxidized carbohydrate moieties;

wherein said modified blood coagulation protein has an oxime linkage between said one or more oxidized carbohydrate moieties and the active aminooxy group on the PSA;

wherein said modified blood coagulation protein has a specific activity of at least 50% relative to an unmodified blood coagulation protein.

[0010b] In one aspect, there is provided a method of conjugating a polysialic acid (PSA) containing an active aminooxy group to an oxidized carbohydrate moiety of a blood coagulation protein, comprising contacting said oxidized carbohydrate moiety with at least one activated PSA under conditions that allow conjugation;

said blood coagulation protein selected from the group consisting of
(i) a blood coagulation protein with FIX biological activity; and
(ii) a blood coagulation protein with FVIII biological activity;

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 (KRuO4); wherein an oxime linkage is formed between the oxidized carbohydrate moiety and the active aminooxy group on the PSA;

said modified blood coagulation protein having a specific activity of at least 50% relative to an unmodified blood coagulation protein.

[0011] In one embodiment of the ~~invention~~disclosure, there is provided a method of conjugating a water soluble polymer to an oxidized carbohydrate moiety of a blood coagulation protein comprising contacting the oxidized carbohydrate moiety with an activated water soluble polymer under conditions that allow conjugation; the blood coagulation protein 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) and ADAMTS 13 protease or a biologically active fragment, derivative or variant thereof; the water soluble polymer containing an active aminooxy group and is selected from the group consisting of polyethylene glycol (PEG), branched PEG, polysialic acid (PSA), 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,

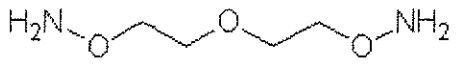
polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, poly(1-hydroxymethylethylene hydroxymethylformal) (PHF), 2-methacryloyloxy-2'-ethyltrimethylammoniumphosphate (MPC); and the carbohydrate moiety oxidized by incubation with a buffer comprising an oxidizing agent selected from 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 water soluble polymer.

[0012] In another embodiment of the ~~invention~~disclosure, the water soluble polymer according to the aforementioned method is PSA. In a related embodiment, the PSA is comprised of about 5-500 or 10 – 300 sialic acid units. In still another embodiment, the blood coagulation protein according to the aforementioned method is FIX. In another embodiment, the blood coagulation protein according to the aforementioned method is FVIIa. In still another embodiment, the blood coagulation protein according to the aforementioned method is FVIII. In yet another embodiment, the aforementioned method is provided wherein the oxidizing agent is sodium periodate (NaIO4). In another embodiment, the oxidized carbohydrate moiety of the blood coagulation protein according to the aforementioned method is located in the activation peptide of the blood coagulation protein.

[0013] In yet another embodiment of the ~~invention~~disclosure, the aforementioned method is provided wherein the PSA is prepared by reacting an activated aminooxy linker with oxidized PSA;

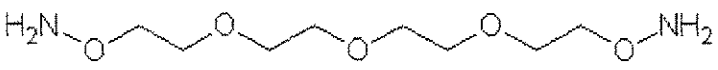
wherein the aminooxy linker is selected from the group consisting of:

a 3-oxa-pentane-1,5-dioxyamine linker of the formula:



and

a 3,6,9-trioxa-undecane-1,11-dioxyamine linker of the formula:



[0014] wherein the PSA is oxidized by incubation with a oxidizing agent to form a terminal aldehyde group at the non-reducing end of the PSA. In still another embodiment, the aforementioned method is provided wherein the activated aminooxy linker comprises 1-50 ethylene glycol units.

In still another embodiment, an aforementioned method is provided wherein the aminooxy linker is 3-oxa-pentane-1,5-dioxyamine. In a related embodiment, the oxidizing agent is $NaIO_4$..

[0015] In another embodiment of the ~~invention~~disclosure, the aforementioned method is provided wherein the contacting of the oxidized carbohydrate moiety with the activated water soluble polymer occurs in a buffer comprising a nucleophilic catalyst selected from the group consisting of aniline and aniline derivatives.

[0016] In yet another embodiment of the ~~invention~~disclosure, an aforementioned method is provided further comprising the step of reducing an oxime linkage in the conjugated blood coagulation protein by incubating the conjugated blood coagulation protein in a buffer comprising a reducing compound selected from the group consisting of sodium cyanoborohydride (NaCNBH3) and ascorbic acid (vitamin C). In a related embodiment the reducing compound is sodium cyanoborohydride (NaCNBH3).

[0017] In another embodiment of the ~~invention~~disclosure, a modified blood coagulation protein produced by an aforementioned method is provided.

[0018] In still another embodiment of the ~~invention~~disclosure, a modified FIX is provided comprising a FIX molecule or a biologically active fragment, derivative or variant thereof; and at least one aminooxy PSA bound to the FIX molecule, wherein said aminooxy PSA is attached to the FIX via one or more carbohydrate moieties.

[0019] In another embodiment of the ~~invention~~disclosure, a modified FVIIa is provided comprising a FVIIa molecule or a biologically active fragment, derivative or variant thereof; and at least one aminooxy PSA bound to the FVIIa molecule, wherein said aminooxy PSA is attached to the FVIIa via one or more carbohydrate moieties.

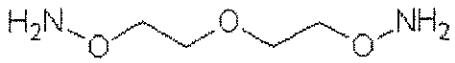
[0020] In still another embodiment of the ~~invention~~disclosure, a modified FVIII is provided comprising a FVIII molecule or a biologically active fragment, derivative or variant thereof; and at least one aminooxy PSA bound to the FVIII molecule, wherein said aminooxy PSA is attached to the FVIII via one or more carbohydrate moieties.

[0021] In still another embodiment of the ~~invention~~disclosure, a modified FIX is provided comprising a FIX molecule or a biologically active fragment, derivative or variant thereof; and at least one aminooxy PEG bound to the FIX molecule, wherein said aminooxy PEG is attached to the FIX via one or more carbohydrate moieties.

[0022] In another embodiment of the ~~invention~~disclosure, a modified FVIIa is provided comprising a FVIIa molecule or a biologically active fragment, derivative or variant thereof; and at least one aminooxy PEG bound to the FVIIa molecule, wherein said aminooxy PEG is attached to the FVIIa via one or more carbohydrate moieties.

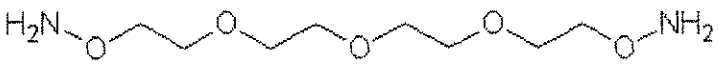
[0023] In still another embodiment of the ~~invention~~disclosure, a modified FVIII is provided comprising a FVIII molecule or a biologically active fragment, derivative or variant thereof; and at least one aminooxy PEG bound to the FVIII molecule, wherein said aminooxy PEG is attached to the FVIII via one or more carbohydrate moieties.

[0024] In yet another embodiment, a water soluble polymer is provided comprising an active aminooxy linker; said water soluble polymer selected from the group consisting of polyethylene glycol (PEG), branched PEG, polysialic acid (PSA), carbohydrate, polysaccharides, pullulane, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, starch, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG), polyoxazoline, poly acryloylmorpholine, 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); said active aminooxy linker is selected from the group consisting of: a 3-oxa-pentane-1,5-dioxyamine linker of the formula:



and

a 3,6,9-trioxa-undecane-1,11-dioxyamine linker of the formula:



[0025] In still another embodiment, the aforementioned method is provided wherein activated aminooxy linker comprises 1-50 ethylene glycol units.

FIGURES

[0026] Figure 1 shows the primary structure of coagulation Factor IX.

[0027] Figure 2 shows the coupling of oxidized rFIX to aminooxy-PSA.