What is claimed:

1. A method of conjugating a water soluble polymer to an oxidized carbohydrate moiety of a therapeutic protein 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); 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;

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~~.

2. ~~A~~ The method of ~~conjugating a water soluble polymer to an oxidized carbohydrate moiety of a therapeutic protein comprising contacting the oxidized carbohydrate moiety with an activated water soluble polymer under conditions that allow conjugation:~~claim 1 wherein

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

line-derived neutrophic factor receptor α1, glial cell line-derived neutrophic factor receptor α2, growth related protein, growth related protein α, growth related protein β, growth related protein γ, heparin binding epidermal growth factor, hepatocyte growth factor, hepatocyte growth factor receptor, hepatoma-derived growth factor, insulin-like growth factor I, insulin-like growth factor receptor, insulin-like growth factor II, insulin-like growth factor binding protein, keratinocyte growth factor, leukemia inhibitory factor, leukemia inhibitory factor receptor α, nerve growth factor nerve growth factor receptor, neuropoietin,neurotrophin-3, neurotrophin-4, oncostatin M (OSM), placenta growth factor, placenta growth factor 2, platelet-derived endothelial cell growth factor, platelet derived growth factor, platelet derived growth factor A chain, platelet derived growth factor AA, platelet derived growth factor AB, platelet derived growth factor B chain, platelet derived growth factor BB, platelet derived growth factor receptor α, platelet derived growth factor receptor β, pre-B cell growth stimulating factor, stem cell factor (SCF), stem cell factor receptor, TNF, TNF0, TNF1, TNF2, transforming growth factor α, transforming growth factor β, transforming growth factor β1, transforming growth factor β1.2, transforming growth factor β2, transforming growth factor β3, transforming growth factor β5, latent transforming growth factor β1, transforming growth factor β binding protein I, transforming growth factor β binding protein II, transforming growth factor β binding protein III, thymic stromal lymphopoietin (TSLP), tumor necrosis factor receptor type I, tumor necrosis factor receptor type II, urokinase-type plasminogen activator receptor, phospholipase-activating protein (PUP), insulin, lectin ricin, prolactin, chorionic gonadotropin, follicle-stimulating hormone, thyroid-stimulating hormone, tissue plasminogen activator, IgG, IgE, IgM, IgA, and IgD, α-galactosidase, β-galactosidase, DNAse, fetuin, leutinizing hormone, estrogen, insulin, albumin, lipoproteins, fetoprotein, transferrin, thrombopoietin, urokinase, integrin, thrombin, leptin, Humira (adalimumab), Prolia (denosumab), Enbrel (etanercept), a protein in Table 1, or a biologically active fragment, derivative or variant thereof.

~~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); 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; and wherein in 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.~~

3. The method according to claim 2 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~~,~~ for example,

~~4. The method of claim 3~~ wherein the initial concentration of the therapeutic protein is about 1.0 mg/ml and the pH is about 6.0.

~~5~~4. The method of claim 2 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~~.~~,

~~6. The method of claim 5 wherein the excess concentration is~~ about 50-fold molar excess.

~~7~~5. The method of claim ~~5~~ 4 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~~.~~; for example,

~~8. The method according to claim 7~~ wherein the conditions comprise a time period of about 120 minutes, a temperature of about 22 °C, the absence of light; and with stirring.

~~9~~6. The method according to claim 2 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~~.~~; for example,

~~10. The method of claim 9~~ wherein 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.

~~11~~7. The method according to claim 2 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~~.~~; for example,

~~12. The method of claim 11~~ wherein 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.

~~13~~8. The method of claim 2 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.

~~14~~9. The method of claim ~~13~~ 8 wherein the quenching agent is L-cysteine~~.~~ and is preferably

~~15. The method of claim 14 wherein the L-cysteine is~~ added to result in a final concentration of about 10 mM and the conditions comprise a time period ofabout 60 minutes, a temperature of about 22°C, the absence of light and with stirring.

~~16~~10. The method of claim 2 comprising:

a) a first step comprising adjusting the pH value of a solution comprising the therapeutic protein to a pH value betweenabout 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~~ the 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.

~~17~~11. The method of claim ~~16~~ 10 wherein 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 °C, 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.

~~18. The method according to claim 2 wherein the water soluble polymer is PSA.~~

~~19. The method according to claim 2 wherein the water soluble polymer is PEG.~~

~~20. The method according to claim 2 wherein the water soluble polymer is HES.~~

~~21. The method according to claim 2 wherein the water soluble polymer is HAS.~~

~~22~~12. The method according to claim ~~18~~ 2 wherein the water soluble polymer is PSA and is comprised of about 10 – 300 sialic acid units.

~~23. The method according to any one of claims 2-22 wherein the therapeutic protein is FIX.~~

~~24. The method according to any one of claims 2-22 wherein the therapeutic protein is FVIIa.~~

~~25. The method according to any one of claims 2-22 wherein the therapeutic protein is FVIII.~~

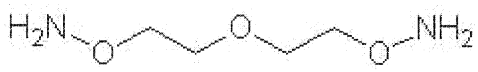
~~26. The method according to any one of claims 2-25 wherein the oxidizing agent is sodium periodate ($NaIO_4$).~~

~~27~~13. The method according to ~~any one of claims 23-25~~claim 2 wherein when the therapeutic protein is a blood coagulation protein, the oxidized carbohydrate moiety of the therapeutic protein is located in the activation peptide of ~~the~~ said blood coagulation protein.

~~28~~14. The method according to claim ~~18~~ 12 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) a 3-oxa-pentane-1,5-dioxyamine linker of the formula:



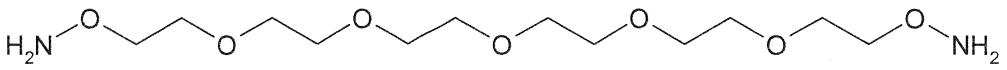
,

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



and

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



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.

~~29. The method according to claim 28 wherein the aminooxy linker is 3-oxa-pentane-1,5-dioxyamine.~~

~~30~~15. The method according to any one of claims 1-~~29~~14 wherein the oxidizing agent is $NaIO_4$.

~~31~~16. The method according to any one of claims 1-~~30~~15 wherein the nucleophilic catalyst is provided at a concentration between about 1 mM and about 50 mM; preferably at a concentration of about 10 mM.

~~32. The method according to claim 31 wherein the nucleophilic catalyst is m-toluidine.~~

~~33. The method according to claim 32 wherein the m-toluidine is present in the conjugation reaction at a concentration of about 10 mM.~~

~~34~~17. The method according to any one of claims 1-~~33~~16 further comprising the step of reducing an oxime linkage in the conjugated therapeutic protein by incubating the conjugated therapeutic protein in a buffer comprising a reducing compound selected from the group consisting of sodium cyanoborohydride ($NaCNBH_3$), ascorbic acid (vitamin C) and $NaBH_3$.

~~35. The method according to claim 34 wherein the reducing compound is sodium cyanoborohydride ($NaCNBH_3$).~~

~~36~~18. The method according to any one of claims 1-~~35~~17 further comprising the step of purifying the conjugated therapeutic protein.; for example;

~~37. The method according to claim 36~~ wherein the conjugated therapeutic protein is purified by a method selected from the group consisting of chromatography, filtration and precipitation.

~~38~~19. The method according to claim ~~37~~ 18 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~~.~~; optionally

~~39. The method of claim 38~~ wherein an anti-chaotropic salt is used in a chromotagraphy loading step and in a chromatography washing step~~.~~; optionally

~~40. The method of claim 38~~ wherein the chromatography takes place in a column~~,~~ such as a

~~41. The method of claim 40 wherein the~~ column ~~comprises~~ comprising a chromatography resin selected from the group consisting of Phenyl-Sepharose FF and Butyl-Sepharose FF~~.~~; and wherein

~~42. The method of claim 41 wherein~~ the resin is preferably present in the column at a bed height of between about 5 cm and about 20 cm~~.~~, preferably

~~43. The method according to claim 42 wherein the bed height is~~ about 10 cm.

~~44~~20. The method of claim ~~40~~ 19 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~~.~~, optionally

~~45. The method according to claim 44~~ wherein the flow rate is about 2 cm/min.

~~46~~21. The method of any one of claims ~~40-45~~19-20 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~~.~~, optionally

~~47. The method according to claim 46~~ wherein the flow rate is about 1 cm/min.

~~48~~22. The method of any one of claims ~~36-47~~18-21 further comprising concentrating the conjugated therapeutic protein by ultra-/diafiltration (UF/DF)~~.~~; optionally

~~49. The method of any one of claims 36-48~~ wherein the final concentration of therapeutic protein is between about 0.5 and about 3 mg/ml.

~~50~~23. The method according to any one of claims ~~36-49~~18-22 wherein the therapeutic protein comprises between about 5 and about 11 water soluble polymer moieties.

~~51~~24. The method of claim 2 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 andabout 6.7 cm/min; further comprising concentrating the conjugated therapeutic protein by ultra-/diafiltration (UF/DF)~~.~~; optionally

~~52. The method of claim 51~~ 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.

~~53. A modified therapeutic protein produced by the method according to any one of claims 1-52.~~

~~54~~25. A method of forming an oxime linkage between an oxidized carbohydrate moiety on a therapeutic protein and an activated water soluble polymer containing an active aminooxy group comprising the steps of:

a) oxidizing a carbohydrate moiety on a therapeutic protein by incubating said protein with an oxidizing agent selected from the group consisting of sodium periodate ($NaIO_4$), lead tetraacetate ($Pb(OAc)_4$) and potassium perruthenate (KRuO4); and

b) forming an oxime linkage between the oxidized carbohydrate moiety of the therapeutic protein and the activated water soluble polymer containing an active aminooxy group in the presence of a nuclephilic catalyst under conditions allowing formation of said oxime linkage;

wherein said water soluble polymer containing an active aminooxy group is selected from the group consisting 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 nucleophilic catalyst is m-toluidine~~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.

55. A method of forming an oxime linkage between an oxidized carbohydrate moiety on a therapeutic protein and an activated water soluble polymer containing an active aminooxy group comprising the steps of:

a) oxidizing a carbohydrate moiety on a therapeutic protein by incubating said protein with an oxidinzing agent selected from the group consisting of sodium periodate ($NaIO_4$), lead tetraacetate ($Pb(OAc)_4$) and potassium perruthenate (KRuO4); and

b) forming an oxime linkage between the oxidized carbohydrate moiety of the therapeutic protein and the activated water soluble polymer containing an an active aminooxy group in the presence of a nuclephilic catalyst under conditions allowing formation of said oxime linkage;

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-3, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-31, IL-32 alpha, IL-33, thrombopoietin (TPO), Ang-1, Ang-2, Ang-4, Ang-Y, angiopoietin-like polypeptide 1 (ANGPTL1), angiopoietin-like polypeptide 2 (ANGPTL2), angiopoietin-like polypeptide 3 (ANGPTL3), angiopoietin-like polypeptide 4 (ANGPTL4), angiopoietin-like polypeptide 5 (ANGPTL5), angiopoietin-like polypeptide 6 (ANGPTL6), angiopoietin-like polypeptide 7 (ANGPTL7), vitronectin, vascular endothelial growth factor (VEGF), angiogenin, activin A, activin B, activin C, bone morphogenic protein 1, bone morphogenic protein 2, bone morphogenic protein 3, bone morphogenic protein 4, bone morphogenic protein 5, bone morphogenic protein 6, bone morphogenic protein 7, bone morphogenic protein 8, bone morphogenic protein 9, bone morphogenic protein 10, bone morphogenic protein 11, bone morphogenic protein 12, bone morphogenic protein 13, bone morphogenic protein 14, bone morphogenic protein 15, bone morphogenic protein receptor IA, bone

morphogenic protein receptor IB, bone morphogenic protein receptor II, brain derived neurotrophic factor, cardiotrophin-1, ciliary neutrophic factor, ciliary neutrophic factor receptor, cripto, cryptic, cytokine-induced neutrophil chemotactic factor 1, cytokine-induced neutrophil, chemotactic factor 2α, cytokine-induced neutrophil chemotactic factor 2β, β endothelial cell growth factor, endothelin 1, epidermal growth factor, epigen, epiregulin, epithelial-derived neutrophil attractant, fibroblast growth factor 4, fibroblast growth factor 5, fibroblast growth factor 6, fibroblast growth factor 7, fibroblast growth factor 8, fibroblast growth factor 8b, fibroblast growth factor 8c, fibroblast growth factor 9, fibroblast growth factor 10, fibroblast growth factor 11, fibroblast growth factor 12, fibroblast growth factor 13, fibroblast growth factor 16, fibroblast growth factor 17, fibroblast growth factor 19, fibroblast growth factor 20, fibroblast growth factor 21, fibroblast growth factor acidic, fibroblast growth factor basic, glial cell line-derived neutrophic factor receptor α1, glial cell line-derived neutrophic factor receptor α2, growth related protein, growth related protein α, growth related protein β, growth related protein γ, heparin binding epidermal growth factor, hepatocyte growth factor, hepatocyte growth factor receptor, hepatoma-derived growth factor, insulin-like growth factor I, insulin-like growth factor receptor, insulin-like growth factor II, insulin-like growth factor binding protein, keratinocyte growth factor, leukemia inhibitory factor, leukemia inhibitory factor receptor α, nerve growth factor nerve growth factor receptor, neuropoietin,neurotrophin-3, neurotrophin-4, oncostatin M (OSM), placenta growth factor, placenta growth factor 2, platelet-derived endothelial cell growth factor, platelet derived growth factor, platelet-derived growth factor A chain, platelet derived growth factor AA, platelet derived growth factor AB, platelet derived growth factor B chain, platelet derived growth factor BB, platelet derived growth factor receptor α, platelet derived growth factor receptor β, pre-B cell growth stimulating factor, stem cell factor (SCF), stem cell factor receptor, TNF, TNF0, TNF1, TNF2, transforming growth factor α, transforming growth factor β, transforming growth factor β1, transforming growth factor β1.2, transforming growth factor β2, transforming growth factor β3, transforming growth factor β5, latent transforming growth factor β1, transforming growth factor β binding protein I, transforming growth factor β binding protein II, transforming growth factor β binding protein III, thymic stromal lymphopoietin (TSLP), tumor necrosis factor receptor type I, tumor necrosis factor receptor type II, urokinase-type plasminogen activator receptor, phospholipase-activating protein (PUP), insulin, lectin ricin, prolactin, chorionic gonadotropin, follicle-stimulating hormone, thyroid-stimulating hormone, tissue plasminogen activator, IgG, IgE, IgM, IgA, and IgD, α-galactosidase, β-galactosidase, DNAse, fetuin, leutinizing hormone, estrogen,

~~insulin, albumin, lipoproteins, fetoprotein, transferrin, thrombopoietin, urokinase, integrin, thrombin, leptin, Humira (adalimumab), Prolia (denosumab), Enbrel (etanercept), a protein from Table 1, or a biologically active fragment, derivative or variant thereof;~~

~~wherein said water soluble polymer containing an active aminooxy group 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 nucleophilic catalyst is 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.~~

~~56~~26. A method of forming a hydrazone linkage between an oxidized carbohydrate moiety on a therapeutic protein and an activated water soluble polymer containing an active hydrazide group comprising the steps of:

a) oxidizing a carbohydrate moiety on a therapeutic protein by incubating said protein with an oxidinzing agent selected from the group consisting of sodium periodate ($NaIO_4$), lead tetraacetate ($Pb(OAc)_4$) and potassium perruthenate (KRuO4); and

b) forming a hydrazone linkage between the oxidized carbohydrate moiety of the therapeutic protein and the activated water soluble polymer containing an an active hydrazide group in the presence of a nuclephilic catalyst under conditions allowing formation of said hydrazone linkage;

wherein said water soluble polymer containing an active hydrazide group 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 nucleophilic catalyst is ~~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~~.

~~57~~27. ~~A~~ The method of ~~forming a hydrazone linkage between an oxidized carbohydrate moiety on a therapeutic protein and an activated water soluble polymer containing an active hydrazide group comprising the steps of:~~

~~a) oxidizing a carbohydrate moiety on a therapeutic protein by incubating said protein with an oxidinzing agent selected from the group consisting of sodium periodate ($NaIO_4$), lead tetraacetate ($Pb(OAc)_4$) and potassium perruthenate (KRuO4); and~~

~~b) forming a hydrazone linkage between the oxidized carbohydrate moiety of the therapeutic protein and the activated water soluble polymer containing an an active hydrazide group in the presence of a nuclephilic catalyst under conditions allowing formation of said hydrazone linkage;~~claim 25 or 26

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

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

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

~~wherein said water soluble polymer containing an active hydrazide group 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 nucleophilic catalyst is 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~~.

~~58~~28. The method according to any one of claims 1-~~52~~24 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 activated 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 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 and precipitation.

~~59~~29. The method ~~according to any one of claims 1-52 wherein the water soluble polymer containing an active aminooxy group is prepared by a method comprising:~~of claim 28 further comprising the step of

~~a) incubating a solution comprising an oxidized water-soluble polymer with an activated 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 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;~~

~~b~~a') incubating a solution comprising the water soluble polymer containing an active aminooxy group of step a) with a reducing agent under conditions that allow the formation of a stable alkoxamine 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 37°C; in the presence or absence of light; and with or without stirring; ~~and~~such that step (a') is conducted after step (a) and before (b).

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

6030. The method ~~according to any one of claims 1-52 wherein the water soluble polymer containing an active aminooxy group is prepared by a method comprising:~~of claim 28 further comprising the step of

~~a) incubating a solution comprising an oxidized water-soluble polymer with an activated 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 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;~~

~~b~~a') incubating a solution comprising the water soluble polymer containing an active aminooxy group of step a) with a nucleophilic catalyst under conditions comprising a time period between 1 minute and 24 hours; a temperature between 2°C and 37°C; in the presence or absence of light; and with or without stirring; ~~and~~such that step (a') is conducted after step (a) and before step (b)

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

6131. The method according to any one of claims 1-28~~52~~ 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 activated 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 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;~~

~~b) incubating a solution comprising the water soluble polymer containing an active aminooxy group of step a) with a nucleophilic catalyst under conditions comprising a time period between 1 minute and 24 hours; a temperature between 2°C and 37°C; in the presence or absence of light; and with or without stirring;~~

~~e~~a'') incubating a solution comprising the water soluble polymer containing an active aminooxy group of step b) with a reducing agent under conditions that allow the formation of a stable alkoxamine 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 37°C; in the presence or absence of light; and with or without stirring; ~~and~~such that step (a'') is conducted after step (a') and before step (b).

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

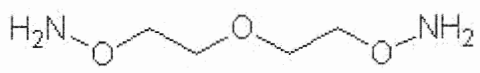
~~62~~32. The method according to any one of claims ~~58-61~~28-31 wherein the oxidized water soluble polymer is selected from the group consisting of polyethylene glycol (PEG), branched PEG, PolyPEG® (Warwick Effect Polymers; Coventry, UK), polysialic acid (PSA), ~~starch, hydroxyalkyl starch (HAS), 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), and wherein said water-soluble polymer is oxidized by incubation with a oxidizing agent to form a terminal aldehyde group at the non-reducing end of the water-soluble polymer.

~~63. The method according to claim 62 wherein the water soluble polymer is PSA.~~

~~64~~33. The method according to claim ~~62~~32 wherein the oxidizing agent is $NaIO_4$.

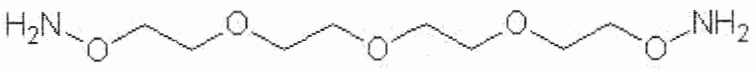
~~65~~34. The method according to any one of claims ~~58-64~~28-33 wherein the aminooxy linker is selected from the group consisting of:

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



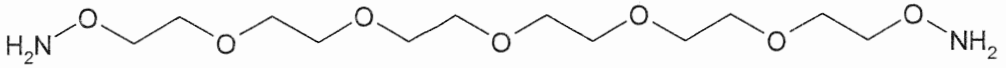
,

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



and

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



.

~~66~~35. The method according to any one of claims ~~59~~ 29 and ~~61~~ 31 wherein the reducing agent is selected from the group consisting of sodium cyanoborohydride ($NaCNBH_3$), ascorbic acid (vitamin C) and $NaBH_3$.

~~67. The method according to claim 66 wherein the reducing agent is sodium cyanoborohydride ($NaCNBH_3$).~~

~~68~~36. The method according to any one of claims ~~60~~ 30 and ~~61~~ 31 wherein the nucleophilic catalyst ~~is 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 is optionally added in an amount to result in a final concentration between about 1.0 mM and about 50 mM nucleophilic catalyst.

~~69. The method according to claim 68 wherein the nucleophilic catalyst is m-toluidine.~~

~~70. The method according to any one of claims 68-69 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.~~

~~71~~37. The method according to any one of claims ~~58-70~~28-36 further comprising concentrating the conjugated therapeutic protein by ultra-/diafiltration (UF/DF).