431, 1987). Long-term dietary treatment of rats has been shown to induce tumors in the spleen (Goodman, DG., et al., J Natl Cancer Inst., 73(1):265-73, 1984). In vitro studies have also shown that aniline has the potential to induce chromosome mutations and has the potentially genotoxic activity (Bombhard E.M. et Herbold B, Critical Reviews in Toxicology 35,783-835, 2005).

[0008] 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 and minimizing the health risks to the patient recipient.

SUMMARY

[0009] The present 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 and the health risks to the patient recipients when the conjugation reaction is catalyzed by a nucleophilic catalyst. In various examples of the disclosure, alternative catalysts to substitute for aniline are provided.

[0009a] In one aspect, the present disclosure refers to a method of preparing a modified therapeutic protein for treating a bleeding disorder, wherein an activated water soluble polymer is conjugated to an oxidized carbohydrate moiety of a therapeutic protein through an oxime linkage to form the modified therapeutic protein; wherein said activated water soluble polymer contains an active aminooxy group and is selected from the group consisting of polyethylene glycol (PEG), branched PEG, PolyPEG® (Warwick Effect Polymers; Coventry, UK), polysialic acid (PSA), carbohydrate, polysaccharides, pullulan, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG), polyoxazoline, polyacryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate, polyvinylpyrrolidone, polyphosphazene, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, and poly(1-hydroxymethylethylene hydroxymethylformal) (PHF); wherein said therapeutic protein is a glycoprotein or a therapeutic protein glycosylated in vitro, and is selected from the group consisting of Factor IX (FIX), Factor VIII (FVIII), Factor VIIa (FVIIa), Von Willebrand Factor (VWF), Factor V (FV), Factor X (FX), Factor XI (FXI), Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF) and a

biologically active fragment, derivative or variant thereof; wherein said oxidized carbohydrate moiety is a 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 activated water soluble polymer; wherein said 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; and wherein said activated water soluble polymer containing an active aminooxy group is prepared by a method comprising: a) incubating a solution comprising an oxidized water-soluble polymer with an aminooxy linker comprising an active aminooxy group under conditions that allow the formation of a stable oxime linkage between the oxidized water-soluble polymer and the activated aminooxy linker, said conditions comprising a time period between 1 minute and 24 hours; a temperature between 2°C and 8°C; in the presence or absence of light, and with or without stirring; thereby forming an activated water-soluble polymer containing an active aminooxy group; and b) purifying the activated water soluble polymer containing an active aminooxy group by a method selected from the group consisting of chromatography, filtration, dialysis, and precipitation, at a temperature between 2°C and 8°C.

[0009b] In one aspect, the present disclosure refers to a method of preparing a modified therapeutic protein for treating a bleeding disorder, wherein an activated water soluble polymer is conjugated to an oxidized carbohydrate moiety of a therapeutic protein through an oxime linkage to form the modified therapeutic protein; wherein said activated water soluble polymer contains an active aminooxy group and is selected from the group consisting of polyethylene glycol (PEG), branched PEG, PolyPEG® (Warwick Effect Polymers; Coventry, UK), polysialic acid (PSA), carbohydrate, polysaccharides, pullulan, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG), polyoxazoline, polyacryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate, polyvinylpyrrolidone, polyphosphazene, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, and poly(1-hydroxymethylethylene hydroxymethylformal) (PHF); wherein said therapeutic protein is a glycoprotein or a therapeutic protein glycosylated in vitro, and is selected from the group consisting of Factor IX (FIX), Factor VIII (FVIII), Factor VIIa

(FVIIa), Von Willebrand Factor (VWF), Factor V (FV), Factor X (FX), Factor XI (FXI), Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF) and a biologically active fragment, derivative or variant thereof; wherein said oxidized carbohydrate moiety is a 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 activated water soluble polymer; wherein said 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; and wherein said activated water soluble polymer containing an active aminooxy group is prepared by a method comprising: a) incubating a solution comprising a non-oxidized water-soluble polymer with an aminooxy linker comprising an active aminooxy group under conditions that allow the formation of a stable oxime linkage between the non-oxidized water-soluble polymer and the activated aminooxy linker, said conditions comprising a time period between 1 minute and 24 hours; a temperature between 22°C and 37°C; in the presence or absence of light, and with or without stirring; thereby forming an activated water-soluble polymer containing an active aminooxy group; and b) purifying the activated water soluble polymer containing an active aminooxy group by a method selected from the group consisting of chromatography, filtration, dialysis, and precipitation.

[0010] The present disclosure also provides an optimized procedure for preparation of various water-soluble polymer-aminooxy linker reagents that can be used to conjugate therapeutic proteins as described herein. Recent NMR studies showed that side reactions at the reducing end of PSA can occur if the preparation of the PSA-aminooxy reagent is performed at room temperature. Thus, in various examples of the present disclusre, the new process is carried out at a temperature between 2-8°C. In one example, the PSA-aminooxy reagent is prepared at 4°C according to the process described herein. In addition, purification of the reagent using a chromatographic purification step (e.g., IEX) at a temperature between 2-8°C is also contemplated by the present disclosure. Unwanted side reactions at the reducing end of the PSA are substantially reduced when the entire process (chemical reaction and purification of the conjugate by IEX) is performed at a temperature between 2-8°C.

[0011] In one example according to the present disclosure, a method of conjugating a water soluble polymer to an oxidized carbohydrate moiety of a therapeutic protein is provided comprising contacting the oxidized carbohydrate moiety with an activated water soluble polymer under conditions that allow conjugation; said water soluble polymer

containing an active aminooxy group and is selected from the group consisting of polyethylene glycol (PEG), branched PEG, PolyPEG® (Warwick Effect Polymers; Coventry, UK), polysialic acid (PSA), starch, hydroxyalkyl starch (HAS), hydroxylethyl 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); wherein the water soluble polymer containing an active aminooxy group is prepared by a method comprising: a) incubating a solution comprising an oxidized water-soluble polymer with an aminooxy linker comprising an active aminooxy group under conditions that allow the formation of a stable oxime linkage between the oxidized water-soluble polymer and the activated aminooxy linker, said conditions comprising a time period between about 1 minute and about 24 hours; a temperature between about 2°C and about 8°C; in the presence or absence of light, and with or without stirring; thereby forming a water-soluble polymer containing an active aminooxy group; and b) purifying the water soluble polymer containing an active aminooxy group by a method selected from the group consisting of chromatography, filtration, dialysis, and precipitation, at a temperature between about 2°C and about 8°C; 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 water soluble polymer; and wherein said 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.

[0012] In another example, the aforementioned method is provided wherein the solution comprising the oxidized water-soluble polymer and the aminooxy linker comprising an active aminooxy group is incubated at 4°C for 1 h in the absence of light with stirring. In another example, the aforementioned method is provided wherein the water soluble polymer containing an active aminooxy group is purified by anion exchange chromatography

at a temperature of 4°C. In another example, an aforementioned method is provided wherein the oxidized water soluble polymer is PSA and is oxidized by incubation with $NaIO_4$.

[0013] In another example of the present disclusre, a method of conjugating a water soluble polymer to an oxidized carbohydrate moiety of a therapeutic protein is provided comprising contacting the oxidized carbohydrate moiety with an activated water soluble polymer under conditions that allow conjugation; said water soluble polymer containing an active aminooxy group and is selected from the group consisting of polyethylene glycol (PEG), branched PEG, PolyPEG® (Warwick Effect Polymers; Coventry, UK), polysialic acid (PSA), starch, hydroxyalkyl starch (HAS), hydroxylethyl 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); wherein the water soluble polymer containing an active aminooxy group is prepared by a method comprising: a) incubating a solution comprising a water-soluble polymer with an aminooxy linker comprising an active aminooxy group under conditions that allow the formation of a stable oxime linkage between the water-soluble polymer and the activated aminooxy linker, said conditions comprising a time period between about 1 minute and about 24 hours; a temperature between about 22°C and about 37°C; in the presence or absence of light, and with or without stirring; thereby forming a water-soluble polymer containing an active aminooxy group; and b) purifying the water soluble polymer containing an active aminooxy group by a method selected from the group consisting of chromatography, filtration, dialysis, and precipitation; 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 water soluble polymer; and wherein said 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.

[0014] In another example, the aforementioned method is provided wherein the solution comprising the water-soluble polymer and the aminooxy linker comprising an active aminooxy group is incubated at 22°C for 2 h in the absence of light with stirring. In another example, the aforementioned method is provided wherein the solution comprising the water-soluble polymer and the aminooxy linker comprising an active aminooxy group is incubated at 22°C for 2 h in the absence of light with stirring; said method further comprising the step of increasing the temperature of the solution to a temperature between about 32°C and about 37°C and incubating for an additional 12-24 hours. In another example, the aforementioned method is provided comprising the additional step of adding an additional amount of aminooxy linker comprising an active aminooxy group immediately prior to increasing the temperature. In another example, the aforementioned method is provided wherein the water soluble polymer containing an active aminooxy group is purified by a method selected from the group consisting of dialysis, ultrafiltration/diafiltration (UF/DF), and chromatography at a temperature of 22°C. In another example, the aforementioned method is provided further comprising the step of purifying the water soluble polymer containing an active aminooxy group by a method selected from the group consisting of dialysis, UF/DF or chromatography at 4°C.

[0015] In various examples of the present disclosure, an aforementioned method is provided wherein the therapeutic 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),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,

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.

[0016] In still another example, the aforementioned method is provided wherein a solution comprising an initial concentration of the therapeutic protein between about 0.3 mg/ml and about 3.0 mg/ml is adjusted to a pH value between about 5.0 and about 8.0 prior to contacting with the activated water soluble polymer. In one example, the the initial concentration of the therapeutic protein is about 1.0 mg/ml and the pH is about 6.0.

[0017] In another example, the aforementioned method is provided wherein the therapeutic protein is contacted by a desired excess concentration of activated water soluble polymer, wherein the excess concentration is between about 1-molar and about 300-molar excess. In one example, the excess concentration is about 50-fold molar excess.

[0018] In another example, the aforementioned method is provided wherein the therapeutic protein is incubated with the activated water soluble polymer under conditions comprising a time period between about 0.5 hours and about 24 hours; a temperature between about 2°C and about 37°C; in the presence or absence of light; and with or without stirring. In one example, the conditions comprise a time period of about 120 minutes, a temperature of about 22 °C, the absence of light; and with stirring.

[0019] In yet another example, an aforementioned method is provided wherein the nucleophilic catalyst is added in an amount to result in a final concentration between about 1.0 mM and about 50 mM nucleophilic catalyst, under conditions comprising a time period between about 0.1 minutes and about 30 minutes; a temperature between about 2°C and about 37°C; in the presence or absence of light; and with or without stirring. In one example, the final concentration of the nucleophilic catalyst is about 10 mM, and the conditions comprise a time period of up to about 15 minutes, a temperature of about 22 °C, the absence of light; and with stirring.

[0020] In yet another example, the aforementioned method is provided wherein the oxidizing agent is added in an amount to result in a final concentration between about 50 μM and about 1000 μM oxidizing agent, under conditions comprising a time period between about 0.1 minutes and 120 minutes; a temperature between about 2°C and about 37°C; in the presence or absence of light; and with or without stirring. In one example, the final concentration of oxidizing agent is about 400 μM, and the conditions comprise a time period of about 10 minutes, a temperature of about 22 °C, the absence of light and with stirring.

[0021] In still another example, the aforementioned method is provided wherein the conjugating the water soluble polymer to the oxidized carbohydrate moiety of the therapeutic protein is stopped by the addition of a quenching agent selected from the group consisting of L-cysteine, methionine, glutathione, glycerol, sodium meta bisulfite ($Na_2S_2O_5$), tryptophane, tyrosine, histidine or derivatives thereof, kresol, imidazol, and combinations thereof; wherein the quenching agent is added in an amount to result in a final concentration between about 1 mM and about 100 mM quenching agent, under conditions comprising a time period between about 5 minutes and about 120 minutes; a temperature between about 2°C and about 37°C; in the presence or absence of light; and with or without stirring. In one example the quenching agent is L-cysteine. In still another example, the L-cysteine is added to result in a final concentration of about 10 mM and the conditions comprise a time period of about 60 minutes, a temperature of about 22°C, the absence of light and with stirring.

[0022] In another example, an aforementioned method is provided comprising**:** a) a first step comprising adjusting the pH value of a solution comprising the therapeutic protein to a pH value between about 5.0 and about 8.0, wherein the therapeutic protein concentration is between about 0.3 mg/ml and about 3.0 mg/ml; b) a second step comprising oxidizing one or more carbohydrates on the therapeutic protein, wherein the oxidizing agent is added to the solution in the first step to result in a final concentration between about 50μM and about 1000μM, under conditions comprising a time period between about 0.1 minutes and about 120 minutes; a temperature between about 2°C and about 37°C; in the presence or absence of light, and with or without stirring; c) a third step comprising contacting the therapeutic protein with a desired excess concentration of activated water soluble polymer, wherein the excess concentration is between about 1-molar excess and about 300-molar excess, under conditions comprising a time period between about 0.5 hours and about 24 hours, a temperature between about 2°C and about 37°C; in the presence or absence of light; and with or without stirring; d) a fourth step comprising adding a nucleophilic catalyst to the solution

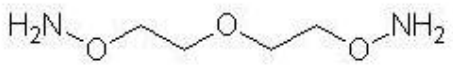
of the third step, wherein the nucleophilic catalyst is added to result in a final concentration between about 1 mM and about 50 mM, under conditions comprising a time period between about 0.1 minutes and about 30 minutes; a temperature between about 2°C and about 37°C; in the presence or absence of light, and with or without stirring; e) a fifth step wherein the therapeutic protein is incubated with the activated water soluble polymer and nucleophilic catalyst under conditions that allow conjugation of the activated water-soluble polymer to one or more oxidized carbohydrates on the therapeutic protein, said conditions comprising a time period between about 0.5 hours and about 24 hours, a temperature between about 2°C and about 37°C; in the presence or absence of light, and with or without stirring; and f) a sixth step wherein the conjugating the water soluble polymer to the one or more oxidized carbohydrates of the therapeutic protein in the fifth step is stopped by the addition of a quenching agent selected from the group consisting of L-cysteine, methionine, glutathione, glycerol, $Na_2S_2O_5$ (sodium meta bisulfite), tryptophane, tyrosine, histidine or derivatives thereof, kresol, imidazol, and combinations thereof; wherein the quenching agent is added to result in a final concentration of about 1 mM and about 100 mM, under conditions comprising a time period between about 5 minutes and about 120 minutes; a temperature between about 2°C and about 37°C; in the presence or absence of light, and with or without stirring. In another embodinment, the initial concentration of the therapeutic protein in the first step is about 1 mg/ml and the pH is about 6.0; wherein the final concentration of oxidizing agent in the second step is about 400 µM, and the conditions in the fifth step comprise a time period of about 10 minutes, a temperature of about 22 ºC, the absence of light and with stirring; wherein the excess concentration in the third step is about 50 molar excess; wherein the conditions in the third step comprise a time period of about 15 minutes, a temperature of about 22 °C, the absence of light and with stirring; wherein the final concentration of the nucleophilic catalyst in the fourth step is about 10 mM, and the conditions in the fourth step comprise a time period of about 15 minutes, a temperature of about 22 °C, the absence of light and with stirring; wherein the conditions of incubating the therapeutic protein with the activated water soluble polymer and nucleophilic catalyst in the fifth step comprise a time period of about 2 hours; a temperature of about 22°C; the absence of light; and with stirring; and wherein the quenching agent in the sixth step is L-cysteine; and wherein the L-cysteine is added to result in a final concentration of about 10 mM and the conditions in the sixth step comprise a time period of about 60 minutes, a temperature of about 22 °C, the absence of light and with stirring. In still another example, the water soluble polymer is PSA. In one example, the water soluble polymer is PEG. In another

example, the water soluble polymer is HES. In yet another example, the water soluble polymer is HAS. In another example the PSA is comprised of about 10 – 300 sialic acid units. In still another example, the therapeutic protein is FIX. In another example, the therapeutic protein is FVIIa. In yet another example, the therapeutic protein is FVIII.

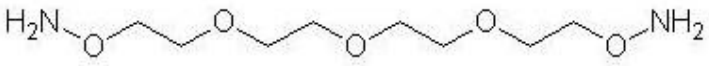
[0023] In another example, an aforementioned method is provided wherein the oxidizing agent is sodium periodate ($NaIO_4$).

[0024] In another example, the aforementioned method is provided wherein the oxidized carbohydrate moiety of the therapeutic protein is located in the activation peptide of the blood coagulation protein.

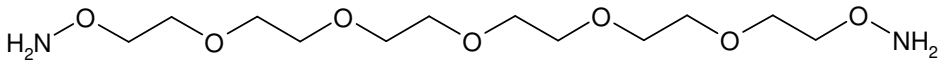
[0025] In another example, the aforementioned method is provided wherein the PSA comprises an activated aminooxy linker selected from the group consisting of: a) a 3-oxa-pentane-1,5-dioxyamine linker of the formula:

[0026]  ,

[0027] b) a 3,6,9-trioxa-undecane-1,11-dioxyamine linker of the formula:

[0028]  and

[0029] c) a 3,6,9,12,15-penatoxa-heptadecane-1,17-dioxyamine linker of the formula:

[0030] 

[0031] wherein the PSA is oxidized by incubation with an oxidizing agent to form a terminal aldehyde group at the non-reducing end of the PSA.

[0032] In another example, the aminooxy linker is 3-oxa-pentane-1,5-dioxyamine.

[0033] In another example, the aforementioned method is provided wherein the nucleophilic catalyst is provided at a concentration between about 1 mM and about 50 mM. In another example, the nucleophilic catalyst is m-toluidine. In still another example, the m-toluidine is present in the conjugation reaction at a concentration of about 10 mM.

[0034] In another example, the aforementioned method is provided further comprising the step of purifying the conjugated therapeutic protein. In one example, the conjugated

therapeutic protein is purified by a method selected from the group consisting of chromatography, filtration and precipitation. In still another example, the chromatography is selected from the group consisting of Hydrophobic Interaction Chromatography (HIC), Ion Exchange chromatography (IEC), Size exclusion chromatography (SEC), Affinity chromatography, and Reversed-phase chromatography. In one example, an anti-chaotropic salt is used in a chromotagraphy loading step and in a chromatography washing step. In still another embomdiment, the chromatography takes place in a column. In one example, the column comprises a chromatography resin selected from the group consisting of Phenyl-Sepharose FF and Butyl-Sepharose FF. In yet another example, the resin is present in the column at a bed height of between about 5 cm and about 20 cm.

[0035] In still another example, the bed height is about 10 cm. In another example, an aforementioned method is provided wherein comprising one or more washing steps wherein flow direction is set to up-flow and wherein the flow rate is between about 0.2 cm/min and about 6.7 cm/min. In one example, the flow rate is about 2 cm/min. In another example, the aforementioned method is provided comprising one or more elution steps wherein flow direction is set to down-flow and wherein the flow rate is between about 0.1 cm/min and about 6.7 cm/min. In one example, the flow rate is about 1 cm/min.

[0036] In yet another example, the aforementioned method is provided further comprising concentrating the conjugated therapeutic protein by ultra-/diafiltration (UF/DF). In another example, the aforementioned method is provided wherein the final concentration of therapeutic protein is between about 0.5 and about 3 mg/ml. In another example, the aforementioned method is provided wherein the therapeutic protein comprises between about 5 and about 11 water soluble polymer moieties.

[0037] In still another example, the aforementioned method is provided wherein the conjugated therapeutic protein is purified using chromatography; wherein an anti-chaotropic salt is used for a loading step and for a washing step; the method comprising one or more washing steps wherein flow direction is set to up-flow and wherein the flow rate is between about 0.2 cm/min and about 6.7 cm/min and one or more elution steps wherein flow direction is set to down-flow and wherein the flow rate is between about 0.2 cm/min and about 6.7 cm/min; further comprising concentrating the conjugated therapeutic protein by ultra-/diafiltration (UF/DF).

[0038] In another example, the aforementioned method is provided wherein the chromatography is hydrophobic interaction chromatography (HIC); wherein the one or more washing steps flow rate is about 2 cm/min; and wherein the one or more elution steps flow rate is about 1 cm/min.

[0039] A modified therapeutic protein produced by an aforementioned method is also provided by the present disclosure.

[0040] In one example of the present disclosure, a method of preparing a water-soluble polymer containing an active aminooxy group is provided comprising: a) incubating a solution comprising an oxidized water-soluble polymer with an aminooxy linker comprising an active aminooxy group under conditions that allow the formation of a stable oxime linkage between the oxidized water-soluble polymer and the activated aminooxy linker, said conditions comprising a time period between about 1 minute and about 24 hours; a temperature between about 2°C and about 8°C; in the presence or absence of light, and with or without stirring; thereby forming a water-soluble polymer containing an active aminooxy group; and b) purifying the water soluble polymer containing an active aminooxy group by a method selected from the group consisting of chromatography, filtration, dialysis, and precipitation, at a temperature between about 2°C and about 8°C; said water soluble polymer is selected from the group consisting of polyethylene glycol (PEG), branched PEG, PolyPEG® (Warwick Effect Polymers; Coventry, UK), polysialic acid (PSA), starch, hydroxyalkyl starch (HAS), hydroxylethyl 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); thereby forming an oxime linkage between the water-soluble polymer and the aminooxy linker.

[0041] In another example, the aforementioned method is provided 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.

[0042] In another example, the aforementioned method is provided wherein the water soluble polymer is PSA In another example, the aforementioned method is provided wherein the water-soluble polymer is PEG. In another example, the aforementioned method is provided wherein the solution comprising the oxidized water-soluble polymer and the aminooxy linker comprising an active aminooxy group is incubated at 4°C for 1 h in the absence of light with stirring. In another example, the aforementioned method is provided wherein the water soluble polymer containing an active aminooxy group is purified by anion exchange chromatography at a temperature of 4°C.

[0043] In yet another example of the present disclosure, a method of preparing a water soluble polymer containing an active aminooxy group is provided comprising: a) incubating a solution comprising a water-soluble polymer with an aminooxy linker comprising an active aminooxy group under conditions that allow the formation of a stable oxime linkage between the water-soluble polymer and the activated aminooxy linker, said conditions comprising a time period between about 1 minute and about 24 hours; a temperature between about 22°C and about 37°C; in the presence or absence of light, and with or without stirring; thereby forming a water-soluble polymer containing an active aminooxy group; and b) purifying the water soluble polymer containing an active aminooxy group by a method selected from the group consisting of chromatography, filtration, dialysis, and precipitation; thereby forming an oxime linkage between the water-soluble polymer and aminooxy linker.

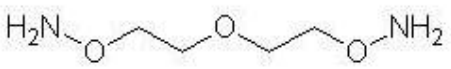
[0044] In another example, the aforementioned method is provided wherein said oxime linkage formation is catalyzed by a nucleophilic catalyst selected from the group consisting of o-amino benzoic acid, m-amino benzoic acid, p-amino benzoic acid, sulfanilic acid, o-aminobenzamide, o-toluidine, m-toluidine, p-toluidine, o-anisidine, m-anisidine, and p-anisidine.

[0045] In another example, the aforementioned method is provided wherein the solution comprising the water-soluble polymer and the aminooxy linker comprising an active aminooxy group is incubated at 22°C for 2 h in the absence of light with stirring. In another example, the aforementioned method is provided wherein the solution comprising the water-soluble polymer and the aminooxy linker comprising an active aminooxy group is incubated at 22°C for 2 h in the absence of light with stirring; said method further comprising the step of increasing the temperature of the solution to a temperature between about 32°C and about 37°C and incubating for an additional 12-24 hours. In another example, the aforementioned method is provided comprising the additional step of adding an additional

amount of aminooxy linker comprising an active aminooxy group immediately prior to increasing the temperature.

[0046] In another example, the aforementioned method is provided wherein the water soluble polymer containing an active aminooxy group is purified by a method selected from the group consisting of dialysis, ultrafiltration/diafiltration (UF/DF), and chromatography at a temperature of 22°C. In another example, the aforementioned method is provided further comprising the step of purifying the water soluble polymer containing an active aminooxy group by a method selected from the group consisting of dialysis, UF/DF or chromatography at 4°C.

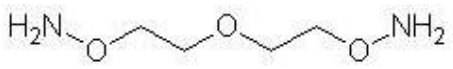
[0047] In still another example of thepresent disclure, a method of preparing a PSA-aminooxy reagent aminooxy group is provided comprising: a) incubating a solution comprising an oxidized PSA with an aminooxy linker comprising an active aminooxy group under conditions that allow the formation of a stable oxime linkage between the oxidized PSA and the activated aminooxy linker, said conditions comprising a time period 1 hour; a temperature of 4°C; in the absence of light, and with stirring; thereby forming a PSA containing an active aminooxy group; and b) purifying the PSA containing an active aminooxy group by anion exchange chromatography, a temperature of 4°C; said activated aminooxy linker is 3-oxa-pentane-1,5-dioxyamine linker of the formula:

[0048] 

;

[0049] thereby forming an oxime linkage between the PSA and the aminooxy linker.

[0050] In yet another example of the present disclosure, a method of preparing a PSA-aminooxy reagent aminooxy group is provided comprising: a) incubating a solution comprising a non-oxidized PSA with an aminooxy linker comprising an active aminooxy group under conditions that allow the formation of a stable oxime linkage between the non-oxidized PSA and the activated aminooxy linker, said conditions comprising a time period 2 hours; a temperature of 22°C; in the absence of light, and with stirring; thereby forming a PSA containing an active aminooxy group; and b) purifying the PSA containing an active aminooxy group by dialysis at a temperature of 22°C; said activated aminooxy linker is 3-oxa-pentane-1,5-dioxyamine linker of the formula:

[0051]  ;

[0052] thereby forming an oxime linkage between the non-oxidized PSA and the aminooxy linker.

In still another example of the present disclosure, a method of conjugating a water soluble polymer to an oxidized carbohydrate moiety of a blood coagulation protein is provided comprising contacting the oxidized carbohydrate moiety with an activated water soluble polymer under conditions that allow conjugation; said 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; said 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 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 water soluble polymer.

FIGURES

[0053] Figure 1 shows the primary structure of coagulation Factor IX (SEQ ID NO: 1).

[0054] Figure 2 shows the coupling of oxidized rFIX to aminooxy-PSA.

[0055] Figure 3 shows the synthesis of the water soluble di-aminoxy linkers 3-oxa-pentane-1,5-dioxyamine and 3,6,9-trioxa-undecane-1,11-dioxyamine.

[0056] Figure 4 shows the preparation of aminooxy-PSA.

What is claimed:

1. A method of preparing a modified therapeutic protein for treating a bleeding disorder, wherein an activated water soluble polymer is conjugated to an oxidized carbohydrate moiety of a therapeutic protein through an oxime linkage to form the modified therapeutic protein;

wherein said activated water soluble polymer contains an active aminooxy group and is selected from the group consisting of polyethylene glycol (PEG), branched PEG, PolyPEG® (Warwick Effect Polymers; Coventry, UK), polysialic acid (PSA), carbohydrate, polysaccharides, pullulan, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG), polyoxazoline, polyacryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate, polyvinylpyrrolidone, polyphosphazene, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, and poly(1-hydroxymethylethylene hydroxymethylformal) (PHF);

wherein said therapeutic protein is a glycoprotein or a therapeutic protein glycosylated in vitro, and is selected from the group consisting of Factor IX (FIX), Factor VIII (FVIII), Factor VIIa (FVIIa), Von Willebrand Factor (VWF), Factor V (FV), Factor X (FX), Factor XI (FXI), Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF) and a biologically active fragment, derivative or variant thereof;

wherein said oxidized carbohydrate moiety is a 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 activated water soluble polymer;

wherein said 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; and

wherein said activated water soluble polymer containing an active aminooxy group is prepared by a method comprising:

a) incubating a solution comprising an oxidized water-soluble polymer with an aminooxy linker comprising an active aminooxy group under conditions that allow the formation of a stable oxime linkage between the oxidized water-soluble polymer and the activated aminooxy linker, said conditions comprising a time period between 1 minute and 24 hours; a temperature between 2°C and 8°C; in the presence or absence of light, and with or without stirring; thereby forming an activated water-soluble polymer containing an active aminooxy group; and

b) purifying the activated water soluble polymer containing an active aminooxy group by a method selected from the group consisting of chromatography, filtration, dialysis, and precipitation, at a temperature between 2°C and 8°C.

2. The method according to claim 1 wherein the solution comprising the oxidized water-soluble polymer and the aminooxy linker comprising an active aminooxy group is incubated at 4°C for 1 h in the absence of light with stirring.

3. The method according to claim 1 wherein the activated water soluble polymer containing an active aminooxy group is purified by anion exchange chromatography at a temperature of 4°C.

4. The method according to claim 1 wherein the oxidized water soluble polymer is oxidized PSA; and wherein the oxidized PSA is a PSA oxidized by incubation with an oxidizing agent to form a terminal aldehyde group at the non-reducing end of the PSA.

5. A method of preparing a modified therapeutic protein for treating a bleeding disorder, wherein an activated water soluble polymer is conjugated to an oxidized

carbohydrate moiety of a therapeutic protein through an oxime linkage to form the modified therapeutic protein;

wherein said activated water soluble polymer contains an active aminooxy group and is selected from the group consisting of polyethylene glycol (PEG), branched PEG, PolyPEG® (Warwick Effect Polymers; Coventry, UK), polysialic acid (PSA), carbohydrate, polysaccharides, pullulan, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG), polyoxazoline, polyacryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate, polyvinylpyrrolidone, polyphosphazene, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, and poly(1-hydroxymethylethylene hydroxymethylformal) (PHF);

wherein said therapeutic protein is a glycoprotein or a therapeutic protein glycosylated in vitro, and is selected from the group consisting of Factor IX (FIX), Factor VIII (FVIII), Factor VIIa (FVIIa), Von Willebrand Factor (VWF), Factor V (FV), Factor X (FX), Factor XI (FXI), Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF) and a biologically active fragment, derivative or variant thereof;

wherein said oxidized carbohydrate moiety is a 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 activated water soluble polymer;

wherein said 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; and

wherein said activated water soluble polymer containing an active aminooxy group is prepared by a method comprising:

a) incubating a solution comprising a non-oxidized water-soluble polymer with an aminooxy linker comprising an active aminooxy group under conditions that allow the formation of a stable oxime linkage between the non-oxidized water-soluble polymer and the

activated aminooxy linker, said conditions comprising a time period between 1 minute and 24 hours; a temperature between 22°C and 37°C; in the presence or absence of light, and with or without stirring; thereby forming an activated water-soluble polymer containing an active aminooxy group; and

b) purifying the activated water soluble polymer containing an active aminooxy group by a method selected from the group consisting of chromatography, filtration, dialysis, and precipitation.

6. The method according to claim 5 wherein the solution comprising the water-soluble polymer and the aminooxy linker comprising an active aminooxy group is incubated at 22°C for 2 h in the absence of light with stirring.

7. The method according to claim 5 wherein the solution comprising the water-soluble polymer and the aminooxy linker comprising an active aminooxy group is incubated at 22°C for 2 h in the absence of light with stirring; said method further comprising the step of increasing the temperature of the solution to a temperature between 32°C and 37°C and incubating for an additional 12-24 hours.

8. The method according to claim 7 comprising the additional step of adding an additional amount of aminooxy linker comprising an active aminooxy group immediately prior to increasing the temperature.

9. The method according to any one of claims 5-8 wherein the water soluble polymer containing an active aminooxy group is purified by a method selected from the group consisting of dialysis, ultrafiltration/diafiltration (UF/DF), and chromatography at a temperature of 22°C.

10. The method according to claim 9 further comprising the step of purifying the water soluble polymer containing an active aminooxy group by a method selected from the group consisting of dialysis, UF/DF and chromatography at 4°C.

11. The method according to any one of claims 1-8 wherein a solution comprising an initial concentration of the therapeutic protein between 0.3 mg/ml and 3.0 mg/ml is

adjusted to a pH value between 5.0 and 8.0 prior to contacting with the activated water soluble polymer.

12. The method of claim 11 wherein the initial concentration of the therapeutic protein is 1.0 mg/ml and the pH is 6.0.

13. The method according to any one of claims 1-8 wherein the therapeutic protein is contacted by a desired excess concentration of activated water soluble polymer, wherein the excess concentration is between 1-fold molar and 300-fold molar excess.

14. The method of claim 13 wherein the excess concentration is 5-fold molar excess or 50-fold molar excess.

15. The method of claim 13 wherein the therapeutic protein is incubated with the activated water soluble polymer under conditions comprising a time period between 0.5 hours and 24 hours; a temperature between 2°C and 37°C; in the presence or absence of light; and with or without stirring.

16. The method according to claim 15 wherein the conditions comprise a time period of 120 minutes, a temperature of 22 °C, the absence of light; and with stirring.

17. The method according to any one of claims 1-8 wherein the nucleophilic catalyst is added in an amount to result in a final concentration between 1.0 mM and 50 mM nucleophilic catalyst, under conditions comprising a time period between 0.1 minutes and 30 minutes; a temperature between 2°C and 37°C; in the presence or absence of light; and with or without stirring.

18. The method according to claim 17 wherein the final concentration of the nucleophilic catalyst is 10 mM, and the conditions comprise a time period of up to 15 minutes, a temperature of 22 °C, the absence of light; and with stirring.

19. The method according to any one of claims 1-8 wherein the oxidizing agent is added in an amount to result in a final concentration between 50 μM and 1000 μM oxidizing agent, under conditions comprising a time period between 0.1 minutes and 120 minutes; a

temperature between 2°C and 37°C; in the presence or absence of light; and with or without stirring.

20. The method according to claim 19 wherein the final concentration of the oxidizing agent is 400 μM, and the conditions comprise a time period of 10 minutes, a temperature of 22 °C, the absence of light and with stirring.

21. The method according to any one of claims 1-8 wherein the conjugating the water soluble polymer to the oxidized carbohydrate moiety of the therapeutic protein is stopped by the addition of a quenching agent selected from the group consisting of L-cysteine, methionine, glutathione, glycerol, sodium meta bisulfite ($Na_2S_2O_5$), tryptophan, tyrosine, histidine or derivatives thereof, kresol, imidazole, and combinations thereof;

wherein the quenching agent is added in an amount to result in a final concentration between 1 mM and 100 mM quenching agent, under conditions comprising a time period between 5 minutes and 120 minutes; a temperature between 2°C and 37°C; in the presence or absence of light; and with or without stirring.

22. The method according to claim 21 wherein the quenching agent is L-cysteine.

23. The method according to claim 22 wherein the L-cysteine is added to result in a final concentration of 10 mM and the conditions comprise a time period of 60 minutes, a temperature of 22°C, the absence of light and with stirring.

24. The method according to any one of claims 1-8 comprising:

a) a first step comprising adjusting the pH value of a solution comprising the therapeutic protein to a pH value between 5.0 and 8.0, wherein the therapeutic protein concentration is between 0.3 mg/ml and 3.0 mg/ml;

b) a second step comprising oxidizing one or more carbohydrates on the therapeutic protein, wherein the oxidizing agent is added to the solution in the first step to result in a final concentration between 50μM and 1000μM, under conditions comprising a time period between 0.1 minutes and 120 minutes; a temperature between 2°C and 37°C; in the presence or absence of light, and with or without stirring;

c) a third step comprising contacting the therapeutic protein with a desired excess concentration of activated water soluble polymer, wherein the excess concentration is

between 1-fold molar excess and 300-fold molar excess, under conditions comprising a time period between 0.5 hours and 24 hours, a temperature between 2°C and 37°C; in the presence or absence of light; and with or without stirring;

d) a fourth step comprising adding a nucleophilic catalyst to the solution of the third step, wherein the nucleophilic catalyst is added to result in a final concentration between 1 mM and 50 mM, under conditions comprising a time period between 0.1 minutes and 30 minutes; a temperature between 2°C and 37°C; in the presence or absence of light, and with or without stirring;

e) a fifth step wherein the therapeutic protein is incubated with the activated water soluble polymer and nucleophilic catalyst in order to allow conjugation of the activated water-soluble polymer to one or more oxidized carbohydrates on the therapeutic protein, said conditions comprising a time period between 0.5 hours and 24 hours, a temperature between 2°C and 37°C; in the presence or absence of light, and with or without stirring; and

f) a sixth step wherein the conjugating the water soluble polymer to the one or more oxidized carbohydrates of the therapeutic protein in the fifth step is stopped by the addition of a quenching agent selected from the group consisting of L-cysteine, methionine, glutathione, glycerol, $Na_2S_2O_5$ (sodium meta bisulfite), tryptophane, tyrosine, histidine or derivatives thereof, kresol, imidazole, and combinations thereof; wherein the quenching agent is added to result in a final concentration of 1 mM and 100 mM, under conditions comprising a time period between 5 minutes and 120 minutes; a temperature between 2°C and 37°C; in the presence or absence of light, and with or without stirring.

25. The method according to any one of claims 1-8 comprising:

a) a first step comprising adjusting the pH value of a solution comprising the therapeutic protein to a pH value of 6.0, wherein the initial concentration of the therapeutic protein is 1.0 mg/ml;

b) a second step comprising oxidizing one or more carbohydrates on the therapeutic protein, wherein the oxidizing agent is added to the solution in the first step to result in a final concentration of 100µM or 400µM, under conditions comprising a time period of 10 minutes; a temperature of 22°C; the absence of light, and with stirring;

c) a third step comprising contacting the therapeutic protein with a desired excess concentration of activated water soluble polymer, wherein the excess concentration is 5-fold molar excess or 50-fold molar excess, under conditions comprising a time period of 15 minutes, a temperature of 22°C; the absence of light; and with stirring;

d) a fourth step comprising adding a nucleophilic catalyst to the solution of the third step, wherein the nucleophilic catalyst is added to result in a final concentration of 10 mM, under conditions comprising a time period of 15 minutes; a temperature of 22°C; the absence of light, and with stirring;

e) a fifth step wherein the therapeutic protein is incubated with the activated water soluble polymer and nucleophilic catalyst in order to allow conjugation of the activated water soluble polymer to one or more oxidized carbohydrates on the therapeutic protein, said conditions comprising a time period of 2 hours, a temperature of 22°C; the absence of light, and with stirring; and

f) a sixth step wherein the conjugating the water soluble polymer to the one or more oxidized carbohydrates of the therapeutic protein in the fifth step is stopped by the addition of a quenching agent L-cysteine; wherein L-cysteine is added to result in a final concentration of 10 mM, under conditions comprising a time period of 60 minutes; a temperature of 22°C; the absence of light, and with stirring.

26. The method according to any one of claims 1-8 wherein the water soluble polymer is PEG.

27. The method according to any one of claims 1-8 wherein the water soluble polymer is PSA.

28. The method according to claim 27 wherein the PSA is comprised of 10 - 300 sialic acid units.

29. The method according to any one of claims 1-8 wherein the therapeutic protein has biological activity of FIX.

30. The method according to any one of claims 1-8 wherein the therapeutic protein has biological activity of FVIIa.

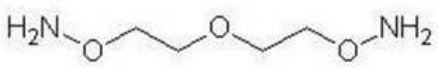
31. The method according to any one of claims 1-8 wherein the therapeutic protein has biological activity of FVIII.

32. The method according to any one of claims 1-8 wherein the oxidizing agent is sodium periodate ($NaIO_4$).

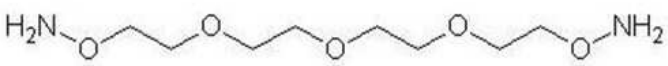
33. The method according to claim 31 wherein the oxidized carbohydrate moiety of the therapeutic protein is located in the activation peptide of the therapeutic protein.

34. The method according to any one of claims 1-8, wherein the activated water soluble polymer is activated PSA, wherein the aminooxy linker is selected from the group consisting of:

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

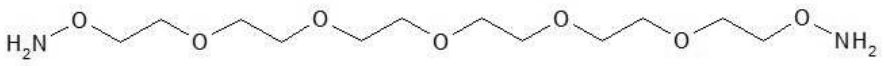


b) a 3,6,9-trioxa-undecane-l,l l-dioxyamine linker of the formula:



; and

c) a 3,6,9,12,15-pentaoxa-heptadecane-l,17-dioxyamine linker of the formula:



.

35. The method according to claim 34 wherein the aminooxy linker is 3-oxa-pentane-1,5-dioxyamine.

36. The method according to any one of claims 1-8 wherein the nucleophilic catalyst is provided at a concentration between 1 mM and 50 mM.

37. The method according to claim 36 wherein the nucleophilic catalyst is m-toluidine.

38. The method according to claim 37 wherein the m-toluidine is present in the conjugation reaction at a concentration of 10 mM.

39. The method according to any one of claims 1-8 further comprising the step of purifying the conjugated therapeutic protein.

40. The method according to claim 39 wherein the conjugated therapeutic protein is purified by a method selected from the group consisting of chromatography, filtration and precipitation.

41. The method according to claim 40 wherein the chromatography is selected from the group consisting of Hydrophobic Interaction Chromatography (HIC), Ion Exchange chromatography (IEC), Size exclusion chromatography (SEC), Affinity chromatography, and Reversed-phase chromatography.

42. The method according to claim 41 wherein an anti-chao tropic salt is used in a chromatography loading step and in a chromatography washing step.

43. The method according to claim 41 wherein the chromatography takes place in a column.

44. The method according to claim 43 wherein the column comprises a chromatography resin selected from the group consisting of Phenyl-Sepharose FF and Butyl-Sepharose FF.

45. The method according to claim 44 wherein the resin is present in the column at a bed height of between 5 cm and 20 cm.

46. The method according to claim 45 wherein the bed height is 10 cm.

47. The method according to claim 43 comprising one or more washing steps wherein flow direction is set to up-flow and wherein the flow rate is between 0.2 cm/min and 6.7 cm/min.

48. The method according to claim 47 wherein the flow rate is 2 cm/min.

49. The method according to claim 43 comprising one or more elution steps wherein flow direction is set to down-flow and wherein the flow rate is between 0.1 cm/min and 6.7 cm/min.

50. The method according to claim 49 wherein the flow rate is 1 cm/min.

51. The method according to claim 39 further comprising concentrating the conjugated therapeutic protein by ultrafiltration/diafiltration (UF/DF).

52. The method according to claim 39 wherein the final concentration of therapeutic protein is between 0.5 and 3 mg/ml.

53. The method according to claim 39 wherein the therapeutic protein comprises between 5 and 11 water soluble polymer moieties.

54. The method according to any one of claims 1-8 wherein the conjugated therapeutic protein is purified using chromatography; wherein an anti-chaotropic salt is used for a loading step and for a washing step; the method comprising one or more washing steps wherein flow direction is set to up-flow and wherein the flow rate is between 0.2 cm/min and 6.7 cm/min and one or more elution steps wherein flow direction is set to down-flow and wherein the flow rate is between 0.2 cm/min and 6.7 cm/min; further comprising concentrating the conjugated therapeutic protein by ultra-/diafiltration (UF/DF).

55. The method according to claim 48 comprising one or more elution steps, wherein the chromatography is hydrophobic interaction chromatography (HIC); wherein the one or more washing steps flow rate is 2 cm/min; and wherein the one or more elution steps flow rate is 1 cm/min.

56. The method according to 24, wherein the steps a) through f) occur in a single vessel.

57. The method according to 25, wherein the steps a) through f) occur in a single vessel.