NUCLEOPHILIC CATALYSTS FOR OXIME LINKAGE

FIELD OF THE INVENTION

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

BACKGROUND OF THE INVENTION

[0002] The preparation of conjugates by forming a covalent linkage between the water soluble polymer and the therapeutic protein can be carried out by a variety of chemical methods. PEGylation of polypeptide drugs protects them in circulation and improves their pharmacodynamic and pharmacokinetic profiles (Harris and Chess, Nat Rev Drug Discov. 2003;2:214-21). The PEGylation process attaches repeating units of ethylene glycol (polyethylene glycol (PEG)) to a polypeptide drug. PEG molecules have a large hydrodynamic volume (5 - 10 times the size of globular proteins), are highly water soluble and hydrated, non-toxic, non-immunogenic and rapidly cleared from the body. PEGylation of molecules can lead to increased resistance of drugs to enzymatic degradation, increased half-life *in vivo*, reduced dosing frequency, decreased immunogenicity, increased physical and thermal stability, increased solubility, increased liquid stability, and reduced aggregation. The first PEGylated drugs were approved by the FDA in the early 1990s. Since then, the FDA has approved several PEGylated drugs for oral, injectable, and topical administration.

[0003] Polysialic acid (PSA), also referred to as colominic acid (CA), is a naturally occurring polysaccharide. It is a homopolymer of N-acetylneuraminic acid with $\alpha(2\rightarrow8)$ ketosidic linkage and contains vicinal diol groups at its non-reducing end. It is negatively charged and a natural constituent of the human body. It can easily be produced from bacteria in large quantities and with pre-determined physical characteristics (US Patent No. 5,846,951). Because the bacterially-produced PSA is chemically and immunologically identical to PSA produced in the human body, bacterial PSA is non-immunogenic, even when coupled to proteins. Unlike some polymers, PSA acid is biodegradable. Covalent coupling of colominic acid to catalase and asparaginase has been shown to increase enzyme stability in the presence of proteolytic enzymes or blood plasma. Comparative studies in vivo with polysialylated and unmodified asparaginase revealed that polysialylation increased the half-life of the enzyme (Fernandes and Gregoriadis, Int J Pharm. 2001;217:215-24).

[0004] Coupling of PEG-derivatives to peptides or proteins is reviewed by Roberts et al. (Adv Drug Deliv Rev 2002;54:459-76). One approach for coupling water soluble polymers

to therapeutic proteins is the conjugation of the polymers via the carbohydrate moieties of the protein. Vicinal hydroxyl (OH) groups of carbohydrates in proteins can be easily oxidized with sodium periodate ($NaIO_4$) to form active aldehyde groups (Rothfus et Smith, J Biol Chem 1963; 238:1402-10; van Lenten et Ashwell, J Biol Chem 1971;246:1889-94). Subsequently the polymer can be coupled to the aldehyde groups of the carbohydrate by use of reagents containing, for example, an active hydrazide group (Wilchek M and Bayer EA, Methods Enzymol 1987;138:429-42). A more recent technology is the use of reagents containing aminooxy groups which react with aldehydes to form oxime linkages (WO 96/40662, WO2008/025856).

[0005] Additional examples describing conjugation of a water soluble polymer to a therapeutic protein are described in WO 06/071801 which teaches the oxidation of carbohydrate moieties in Von Willebrand factor and subsequent coupling to PEG using hydrazide chemistry; US Publication No. 2009/0076237 which teaches the oxidation of rFVIII and subsequent coupling to PEG and other water soluble polymers (e.g. PSA, HES, dextran) using hydrazide chemistry; WO 2008/025856 which teaches oxidation of different coagulation factors, e.g. rFIX, FVIII and FVIIa and subsequent coupling to e.g., PEG, using aminooxy chemistry by forming an oxime linkage; and US Patent No. 5,621,039 which teaches the oxidation of FIX and subsequent coupling to PEG using hydrazide chemistry.

[0006] Recently, an improved method was described comprising mild periodate oxidation of sialic acids to generate aldehydes followed by reaction with an aminooxy group containing reagent in the presence of catalytic amounts of aniline (Dirksen A., and Dawson PE, Bioconjugate Chem. 2008;19,2543-8; and Zeng Y et al., Nature Methods 2009;6:207-9). The aniline catalysis dramatically accelerates the oxime ligation, allowing the use of very low concentrations of the reagent. The use of nucelophilic catalysts are also described in Dirksen, A., et al., J Am Chem Soc., 128:15602-3 (2006); Dirksen, A., et al., Angew chem. Int Ed., 45:7581-4 (2006); Kohler, J.J., ChemBioChem., 10:2147-50 (2009); Giuseppone, N., et al., J Am Chem Soc., 127:5528-39 (2005); and Thygesen, M.B., et al., J Org Chem., 75:1752-5 (2010).

[0007] Although aniline catalysis can accelerate the oxime ligation allowing short reaction times and the use of low concentrations of the aminooxy reagent, aniline has toxic properties that must be considered when, for example, the conjugated therapeutic protein to form the basis of a pharmaceutical. For example, aniline has been shown to induce methemoglobinemia (Harrison, J.H.., and Jollow, D.J., Molecular Pharmacology, 32(3) 423-

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 OF THE INVENTION

[0009] The present invention 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 embodiments of the invention, alternative catalysts to substitute for aniline are provided.

[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 embodiments of the present disclusre, the new process is carried out at a temperature between 2-8°C. In one embodiment, 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 embodiment 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 embodiment, 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 embodiment, 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 embodiment, an aforementioned method is provided wherein the oxidized water soluble polymer is PSA and is oxidized by incubation with $NaIO_4$.

[0013] In another embodiment 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 embodiment, 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 embodiment, 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 embodiment, 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 embodiment, 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 embodiment, 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 embodiments 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,

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.

[0016] In still another embodiment, 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 embodiment, the the initial concentration of the therapeutic protein is about 1.0 mg/ml and the pH is about 6.0.

[0017] In another embodiment, 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 embodiment, the excess concentration is about 50-fold molar excess.

[0018] In another embodiment, 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 embodiment, 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 embodiment, 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 embodiment, 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 embodiment, 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 embodiment, 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 embodiment, 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 embodiment the quenching agent is L-cysteine. In still another embodiment, 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 embodiment, 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

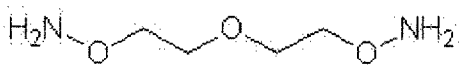
of the third step, wherein the nucleophilic catalyst is added to result in a final concentration between about 1 mM and about 50 mM, under conditions comprising a time period between about 0.1 minutes and about 30 minutes; a temperature between about 2°C and about 37°C; in the presence or absence of 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 °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. In still another embodiment, the water soluble polymer is PSA. In one embodiment, the water soluble polymer is PEG. In another

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

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

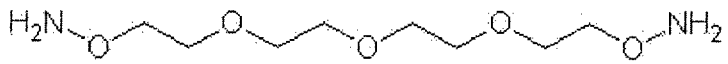
[0024] In another embodiment, the aforementioned method is provided wherein the oxidized carbohydrate moiety of the therapeutic protein is located in the activation peptide of the blood coagulation protein.

[0025] In another embodiment, 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] 

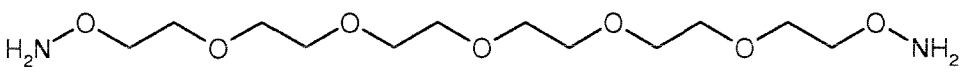
,

[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 embodiment, the aminooxy linker is 3-oxa-pentane-1,5-dioxyamine.

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

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

therapeutic protein is purified by a method selected from the group consisting of chromatography, filtration and precipitation. In still another embodiment, 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 embodiment, 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 embodiment, the column comprises a chromatography resin selected from the group consisting of Phenyl-Sepharose FF and Butyl-Sepharose FF. In yet another embodiment, the resin is present in the column at a bed height of between about 5 cm and about 20 cm.

[0035] In still another embodiment, the bed height is about 10 cm. In another embodiment, 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 embodiment, the flow rate is about 2 cm/min. In another embodiment, 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 embodiment, the flow rate is about 1 cm/min.

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

[0037] In still another embodiment, 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 embodiment, 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 embodiment 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 embodiment, 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 embodiment, the aforementioned method is provided wherein the water soluble polymer is PSA In another embodiment, the aforementioned method is provided wherein the water-soluble polymer is PEG. In another embodiment, 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 embodiment, 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 embodiment 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.

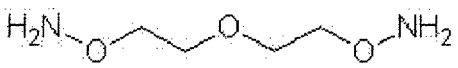
[0044] In another embodiment, the aforementioned method is provided wherein said oxime linkage formation is catalyzed by a nucleophilic catalyst selected from the group consisting of o-amino benzoic acid, m-amino benzoic acid, p-amino benzoic acid, sulfanilic acid, o-aminobenzamide, o-toluidine, m-toluidine,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

[0045] In another embodiment, 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 embodiment, 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 embodiment, 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 embodiment, 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 embodiment, 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.

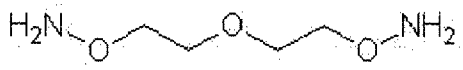
[0047] In still another embodiment of thepresent disclure, a method of preparing a PSA-aminooxy reagent aminooxy group is provided comprising: a) incubating a solution comprising an oxidized PSA with an aminooxy linker comprising an active aminooxy group under conditions that allow the formation of a 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 embodiment 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 embodiment 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.

[0057] Figure 5 shows the visualization of PSA-FIX conjugates prepared in the presence of different catalysts by SDS PAGE. a) Comparison of aniline with m-toluidine using different concentrations; b) Comparison of aniline with o-aminobenzoic acid, m-aminobenzoic acid, p-aminobenzoic acid, p-aminobenzamide and sulfanilic acid; c) Comparison of aniline and m-toluidine with o-anisidine and m-anisidine.

[0058] Figure 6 shows percent of polysialylation with various nucleophilic catalysts.

DETAILED DESCRIPTION OF THE INVENTION

[0059] The pharmacological and immunological properties of therapeutic proteins can be improved by chemical modification and conjugation with polymeric compounds such as polyethylene glycol (PEG), branched PEG, polysialic acid (PSA), 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). The properties of the resulting conjugates generally strongly depend on the structure and the size of the polymer. Thus, polymers with a defined and narrow size distribution are usually preferred in the art. Synthetic polymers like PEG can be manufactured easily with a narrow size distribution, while PSA can be purified in such a manner that results in a final PSA preparation with a narrow size distribution. In addition PEGylation reagents with defined polymer chains and narrow size distribution are on the market and commercially available for a reasonable price.

[0060] The addition of a soluble polymer, such as through polysialylation, is one approach to improve the properties of therapeutic proteins such as the blood coagulation protein FIX, as well as other coagulation proteins (e.g., VWF, FVIIa (see, e.g., US 2008/0221032A1, incorporated herein by reference) and FVIII).

THERAPEUTIC PROTEINS

[0061] In certain embodiments of the invention, the aforementioned polypeptides and polynucleotides are exemplified by the following therapeutic proteins: enzymes, antigens, antibodies, receptors, blood coagulation proteins, growth factors, hormones, and ligands. In

certain embodiments, the therapeutic protein is a blood coagulation protein such as Factor IX (FIX), Factor VIII (FVIII), Factor VIIa (FVIIa), Von Willebrand Factor (VWF), Factor FV (FV), Factor X (FX), Factor XI (FXI), Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF) or ADAMTS 13 protease. In one embodiment, a therapeutic protein according to the invention is a glycoprotein or, in various embodiments, a protein that is not naturally glycosylated in vivo (i.e., a protein that does not contain a natural glycosylation site or a protein that is not glycosylated in a host cell prior to purification).

[0062] In certain embodiments, the therapeutic protein is immunoglobulins, cytokines such IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, colony stimulating factor-1 (CSF-1), M-CSF, SCF, GM-CSF, granulocyte colony stimulating factor (G-CSF), EPO, interferon-alpha (IFN-alpha), consensus interferon, IFN-beta, IFN-gamma, IFN-omega, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-31, IL-32 alpha, IL-33, thrombopoietin (TPO), angiopoietins, for example Ang-1, Ang-2, Ang-4, Ang-Y, the human angiopoietin-like polypeptides ANGPTL1 through 7, vitronectin, vascular endothelial growth factor (VEGF), angiogenin, activin A, activin B, activin C, bone morphogenic protein-1, bone morphogenic protein-2, bone morphogenic protein-3, bone morphogenic protein-4, bone morphogenic protein-5, bone morphogenic protein-6, bone morphogenic protein-7, bone morphogenic protein-8, bone morphogenic protein-9, bone morphogenic protein-10, bone morphogenic protein-11, bone morphogenic protein-12, bone morphogenic protein-13, bone morphogenic protein-14, bone morphogenic protein-15, bone morphogenic protein receptor IA, bone morphogenic protein receptor IB, bone morphogenic protein receptor II, brain derived neurotrophic factor, cardiotrophin-1, ciliary neutrophic factor, ciliary neutrophic factor receptor, cripto, cryptic, cytokine-induced neutrophil chemotactic factor 1, cytokine-induced neutrophil, chemotactic factor 2α, cytokine-induced neutrophil chemotactic factor 2β, β endothelial cell growth factor, endothelin 1, epidermal growth factor, epigen, epiregulin, epithelial-derived neutrophil attractant, fibroblast growth factor 4, fibroblast growth factor 5, fibroblast growth factor 6, fibroblast growth factor 7, fibroblast growth factor 8, fibroblast growth factor 8b, fibroblast growth factor 8c, fibroblast growth factor 9, fibroblast growth factor 10, fibroblast growth factor 11, fibroblast growth factor 12, fibroblast growth factor 13, fibroblast growth factor 16, fibroblast growth factor 17, fibroblast growth factor 19, fibroblast growth factor 20, fibroblast growth factor 21, fibroblast growth factor acidic, fibroblast growth factor basic, glial cell line-derived neutrophic factor receptor α1, glial cell line-derived neutrophic factor receptor

α2, growth related protein, growth related protein α, growth related protein β, growth related protein γ, heparin binding epidermal growth factor, hepatocyte growth factor, hepatocyte growth factor receptor, hepatoma-derived growth factor, insulin-like growth factor I, insulin-like growth factor receptor, insulin-like growth factor II, insulin-like growth factor binding protein, keratinocyte growth factor, leukemia inhibitory factor, leukemia inhibitory factor receptor α, nerve growth factor nerve growth factor receptor, neuropoietin,neurotrophin-3, neurotrophin-4, oncostatin M (OSM), placenta growth factor, placenta growth factor 2, platelet-derived endothelial cell growth factor, platelet derived growth factor, platelet derived growth factor A chain, platelet derived growth factor AA, platelet derived growth factor AB, platelet derived growth factor B chain, platelet derived growth factor BB, platelet derived growth factor receptor α, platelet derived growth factor receptor β, pre-B cell growth stimulating factor, stem cell factor (SCF), stem cell factor receptor, TNF, including TNF0, TNF1, TNF2, transforming growth factor α, transforming growth factor β, transforming growth factor β1, transforming growth factor β1.2, transforming growth factor β2, transforming growth factor β3, transforming growth factor β5, latent transforming growth factor β1, transforming growth factor β binding protein I, transforming growth factor β binding protein II, transforming growth factor β binding protein III, thymic stromal lymphopoietin (TSLP), tumor necrosis factor receptor type I, tumor necrosis factor receptor type II, urokinase-type plasminogen activator receptor, vascular endothelial growth factor, and chimeric proteins and biologically or immunologically active fragments thereof.

[0063] In certain embodiments, the therapeutic protin is alpha-, beta-, and gamma-interferons, colony stimulating factors including granulocyte colony stimulating factors, fibroblast growth factors, platelet derived growth factors, phospholipase-activating protein (PUP), insulin, plant proteins such as lectins and ricins, tumor necrosis factors and related alleles, soluble forms of tumor necrosis factor receptors, interleukin receptors and soluble forms of interleukin receptors, growth factors such as tissue growth factors, such as TGFαs or TGFβs and epidermal growth factors, hormones, somatomedins, pigmentary hormones, hypothalamic releasing factors, antidiuretic hormones, prolactin, chorionic gonadotropin, follicle-stimulating hormone, thyroid-stimulating hormone, tissue plasminogen activator, and immunoglobulins such as IgG, IgE, IgM, IgA, and IgD, a galactosidase, α-galactosidase, β-galactosidase, DNAse, fetuin, leutinizing hormone, estrogen, corticosteroids, insulin, albumin, lipoproteins, fetoprotein, transferrin, thrombopoietin, urokinase, DNase, integrins, thrombin, hematopoietic growth actors, leptin, glycosidases, Humira (adalimumab), Prolia

(denosumab), Enbrel (etanercept), and fragments thereof, or any fusion proteins comprising any of the above mentioned proteins or fragments thereof. In addition to the aforementioned proteins, the following Table 1 provides therapeutic proteins contemplated by the present invention:

Table 1

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

[0064] The therapeutic proteins provided herein should not be considered to be exclusive. Rather, as is apparent from the disclosure provided herein, the methods of the invention are applicable to any protein wherein attachment of a water soluble polymer is desired according to the invention. For example, therapeutic proteins are described in US 2007/0026485, incorporated herein by reference in its entirety.

Blood coagulation proteins

[0065] In one aspect, the starting material of the present invention is a blood coagulation protein, which can be derived from human plasma, or produced by recombinant engineering techniques, as described in patents US Patent No. 4,757,006; US Patent No. 5,733,873; US Patent No. 5,198,349; US Patent No. 5,250,421; US Patent No. 5,919,766; and EP 306 968.

[0066] Therapeutic polypeptides such as blood coagulation proteins including Factor IX (FIX), Factor VIII (FVIII), Factor VIIa (FVIIa), Von Willebrand Factor (VWF), Factor FV (FV), Factor X (FX), Factor XI (FXI), Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF) and ADAMTS 13 protease are rapidly degraded by proteolytic enzymes and neutralized by antibodies. This reduces their half-life and circulation time, thereby limiting their therapeutic effectiveness. Relatively high doses and frequent administration are necessary to reach and sustain the desired therapeutic or prophylactic effect of these coagulation proteins. As a consequence, adequate dose regulation is difficult to obtain and the need of frequent intravenous administrations imposes restrictions on the patient's way of living.

[0067] As described herein, blood coagulation proteins including, but not limited to, Factor IX (FIX), Factor VIII (FVIII), Factor VIIa (FVIIa), Von Willebrand Factor (VWF), Factor FV (FV), Factor X (FX), Factor XI, Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF) and ADAMTS 13 protease are contemplated by the invention. As used herein, the term "blood coagulation protein" refers to any Factor IX (FIX), Factor VIII (FVIII), Factor VIIa (FVIIa), Von Willebrand Factor (VWF), Factor FV (FV), Factor X (FX), Factor XII (FXII), thrombin (FII), protein C, protein S, tPA, PAI-1, tissue factor (TF) and ADAMTS 13 protease which exhibits biological activity that is associated with that particular native blood coagulation protein.

[0068] The blood coagulation cascade is divided into three distinct segments: the intrinsic, extrinsic, and common pathways (Schenone et al., Curr Opin Hematol. 2004;11:272-7). The cascade involves a series of serine protease enzymes (zymogens) and protein cofactors. When required, an inactive zymogen precursor is converted into the active form, which consequently converts the next enzyme in the cascade.

[0069] The intrinsic pathway requires the clotting factors VIII, IX, X, XI, and XII. Initiation of the intrinsic pathway occurs when prekallikrein, high-molecular-weight kininogen, factor XI (FXI) and factor XII (FXII) are exposed to a negatively charged surface. Also required are calcium ions and phospholipids secreted from platelets.

[0070] The extrinsic pathway is initiated when the vascular lumen of blood vessels is damaged. The membrane glycoprotein tissue factor is exposed and then binds to circulating factor VII (FVII) and to small preexisting amounts of its activated form FVIIa. This binding facilitates full conversion of FVII to FVIIa and subsequently, in the presence of calcium and phospholipids, the conversion of factor IX (FIX) to factor IXa (FIXa) and factor X (FX) to factor Xa (FXa). The association of FVIIa with tissue factor enhances the proteolytic activity by bringing the binding sites of FVII for the substrate (FIX and FX) into closer proximity and by inducing a conformational change, which enhances the enzymatic activity of FVIIa.

[0071] The activation of FX is the common point of the two pathways. Along with phospholipid and calcium, factors Va (FVa) and Xa convert prothrombin to thrombin (prothrombinase complex), which then cleaves fibrinogen to form fibrin monomers. The monomers polymerize to form fibrin strands. Factor XIIIa (FXIIIa) covalently bonds these strands to one another to form a rigid mesh.

[0072] Conversion of FVII to FVIIa is also catalyzed by a number of proteases, including thrombin, FIXa, FXa, factor XIa (FXIa), and factor XIIa (FXIIa). For inhibition of the early phase of the cascade, tissue factor pathway inhibitor targets FVIIa/tissue factor/FXa product complex.

Factor VIIa

[0073] FVII (also known as stable factor or proconvertin) is a vitamin K–dependent serine protease glycoprotein with a pivotal role in hemostasis and coagulation (Eigenbrot, Curr Protein Pept Sci. 2002;3:287-99).

[0074] FVII is synthesized in the liver and secreted as a single-chain glycoprotein of 48 kD. FVII shares with all vitamin K–dependent serine protease glycoproteins a similar protein domain structure consisting of an amino-terminal gamma-carboxyglutamic acid (Gla) domain with 9-12 residues responsible for the interaction of the protein with lipid membranes, a carboxy-terminal serine protease domain (catalytic domain), and two epidermal growth factor–like domains containing a calcium ion binding site that mediates interaction with tissue factor. Gamma-glutamyl carboxylase catalyzes carboxylation of Gla residues in the amino-terminal portion of the molecule. The carboxylase is dependent on a reduced form of vitamin K for its action, which is oxidized to the epoxide form. Vitamin K epoxide reductase is required to convert the epoxide form of vitamin K back to the reduced form.

[0075] The major proportion of FVII circulates in plasma in zymogen form, and activation of this form results in cleavage of the peptide bond between arginine 152 and isoleucine 153. The resulting activated FVIIa consists of a NH2-derived light chain (20 kD) and a COOH terminal–derived heavy chain (30 kD) linked via a single disulfide bond (Cys 135 to Cys 262). The light chain contains the membrane-binding Gla domain, while the heavy chain contains the catalytic domain.

[0076] The plasma concentration of FVII determined by genetic and environmental factors is about 0.5 mg/mL (Pinotti et al., Blood. 2000;95:3423-8). Different FVII genotypes can result in several-fold differences in mean FVII levels. Plasma FVII levels are elevated during pregnancy in healthy females and also increase with age and are higher in females and in persons with hypertriglyceridemia. FVII has the shortest half-life of all procoagulant factors (3-6 h). The mean plasma concentration of FVIIa is 3.6 ng/mL in healthy individuals and the circulating half-life of FVIIa is relatively long (2.5 h) compared with other coagulation factors.

[0077] Hereditary FVII deficiency is a rare autosomal recessive bleeding disorder with a prevalence estimated to be 1 case per 500,000 persons in the general population (Acharya et al., J Thromb Haemost. 2004;2248-56). Acquired FVII deficiency from inhibitors is also very rare. Cases have also been reported with the deficiency occurring in association with drugs such as cephalosporins, penicillins, and oral anticoagulants. Furthermore, acquired FVII deficiency has been reported to occur spontaneously or with other conditions, such as myeloma, sepsis, aplastic anemia, with interleukin-2 and antithymocyte globulin therapy.

[0078] Reference polynucleotide and polypeptide sequences include, e.g., GenBank Accession Nos. J02933 for the genomic sequence, M13232 for the cDNA (Hagen et al. PNAS 1986; 83: 2412-6), and P08709 for the polypeptide sequence (references incorporated herein in their entireties). A variety of polymorphisms of FVII have been described, for example see Sabater-Lleal et al. (Hum Genet. 2006; 118:741-51) (reference incorporated herein in its entirety).

Factor IX

[0079] FIX is a vitamin K-dependent plasma protein that participates in the intrinsic pathway of blood coagulation by converting FX to its active form in the presence of calcium ions, phospholipids and FVIIIa. The predominant catalytic capability of FIX is as a serine protease with specificity for a particular arginine-isoleucine bond within FX. Activation of FIX occurs by FXIa which causes excision of the activation peptide from FIX to produce an activated FIX molecule comprising two chains held by one or more disulphide bonds. Defects in FIX are the cause of recessive X-linked hemophilia B.

[0080] Hemophilia A and B are inherited diseases characterized by deficiencies in FVIII and FIX polypeptides, respectively. The underlying cause of the deficiencies is frequently the result of mutations in FVIII and FIX genes, both of which are located on the X chromosome. Traditional therapy for hemophilias often involves intravenous administration of pooled plasma or semi-purified coagulation proteins from normal individuals. These preparations can be contaminated by pathogenic agents or viruses, such as infectious prions, HIV, parvovirus, hepatitis A, and hepatitis C. Hence, there is an urgent need for therapeutic agents that do not require the use of human serum.

[0081] The level of the decrease in FIX activity is directly proportional to the severity of hemophilia B. The current treatment of hemophilia B consists of the replacement of the missing protein by plasma-derived or recombinant FIX (so-called FIX substitution or replacement treatment or therapy).

[0082] Polynucleotide and polypeptide sequences of FIX can be found for example in the UniProtKB/Swiss-Prot Accession No. P00740, US Pat. No. 6,531,298 and in Figure 1 (SEQ ID NO: 1).

Factor VIII

[0083] Coagulation factor VIII (FVIII) circulates in plasma at a very low concentration and is bound non-covalently to Von Willebrand factor (VWF). During hemostasis, FVIII is separated from VWF and acts as a cofactor for activated factor IX (FIXa)-mediated FX activation by enhancing the rate of activation in the presence of calcium and phospholipids or cellular membranes.

[0084] FVIII is synthesized as a single-chain precursor of approximately 270-330 kD with the domain structure A1-A2-B-A3-C1-C2. When purified from plasma (e.g., "plasma-derived" or "plasmatic"), FVIII is composed of a heavy chain (A1-A2-B) and a light chain (A3-C1-C2). The molecular mass of the light chain is 80 kD whereas, due to proteolysis within the B domain, the heavy chain is in the range of 90-220 kD.

[0085] FVIII is also synthesized as a recombinant protein for therapeutic use in bleeding disorders. Various in vitro assays have been devised to determine the potential efficacy of recombinant FVIII (rFVIII) as a therapeutic medicine. These assays mimic the in vivo effects of endogenous FVIII. In vitro thrombin treatment of FVIII results in a rapid increase and subsequent decrease in its procoagulant activity, as measured by in vitro assays. This activation and inactivation coincides with specific limited proteolysis both in the heavy and the light chains, which alter the availability of different binding epitopes in FVIII, e.g. allowing FVIII to dissociate from VWF and bind to a phospholipid surface or altering the binding ability to certain monoclonal antibodies.

[0086] The lack or dysfunction of FVIII is associated with the most frequent bleeding disorder, hemophilia A. The treatment of choice for the management of hemophilia A is replacement therapy with plasma derived or rFVIII concentrates. Patients with severe hemophilia A with FVIII levels below 1 %, are generally on prophylactic therapy with the aim of keeping FVIII above 1% between doses. Taking into account the average half-lives of the various FVIII products in the circulation, this result can usually be achieved by giving FVIII two to three times a week.

[0087] Reference polynucleotide and polypeptide sequences include, e.g., UniProtKB/Swiss-Prot P00451 (FA8_HUMAN); Gitschier J et al., Characterization of the human Factor VIII gene, Nature, 312(5992): 326-30 (1984); Vehar GH et al., Structure of human Factor VIII, Nature, 312(5992):337-42 (1984); Thompson AR. Structure and Function of the Factor VIII gene and protein, Semin Thromb Hemost, 2003:29;11-29 (2002).

Von Willebrand Factor

[0088] Von Willebrand factor (VWF) is a glycoprotein circulating in plasma as a series of multimers ranging in size from about 500 to 20,000 kD. Multimeric forms of VWF are composed of 250 kD polypeptide subunits linked together by disulfide bonds. VWF mediates initial platelet adhesion to the sub-endothelium of the damaged vessel wall. Only the larger multimers exhibit hemostatic activity. It is assumed that endothelial cells secrete large polymeric forms of VWF and those forms of VWF which have a low molecular weight (low molecular weight VWF) arise from proteolytic cleavage. The multimers having large molecular masses are stored in the Weibel-Pallade bodies of endothelial cells and liberated upon stimulation.

[0089] VWF is synthesized by endothelial cells and megakaryocytes as prepro-VWF that consists to a large extent of repeated domains. Upon cleavage of the signal peptide, pro-VWF dimerizes through disulfide linkages at its C-terminal region. The dimers serve as protomers for multimerization, which is governed by disulfide linkages between the free end termini. The assembly to multimers is followed by the proteolytic removal of the propeptide sequence (Leyte et al., Biochem. J. 274 (1991), 257-261).

[0090] The primary translation product predicted from the cloned cDNA of VWF is a 2813-residue precursor polypeptide (prepro-VWF). The prepro-VWF consists of a 22 amino acid signal peptide and a 741 amino acid propeptide, with the mature VWF comprising 2050 amino acids (Ruggeri Z.A., and Ware, J., FASEB J., 308-316 (1993).

[0091] Defects in VWF are causal to Von Willebrand disease (VWD), which is characterized by a more or less pronounced bleeding phenotype. VWD type 3 is the most severe form, in which VWF is completely missing, and VWD type 1 relates to a quantitative loss of VWF and its phenotype can be very mild. VWD type 2 relates to qualitative defects of VWF and can be as severe as VWD type 3. VWD type 2 has many sub forms, some being associated with the loss or the decrease of high molecular weight multimers. Von Willebrand disease type 2a (VWD-2A) is characterized by a loss of both intermediate and large multimers. VWD-2B is characterized by a loss of highest-molecular-weight multimers. Other diseases and disorders related to VWF are known in the art.

[0092] The polynucleotide and amino acid sequences of prepro-VWF are available at GenBank Accession Nos. NM_000552 and NP_000543, respectively.

[0093] Other blood coagulation proteins according to the present invention are described in the art, e.g. Mann KG, Thromb Haemost, 1999;82:165-74.

A. Polypeptides

[0094] In one aspect, the starting material of the present invention is a protein or polypeptide. As described herein, the term therapeutic protein refers to any therapeutic protein molecule which exhibits biological activity that is associated with the therapeutic protein. In one embodiment of the invention, the therapeutic protein molecule is a full-length protein.

[0095] Therapeutic protein molecules contemplated include full-length proteins, precursors of full length proteins, biologically active subunits or fragments of full length proteins, as well as biologically active derivatives and variants of any of these forms of therapeutic proteins. Thus, therapeutic protein include those that (1) have an amino acid sequence that has greater than about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% or greater amino acid sequence identity, over a region of at least about 25, about 50, about 100, about 200, about 300, about 400, or more amino acids, to a polypeptide encoded by a referenced nucleic acid or an amino acid sequence described herein; and/or (2) specifically bind to antibodies, e.g., polyclonal or monoclonal antibodies, generated against an immunogen comprising a referenced amino acid sequence as described herein, an immunogenic fragment thereof, and/or a conservatively modified variant thereof.

[0096] According to the present invention, the term "recombinant therapeutic protein" includes any therapeutic protein obtained via recombinant DNA technology. In certain embodiments, the term encompasses proteins as described herein.

[0097] As used herein, "endogenous therapeutic protein" includes a therapeutic protein which originates from the mammal intended to receive treatment. The term also includes therapeutic protein transcribed from a transgene or any other foreign DNA present in said mammal. As used herein, "exogenous therapeutic protein " includes a blood coagulation protein which does not originate from the mammal intended to receive treatment.

[0098] As used herein, "plasma-derived blood coagulation protein " or "plasmatic" includes all forms of the protein found in blood obtained from a mammal having the property participating in the coagulation pathway.

[0099] As used herein "biologically active derivative" or "biologically active variant" includes any derivative or variant of a molecule having substantially the same functional and/or biological properties of said molecule, such as binding properties, and/or the same structural basis, such as a peptidic backbone or a basic polymeric unit.

[00100] An "analog," such as a "variant" or a "derivative," is a compound substantially similar in structure and having the same biological activity, albeit in certain instances to a differing degree, to a naturally-occurring molecule. For example, a polypeptide variant refers to a polypeptide sharing substantially similar structure and having the same biological activity as a reference polypeptide. Variants or analogs differ in the composition of their amino acid sequences compared to the naturally-occurring polypeptide from which the analog is derived, based on one or more mutations involving (i) deletion of one or more amino acid residues at one or more termini of the polypeptide and/or one or more internal regions of the naturally-occurring polypeptide sequence (e.g., fragments), (ii) insertion or addition of one or more amino acids at one or more termini (typically an "addition" or "fusion") of the polypeptide and/or one or more internal regions (typically an "insertion") of the naturally-occurring polypeptide sequence or (iii) substitution of one or more amino acids for other amino acids in the naturally-occurring polypeptide sequence. By way of example, a "derivative" is a type of analog and refers to a polypeptide sharing the same or substantially similar structure as a reference polypeptide that has been modified, e.g., chemically.

[00101] A variant polypeptide is a type of analog polypeptide and includes insertion variants, wherein one or more amino acid residues are added to a therapeutic protein amino acid sequence of the invention. Insertions may be located at either or both termini of the protein, and/or may be positioned within internal regions of the therapeutic protein amino acid sequence. Insertion variants, with additional residues at either or both termini, include for example, fusion proteins and proteins including amino acid tags or other amino acid labels. In one aspect, the blood coagulation protein molecule optionally contains an N-terminal Met, especially when the molecule is expressed recombinantly in a bacterial cell such as E. coli.

[00102] In deletion variants, one or more amino acid residues in a therapeutic protein polypeptide as described herein are removed. Deletions can be effected at one or both termini of the therapeutic protein polypeptide, and/or with removal of one or more residues within the therapeutic protein amino acid sequence. Deletion variants, therefore, include fragments of a therapeutic protein polypeptide sequence.

[00103] In substitution variants, one or more amino acid residues of a therapeutic protein polypeptide are removed and replaced with alternative residues. In one aspect, the substitutions are conservative in nature and conservative substitutions of this type are well known in the art. Alternatively, the invention embraces substitutions that are also non-conservative. Exemplary conservative substitutions are described in Lehninger, [Biochemistry, 2nd Edition; Worth Publishers, Inc., New York (1975), pp.71-77] and are set out immediately below.

CONSERVATIVE SUBSTITUTIONS

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

[00104] Alternatively, exemplary conservative substitutions are set out immediately below.

CONSERVATIVE SUBSTITUTIONS II

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

B. Polynucleotides

[00105] Nucleic acids encoding a therapeutic protein of the invention include, for example and without limitation, genes, pre-mRNAs, mRNAs, cDNAs, polymorphic variants, alleles, synthetic and naturally-occurring mutants.

[00106] Polynucleotides encoding a therapeutic protein of the invention also include, without limitation, those that (1) specifically hybridize under stringent hybridization conditions to a nucleic acid encoding a referenced amino acid sequence as described herein, and conservatively modified variants thereof; (2) have a nucleic acid sequence that has greater than about 95%, about 96%, about 97%, about 98%, about 99%, or higher nucleotide sequence identity, over a region of at least about 25, about 50, about 100, about 150, about 200, about 250, about 500, about 1000, or more nucleotides (up to the full length sequence of

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

[00107] A "naturally-occurring" polynucleotide or polypeptide sequence is typically derived from a mammal including, but not limited to, primate, e.g., human; rodent, e.g., rat, mouse, hamster; cow, pig, horse, sheep, or any mammal. The nucleic acids and proteins of the invention can be recombinant molecules (e.g., heterologous and encoding the wild type sequence or a variant thereof, or non-naturally occurring).

C. Production of therapeutic proteins

[00108] Production of a therapeutic protein includes any method known in the art for (i) the production of recombinant DNA by genetic engineering, (ii) introducing recombinant DNA into prokaryotic or eukaryotic cells by, for example and without limitation, transfection, electroporation or microinjection, (iii) cultivating said transformed cells, (iv) expressing therapeutic protein, e.g. constitutively or upon induction, and (v) isolating said blood coagulation protein, e.g. from the culture medium or by harvesting the transformed cells, in order to obtain purified therapeutic protein.

[00109] In other aspects, the therapeutic protein is produced by expression in a suitable prokaryotic or eukaryotic host system characterized by producing a pharmacologically acceptable blood coagulation protein molecule. Examples of eukaryotic cells are mammalian cells, such as CHO, COS, HEK 293, BHK, SK-Hep, and HepG2.

[00110] A wide variety of vectors are used for the preparation of the therapeutic protein and are selected from eukaryotic and prokaryotic expression vectors. Examples of vectors for prokaryotic expression include plasmids such as, and without limitation, pRSET, pET, and pBAD, wherein the promoters used in prokaryotic expression vectors include one or more of, and without limitation, lac, trc, trp, recA, or araBAD. Examples of vectors for eukaryotic expression include: (i) for expression in yeast, vectors such as, and without limitation, pAO, pPIC, pYES, or pMET, using promoters such as, and without limitation, AOX1, GAP,

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

D. Administration

[00111] In one embodiment a conjugated therapeutic protein of the present invention may be administered by injection, such as intravenous, intramuscular, or intraperitoneal injection.

[00112] To administer compositions comprising a conjugated therapeutic protein of the present invention to human or test animals, in one aspect, the compositions comprise one or more pharmaceutically acceptable carriers. The terms "pharmaceutically" or "pharmacologically acceptable" refer to molecular entities and compositions that are stable, inhibit protein degradation such as aggregation and cleavage products, and in addition do not produce allergic, or other adverse reactions when administered using routes well-known in the art, as described below. "Pharmaceutically acceptable carriers" include any and all clinically useful solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like, including those agents disclosed above.

[00113] As used herein, "effective amount" includes a dose suitable for treating a disease or disorder or ameliorating a symptom of a disease or disorder. In one embodiment, "effective amount" includes a dose suitable for treating a mammal having a bleeding disorder as described herein.

[00114] The compositions may be administered orally, topically, transdermally, parenterally, by inhalation spray, vaginally, rectally, or by intracranial injection. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intracisternal injection, or infusion techniques. Administration by intravenous, intradermal, intramuscular, intramammary, intraperitoneal, intrathecal, retrobulbar, intrapulmonary injection and or surgical implantation at a particular site is contemplated as well. Generally, compositions are essentially free of pyrogens, as well as other impurities that could be harmful to the recipient.

[00115] Single or multiple administrations of the compositions can be carried out with the dose levels and pattern being selected by the treating physician. For the prevention or treatment of disease, the appropriate dosage will depend on the type of disease to be treated, as described above, the severity and course of the disease, whether drug is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the drug, and the discretion of the attending physician.

[00116] The present invention also relates to a pharmaceutical composition comprising an effective amount of a conjugated therapeutic protein as defined herein. The pharmaceutical composition may further comprise a pharmaceutically acceptable carrier, diluent, salt, buffer, or excipient. The pharmaceutical composition can be used for treating the above-defined bleeding disorders. The pharmaceutical composition of the invention may be a solution or a lyophilized product. Solutions of the pharmaceutical composition may be subjected to any suitable lyophilization process.

[00117] As an additional aspect, the invention includes kits which comprise a composition of the invention packaged in a manner which facilitates its use for administration to subjects. In one embodiment, such a kit includes a compound or composition described herein (e.g., a composition comprising a conjugated therapeutic protein), packaged in a container such as a sealed bottle or vessel, with a label affixed to the container or included in the package that describes use of the compound or composition in practicing the method. In one embodiment, the kit contains a first container having a composition comprising a conjugated therapeutic protein and a second container having a physiologically acceptable reconstitution solution for the composition in the first container. In one aspect, the compound or composition is packaged in a unit dosage form. The kit may further include a device suitable for administering the composition according to a specific route of administration. Preferably, the kit contains a label that describes use of the therapeutic protein or peptide composition.

WATER SOLUBLE POLYMERS

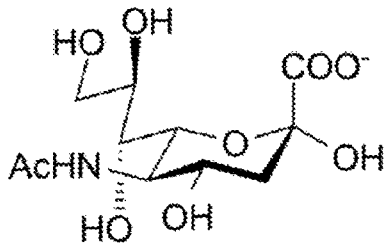
[00118] In one aspect, a therapeutic protein derivative (i.e., a conjugated therapeutic protein) molecule provided is bound to a water-soluble polymer including, but not limited to, polyethylene glycol (PEG), branched PEG, polysialic acid (PSA), hydroxyalkyl starch (HAS), hydroxylethyl starch (HES), carbohydrate, polysaccharides, pullulane, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, starch, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG)

polyoxazoline, poly acryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate, polyvinylpyrrolidone, polyphosphazene, polyoxazoline, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, poly(1-hydroxymethylethylene hydroxymethylformal) (PHF), 2-methacryloyloxy-2'-ethyltrimethylammoniumphosphate (MPC). In one embodiment of the invention, the water soluble polymer is consisting of sialic acid molecule having a molecular weight range of 350 to 120,000, 500 to 100,000,1000 to 80,000, 1500 to 60,000, 2,000 to 45,000 Da, 3,000 to 35,000 Da, and 5,000 to 25,000 Da. The coupling of the water soluble polymer can be carried out by direct coupling to the protein or via linker molecules. One example of a chemical linker is MBPH (4-[4-N-Maleimidophenyl]butyric acid hydrazide) containing a carbohydrate-selective hydrazide and a sulfhydryl-reactive maleimide group (Chamow et al., J Biol Chem 1992;267:15916-22). Other exemplary and preferred linkers are described below.

[00119] In one embodiment, the derivative retains the full functional activity of native therapeutic protein products, and provides an extended half-life in vivo, as compared to native therapeutic protein products. In another embodiment, the derivative retains at least 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44. 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56,57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, or 150 percent (%) biological activity relative to native blood coagulation protein. In a related aspect, the biological activities of the derivative and native blood coagulation protein are determined by the ratios of chromogenic activity to blood coagulation factor antigen value (blood coagulation factor:Chr: blood coagulation factor:Ag). In still another embodiment of the invention, the half-life of the construct is decreased or increased 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, or 10-fold relative to the in vivo half-life of native therapeutic protein.

A. Sialic acid and PSA

[00120] PSAs consist of polymers (generally homopolymers) of N-acetylneuraminic acid. The secondary amino group normally bears an acetyl group, but it may instead bear a glycolyl group. Possible substituents on the hydroxyl groups include acetyl, lactyl, ethyl, sulfate, and phosphate groups.



N-Acetylneuraminic acid
Neu5Ac

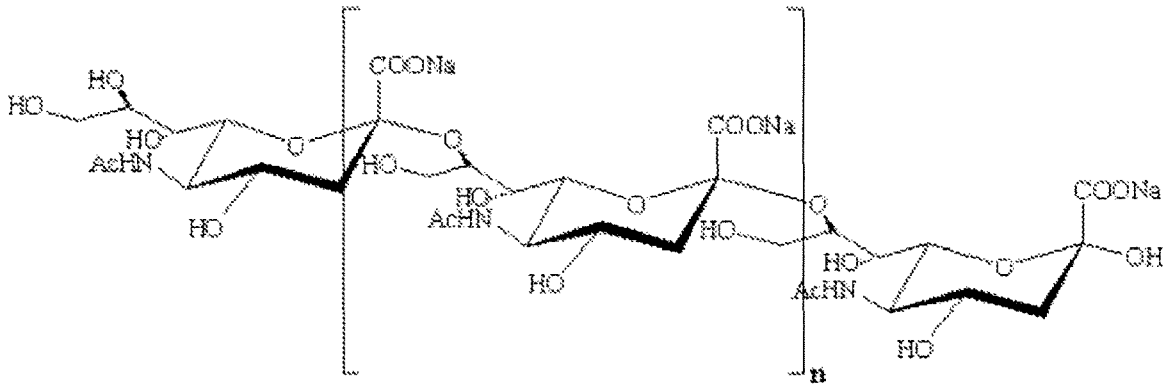
[00121] Structure of sialic acid (N-acetylneuraminic acid)

[00122] PSAs and mPSAs generally comprise linear polymers consisting essentially of N-acetylneuraminic acid moieties linked by 2,8- or 2,9- glycosidic linkages or combinations of these (e.g. alternating 2,8- and 2,9- linkages). In particularly preferred PSAs and mPSAs, the glycosidic linkages are α-2,8. Such PSAs and mPSAs are conveniently derived from colominic acids, and are referred to herein as "CAs" and "mCAs". Typical PSAs and mPSAs comprise at least 2, preferably at least 5, more preferably at least 10 and most preferably at least 20 N-acetylneuraminic acid moieties. Thus, they may comprise from 2 to 300 N-acetylneuraminic acid moieties, preferably from 5 to 200 N-acetylneuraminic acid moieties, or most preferably from 10 to 100 N-acetylneuraminic acid moieties. PSAs and CAs preferably are essentially free of sugar moieties other than N-acetylneuraminic acid. Thus PSAs and CAs preferably comprise at least 90 %, more preferably at least 95 % and most preferably at least 98 % N-acetylneuraminic acid moieties.

[00123] Where PSAs and CAs comprise moieties other than N-acetylneuraminic acid (as, for example in mPSAS and mCAs) these are preferably located at one or both of the ends of the polymer chain. Such "other" moieties may, for example, be moieties derived from terminal N-acetylneuraminic acid moieties by oxidation or reduction.

[00124] For example, WO-A-0187922 describes such mPSAs and mCAs in which the non-reducing terminal N-acetylneuraminic acid unit is converted to an aldehyde group by reaction with sodium periodate. Additionally, WO 2005/016974 describes such mPSAs and mCAs in which the reducing terminal N-acetylneuraminic acid unit is subjected to reduction to reductively open the ring at the reducing terminal N-acetylneuraminic acid unit, whereby a vicinal diol group is formed, followed by oxidation to convert the vicinal diol group to an aldehyde group.

[00125] Sialic acid rich glycoproteins bind selectin in humans and other organisms. They play an important role in human influenza infections. E.g., sialic acid can hide mannose antigens on the surface of host cells or bacteria from mannose-binding lectin. This prevents activation of complement. Sialic acids also hide the penultimate galactose residue thus preventing rapid clearance of the glycoprotein by the galactose receptor on the hepatic parenchymal cells.



[00126] Structure of colominic acid (homopolymer of N-acetylneuraminic acid)

[00127] Colominic acids (a sub-class of PSAs) are homopolymers of N-acetylneuraminic acid (NANA) with α (2→8) ketosidic linkage, and are produced, inter alia, by particular strains of Escherichia coli possessing K1 antigen. Colominic acids have many physiological functions. They are important as a raw material for drugs and cosmetics.

[00128] Comparative studies in vivo with polysialylated and unmodified asparaginase revealed that polysialylation increased the half-life of the enzyme (Fernandes and Gregoriadis, Biochimica Biophysica Acta 1341: 26-34, 1997).

[00129] As used herein, "sialic acid moieties" includes sialic acid monomers or polymers ("polysaccharides") which are soluble in an aqueous solution or suspension and have little or no negative impact, such as side effects, to mammals upon administration of the PSA-blood coagulation protein conjugate in a pharmaceutically effective amount. The polymers are characterized, in one aspect, as having 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, or 500 sialic acid units. In certain aspects, different sialic acid units are combined in a chain.

[00130] In one embodiment of the invention, the sialic acid portion of the polysaccharide compound is highly hydrophilic, and in another embodiment the entire compound is highly hydrophilic. Hydrophilicity is conferred primarily by the pendant carboxyl groups of the sialic acid units, as well as the hydroxyl groups. The saccharide unit may contain other functional groups, such as, amine, hydroxyl or sulphate groups, or combinations thereof. These groups may be present on naturally-occurring saccharide compounds, or introduced into derivative polysaccharide compounds.

[00131] The naturally occurring polymer PSA is available as a polydisperse preparation showing a broad size distribution (e.g. Sigma C-5762) and high polydispersity (PD). Because the polysaccharides are usually produced in bacteria carrying the inherent risk of copurifying endotoxins, the purification of long sialic acid polymer chains may raise the probability of increased endotoxin content. Short PSA molecules with 1-4 sialic acid units can also be synthetically prepared (Kang SH et al., Chem Commun. 2000;227-8; Ress DK and Linhardt RJ, Current Organic Synthesis. 2004;1:31-46), thus minimizing the risk of high endotoxin levels. However PSA preparations with a narrow size distribution and low polydispersity, which are also endotoxin-free, can now be manufactured. Polysaccharide compounds of particular use for the invention are, in one aspect, those produced by bacteria. Some of these naturally-occurring polysaccharides are known as glycolipids. In one embodiment, the polysaccharide compounds are substantially free of terminal galactose units.

B. Polyethylene glycol (PEG) and Pegylation

[00132] In certain aspects, therapeutic proteins are conjugated to a water soluble polymer by any of a variety of chemical methods (Roberts JM et al., Advan Drug Delivery Rev 2002;54:459-76). For example, in one embodiment a therapeutic protein is modified by the conjugation of PEG to free amino groups of the protein using N-hydroxysuccinimide (NHS) esters. In another embodiment the water soluble polymer, for example PEG, is coupled to free SH groups using maleimide chemistry or the coupling of PEG hydrazides or PEG amines to carbohydrate moieties of the therapeutic protein after prior oxidation.

[00133] The conjugation is in one aspect performed by direct coupling (or coupling via linker systems) of the water soluble polymer to a therapeutic protein under formation of stable bonds. In addition degradable, releasable or hydrolysable linker systems are used in certain aspects the present invention (Tsubery et al. J Biol Chem 2004;279:38118-24 /

Greenwald et al., J Med Chem 1999;42:3657-67 / Zhao et al., Bioconj Chem 2006;17:341-51 / WO2006/138572A2 / US7259224B2 / US7060259B2).

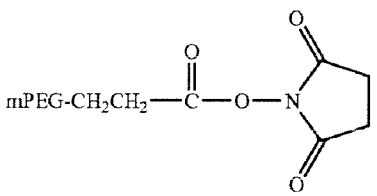
[00134] In one embodiment of the invention, a therapeutic protein is modified via lysine residues by use of polyethylene glycol derivatives containing an active N-hydroxysuccinimide ester (NHS) such as succinimidyl succinate, succinimidyl glutarate or succinimidyl propionate. These derivatives react with the lysine residues of the therapeutic protein under mild conditions by forming a stable amide bond. In one embodiment of the invention, the chain length of the PEG derivative is 5,000 Da. Other PEG derivatives with chain lengths of 500 to 2,000 Da, 2,000 to 5,000 Da, greater than 5,000 up to 10,000 Da or greater than 10,000 up to 20,000 Da, or greater than 20,000 up to 150,000 Da are used in various embodiments, including linear and branched structures.

[00135] Alternative methods for the PEGylation of amino groups are, without limitation, the chemical conjugation with PEG carbonates by forming urethane bonds, or the reaction with aldehydes or ketones by reductive amination forming secondary amide bonds.

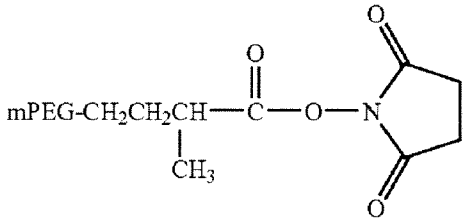
[00136] In one embodiment of the present invention a therapeutic protein molecule is chemically modified using PEG derivatives that are commercially available. These PEG derivatives in alternative aspects have linear or branched structures. Examples of PEG-derivatives containing NHS groups are listed below.

[00137] The following PEG derivatives are non-limiting examples of those commercially available from Nektar Therapeutics (Huntsville, Ala.; see www.nektar.com/PEG reagent catalog; Nektar Advanced PEGylation, price list 2005-2006):

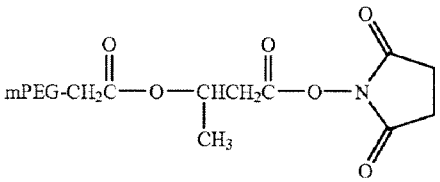
mPEG-Succinimidyl propionate (mPEG-SPA)



mPEG-Succinimidyl α-methylbutanoate (mPEG-SMB)

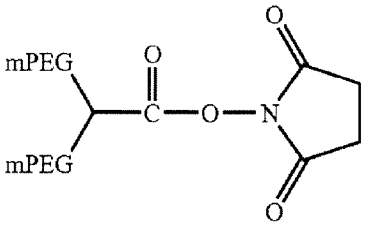


mPEG-CM-HBA-NHS (CM=carboxymethyl; HBA=Hydroxy butyric acid)



Structure of a Branched PEG-derivative (Nektar Therapeutics):

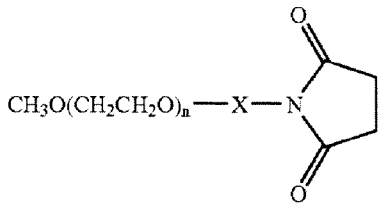
Branched PEG N-Hydroxysuccinimide (mPEG2-NHS)



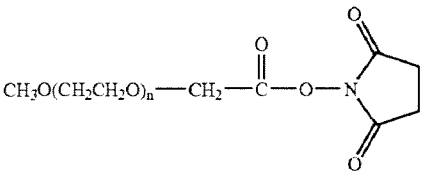
[00138] This reagent with branched structure is described in more detail by Kozlowski et al. (BioDrugs 2001;5:419-29).

[00139] Other non-limiting examples of PEG derivatives are commercially available from NOF Corporation (Tokyo, Japan; see www.nof.co.jp/english: Catalogue 2005)

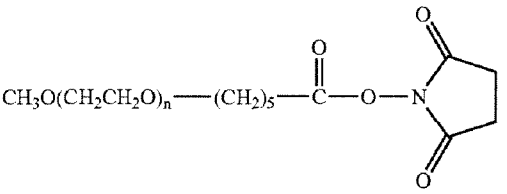
General Structure of Linear PEG-derivatives (NOF Corp.):



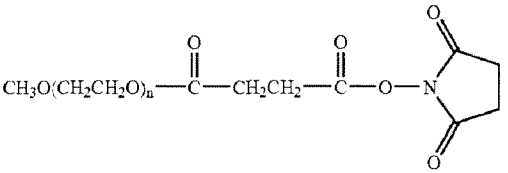
X=carboxymethyl



X=carboxypentyl

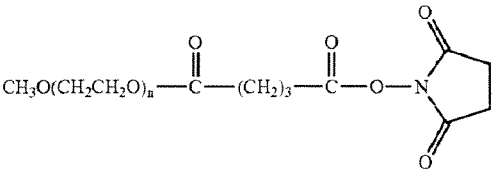


x=succinate



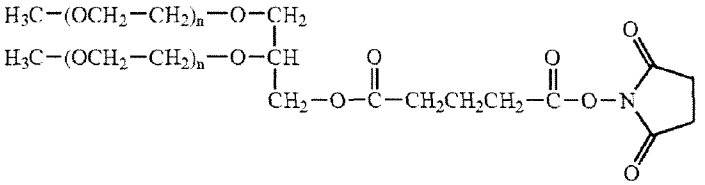
mPEG Succinimidyl succinate

x=glutarate

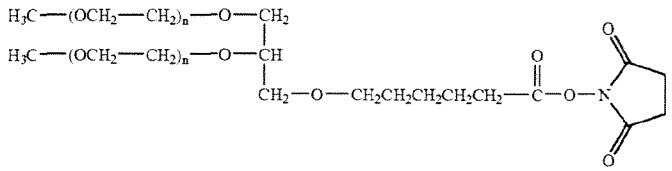


mPEG Succinimidyl glutarate

Structures of Branched PEG-derivatives (NOF Corp.): 2,3-Bis(methylpolyoxyethylene-oxy)-1-(1,5-dioxo-5-succinimidyloxy, pentyloxy)propane



2,3-Bis(methylpolyoxyethylene-oxy)-1-(succinimidyl carboxypentyloxy)propane



[00140] These propane derivatives show a glycerol backbone with a 1,2 substitution pattern. In the present invention branched PEG derivatives based on glycerol structures with 1,3 substitution or other branched structures described in US2003/0143596A1 are also contemplated.

[00141] PEG derivatives with degradable (for example, hydrolysable) linkers as described by Tsubery et al. (J Biol Chem 2004;279:38118-24) and Shechter et al. (WO04089280A3) are also contemplated.

[00142] Surprisingly, the PEGylated therapeutic protein of this invention exhibits functional activity, combined with an extended half-life in vivo. In addition the PEGylated rFVIII, FVIIa, FIX, or other blood coagulation factor seems to be more resistant against thrombin inactivation.

C. Hydroxyalkyl starch (HAS) and hydroxylethyl starch (HES)

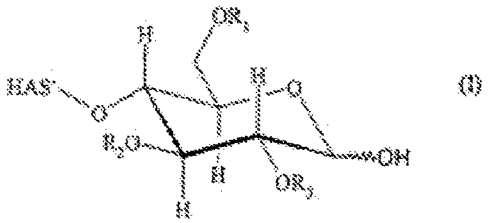
[00143] In various embodiments of the present invention, a therapeutic protein molecule is chemically modified using hydroxyalkyl starch (HAS) or hydroxylethyl starch (HES) or derivatives thereof.

[00144] HES is a derivative of naturally occurring amylopectin and is degraded by alpha-amylase in the body. HES is a substituted derivative of the carbo- hydrate polymer amylopectin, which is present in corn starch at a concentration of up to 95 % by weight. HES exhibits advantageous biological properties and is used as a blood volume replacement agent and in hemodilution therapy in the clinics (Sommermeyer et al., 1987, Krankenhauspharmazie, 8 (8), 271-278; and Weidler et al., 1991, Arzneim.-Forschung/Drug Res. g 419 494-498).

[00145] Amylopectin consists of glucose moieties, wherein in the main chain alpha-1,4-glycosidic bonds are present and at the branching sites alpha-1, 6-glycosidic bonds are found. The physical-chemical properties of this molecule are mainly determined by the type of

glycosidic bonds. Due to the nicked alpha-1,4-glycosidic bond, helical structures with about six glucose-monomers per turn are produced. The physico- chemical as well as the biochemical properties of the polymer can be modified via substitution. The introduction of a hydroxyethyl group can be achieved via alkaline hydroxyethylation. By adapting the reaction conditions it is possible to exploit the different reactivity of the respective hydroxy group in the unsubstituted glucose monomer with respect to a hydroxyethylation. Owing to this fact, the skilled person is able to influence the substitution pattern to a limited extent.

[00146] HAS refers to a starch derivative which has been substituted by at least one hydroxyalkyl group. Therefore, the term hydroxyalkyl starch is not limited to compounds where the terminal carbohydrate moiety comprises hydroxyalkyl groups R1, R2, and/or R3, but also refers to compounds in which at least one hydroxy group present anywhere, either in the terminal carbohydrate moiety and/or in the remaining part of the starch molecule, HAS', is substituted by a hydroxyalkyl group Rl, R2, or R3.



(I)

[00147] The alkyl group may be a linear or branched alkyl group which may be suitably substituted. Preferably, the hydroxyalkyl group contains 1 to 10 carbon atoms, more preferably from 1 to 6 carbon atoms, more preferably from 1 to 4 carbon atoms, and even more preferably 2-4 carbon atoms. "Hydroxyalkyl starch" therefore preferably comprises hydroxyethyl starch, hydroxypropyl starch and hydroxybutyl starch, wherein hydroxyethyl starch and hydroxypropyl starch are particularly preferred.

[00148] Hydroxyalkyl starch comprising two or more different hydroxyalkyl groups is also comprised in the present invention. The at least one hydroxyalkyl group comprised in HAS may contain two or more hydroxy groups. According to one embodiment, the at least one hydroxyalkyl group comprised HAS contains one hydroxy group.

[00149] The term HAS also includes derivatives wherein the alkyl group is mono-or polysubstituted. In one embodiment, the alkyl group is substituted with a halogen, especially fluorine, or with an aryl group, provided that the HAS remains soluble in water. Furthermore, the terminal hydroxy group a of hydroxyalkyl group may be esterified or etherified. HAS derivatives are described in WO/2004/024776, which is incorporated by reference in its entirety.

D. Methods of attachment

[00150] A therapeutic protein may be covalently linked to the polysaccharide compounds by any of various techniques known to those of skill in the art. In various aspects of the invention, sialic acid moieties are bound to a therapeutic protein, e.g., FIX, FVIII, FVIIa or VWF, for example by the method described in US Patent No. 4,356,170, which is herein incorporated by reference.

[00151] Other techniques for coupling PSA to polypeptides are also known and contemplated by the invention. For example, US Publication No. 2007/0282096 describes conjugating an amine or hydrazide derivative of, e.g., PSA, to proteins. In addition, US Publication No. 2007/0191597 describes PSA derivatives containing an aldehyde group for reaction with substrates (e.g., proteins) at the reducing end. These references are incorporated by reference in their entireties.

[00152] Various methods are disclosed at column 7, line 15, through column 8, line 5 of U.S. Patent No. 5,846,951 (incorporated by reference in its entirety). Exemplary techniques include linkage through a peptide bond between a carboxyl group on one of either the blood coagulation protein or polysaccharide and an amine group of the blood coagulation protein or polysaccharide, or an ester linkage between a carboxyl group of the blood coagulation protein or polysaccharide and a hydroxyl group of the therapeutic protein or polysaccharide. Another linkage by which the therapeutic protein is covalently bonded to the polysaccharide compound is via a Schiff base, between a free amino group on the blood coagulation protein being reacted with an aldehyde group formed at the non-reducing end of the polysaccharide by periodate oxidation (Jennings HJ and Lugowski C, J Immunol. 1981;127:1011-8; Fernandes AI and Gregoriadis G, Biochim Biophys Acta. 1997;1341;26-34). The generated Schiff base is in one aspect stabilized by specific reduction with $NaCNBH_3$ to form a secondary amine. An alternative approach is the generation of terminal free amino groups in the PSA by reductive amination with NH_4Cl after prior oxidation. Bifunctional reagents can

be used for linking two amino or two hydroxyl groups. For example, PSA containing an amino group is coupled to amino groups of the protein with reagents like BS3 (Bis(sulfosuccinimidyl)suberate / Pierce, Rockford, IL). In addition heterobifunctional cross linking reagents like Sulfo-EMCS (N-ε-Maleimidocaproyloxy) sulfosuccinimide ester / Pierce) is used for instance to link amine and thiol groups.

[00153] In another approach, a PSA hydrazide is prepared and coupled to the carbohydrate moiety of the protein after prior oxidation and generation of aldehyde functions.

[00154] As described above, a free amine group of the therapeutic protein reacts with the 1-carboxyl group of the sialic acid residue to form a peptidyl bond or an ester linkage is formed between the 1-carboxylic acid group and a hydroxyl or other suitable active group on a blood coagulation protein. Alternatively, a carboxyl group forms a peptide linkage with deacetylated 5-amino group, or an aldehyde group of a molecule of a therapeutic protein forms a Schiff base with the N-deacetylated 5-amino group of a sialic acid residue.

[00155] Alternatively, the polysaccharide compound is associated in a non-covalent manner with a therapeutic protein. For example, the polysaccharide compound and the pharmaceutically active compound are in one aspect linked via hydrophobic interactions. Other non-covalent associations include electrostatic interactions, with oppositely charged ions attracting each other.

[00156] In various embodiments, the therapeutic protein is linked to or associated with the polysaccharide compound in stoichiometric amounts (e.g., 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:7, 1:8, 1:9, or 1:10, etc.). In various embodiments, 1-6, 7-12 or 13-20 polysaccharides are linked to the blood coagulation protein. In still other embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more polysaccharides are linked to the blood coagulation protein.

[00157] In various embodiments, the therapeutic protein is modified to introduce glycosylation sites (i.e., sites other than the native glycosylation sites). Such modification may be accomplished using standard molecular biological techniques known in the art. Moreover, the therapeutic protein, prior to conjugation to a water soluble polymer via one or more carbohydrate moieties, may be glycosylated in vivo or in vitro. These glycosylated sites can serve as targets for conjugation of the proteins with water soluble polymers (US Patent Application No. 20090028822, US Patent Application No. 2009/0093399, US Patent Application No. 2009/0081188, US Patent Application No. 2007/0254836, US Patent

Application No. 2006/0111279, and DeFrees S. et al., Glycobiology, 2006, 16, 9, 833-43). For example, a protein that is not naturally glycoslyated in vivo (e.g., a protein that is not a glycoprotein) may be modified as described above.

E. Aminooxy linkage

[00158] In one embodiment of the invention, the reaction of hydroxylamine or hydroxylamine derivatives with aldehydes (e.g., on a carbohydrate moiety following oxidation by sodium periodate) to form an oxime group is applied to the preparation of conjugates of blood coagulation protein. For example, a glycoprotein (e.g., a therapeutic protein according to the present invention) is first oxidized with a oxidizing agent such as sodium periodate ($NaIO_4$) (Rothfus JA et Smith EL., J Biol Chem 1963, 238, 1402-10; and Van Lenten L and Ashwell G., J Biol Chem 1971, 246, 1889-94). The periodate oxidation of glycoproteins is based on the classical Malaprade reaction described in 1928, the oxidation of vicinal diols with periodate to form an active aldehyde group (Malaprade L., Analytical application, Bull Soc Chim France, 1928, 43, 683-96). Additional examples for such an oxidizing agent are lead tetraacetate (Pb(OAc)4), manganese acetate (MnO(Ac)3), cobalt acetate (Co(OAc)2), thallium acetate (TlOAc), cerium sulfate (Ce(SO4)2) (US 4,367,309) or potassium perruthenate (KRuO4) (Marko et al., J Am Chem Soc 1997,119, 12661-2). By "oxidizing agent" a mild oxidizing compound which is capable of oxidizing vicinal diols in carbohydrates, thereby generating active aldehyde groups under physiological reaction conditions is meant.

[00159] The second step is the coupling of the polymer containing an aminooxy group to the oxidized carbohydrate moiety to form an oxime linkage. In one embodiment of the invention, this step can be carried out in the presence of catalytic amounts of the nucleophilic catalyst aniline or aniline derivatives (Dirksen A et Dawson PE, Bioconjugate Chem. 2008; Zeng Y et al., Nature Methods 2009;6:207-9). The aniline catalysis dramatically accelerates the oxime ligation allowing the use of very low concentrations of the reagents. In another embodiment of the invention the oxime linkage is stabilized by reduction with $NaCNBH_3$ to form an alkoxyamine linkage (Figure 2). Additional catalysts are described below.

[00160] Additional information on aminooxy technology can be found in the following references, each of which is incorporated in their entireties: EP 1681303A1 (HASylated erythropoietin); WO 2005/014024 (conjugates of a polymer and a protein linked by an oxime linking group); WO96/40662 (aminooxy-containing linker compounds and their application

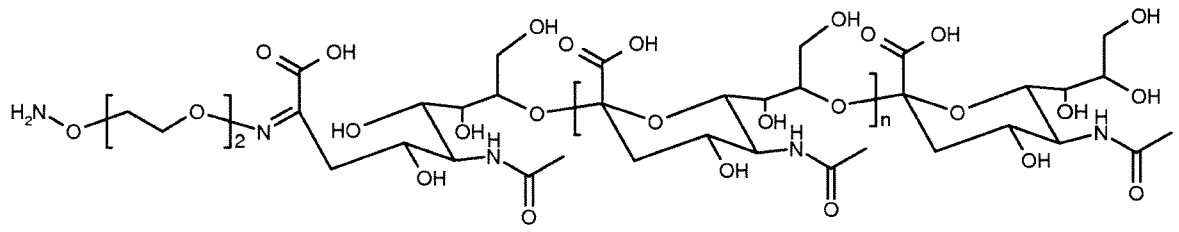
in conjugates); WO 2008/025856 (Modified proteins); Peri F et al., Tetrahedron 1998, 54, 12269-78; Kubler-Kielb J et. Pozsgay V., J Org Chem 2005, 70, 6887-90; Lees A et al., Vaccine 2006, 24(6), 716-29; and Heredia KL et al., Macromoecules 2007, 40(14), 4772-9.

[00161] Numerous methods of coupling a water-soluble polymer to an aminooxy linker are contemplated by the present disclosure. For example, coupling of a linker to either the reducing or non-reducing end of a water-soluble polymer such as PSA is described herein. The coupling site (e.g., reducing end versus non-reducing end) is determined by one or more conditions (e.g., time and temperature) of the coupling process as well as the state (e.g., native versus oxidized) of the water-soluble polymer. In one embodiment, an oxidized water-soluble polymer such as PSA is coupled at it's non-reducing end to an aminooxy linker by performing the coupling reaction at a reduced temperature (e.g., between 2-8°C). In another embodiment, a native (e.g., non-oxidized) water-soluble polymer such as PSA is coupled at it's reducing end to an aminooxy linker by performing the coupling reaction at a higher temperature (e.g., between 22-37°C). The aforementioned embodiments are described in more detail below and in the Examples.

[00162] As described herein, the reaction of oxidized PSA with a diaminooxy linker shows two reactions: a "quick reaction" of the aldehyde group at the non-reducing end, and a "slow reaction" at the reducing end. If native PSA (which is not oxidized and does not contain an active aldehyde group) is reacted with the reducing end at room temperature, a derivatized PSA can be observed. Thus, in various embodiments, in order to minimize an unwanted side reaction at the reducing end of a water-soluble polymer such as PSA, the PSA-aminooxy linker reagent preparation is performed at a temperature between 2-8°C.

[00163] In still another embodiment of the present disclosure, the derivatization of native PSA at the reducing end is provided. As described herein, native PSA (which is not oxidized by $NaIO_4$ and thus does not contain a free aldehyde group at its non-reducing end) is reacted with a diaminooxy linker at room temperature, a derivatization of the PSA at its reducing end can be observed. This coupling occurs through ring opening at the reducing end and subsequent oxime formation (the actual side reaction described above and the cause for the presence of by-product in the aminooxy-PSA reagent). This reaction can be performed with native PSA yielding in a degree of modification up to approximately 70%.

[00164] As a main product the following structure was determimed by 13C NMR spectroscopy



The reaction can be transferred to other carbohydrates like dextran and starch or other polysaccharides containing reducing end groups. The use of a nucleophilic catalyst like m-toluidine or aniline is also contemplated. Thus, preparation of aminooxy-PSA reagents using native PSA (i.e. without prior oxidation), which can then be used for chemical modification of therapeutic proteins, is provided herein.

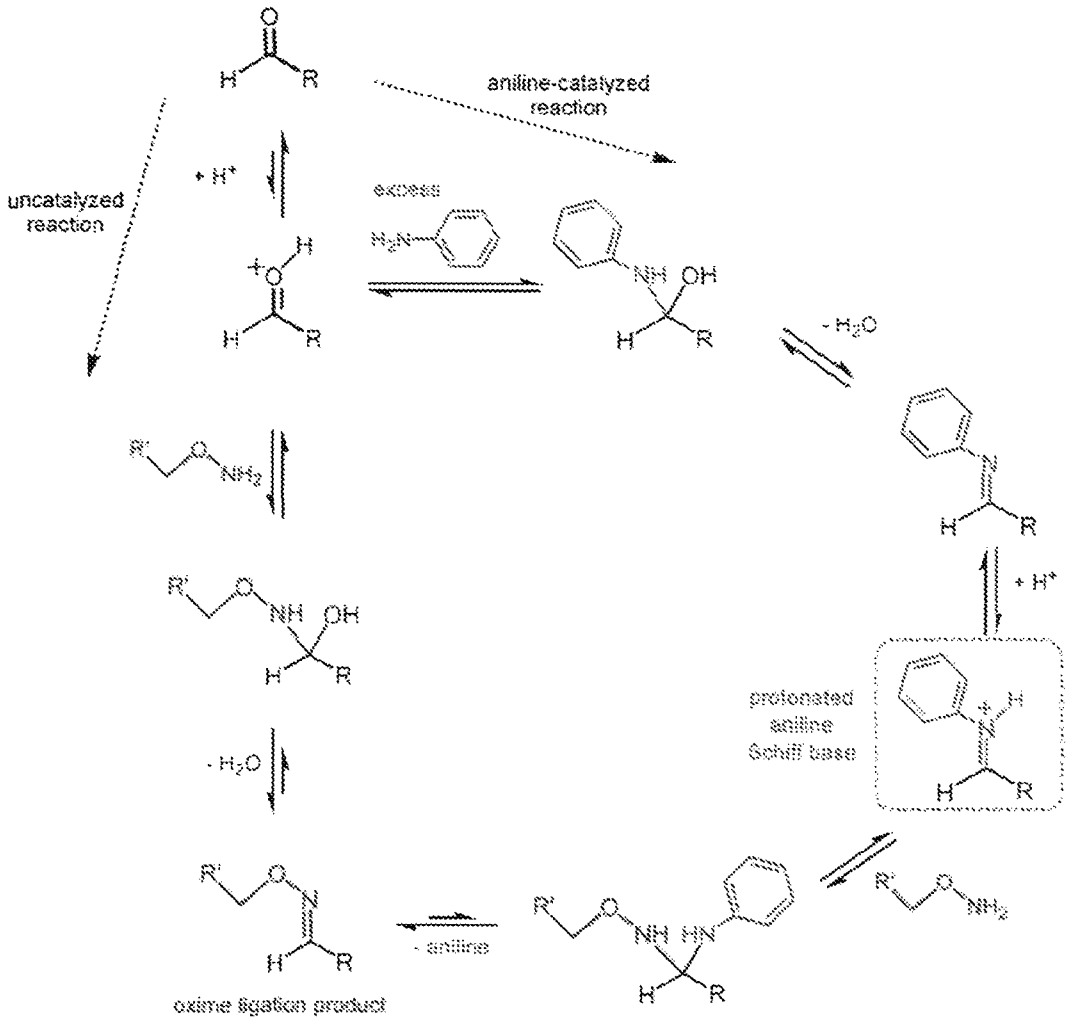
[00165] Thus, in various embodiments of the present disclosure, methods are provided wherein the conditions of coupling, (e.g., 2-8°C incubation temperature) a diaminooxy linker to a water soluble polymer such as oxidized PSA, favor the coupling to either the non-reducing end or, in one alternative, wherein the conditions of coupling (e.g., room temperature incubation) a diaminooxy linker to a water soluble polymer such as native, non-oxidized PSA, favor the coupling to either the reducing end.

[00166] In various embodiments of the invention, the water soluble polymer which is linked according to the aminooxy technology described herein to an oxidized carbohydrate moiety of a therapeutic protein (e.g., FVIII, FVIIa, or FIX) include, but are not limited to polyethylene glycol (PEG), branched PEG, polysialic acid (PSA), carbohydrate, polysaccharides, pullulane, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, starch, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG) polyoxazoline, poly acryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate, polyvinylpyrrolidone, polyphosphazene, polyoxazoline, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, poly(1-hydroxymethylethylene hydroxymethylformal) (PHF), 2-methacryloyloxy-2'-ethyltrimethylammoniumphosphate (MPC).

NUCLEOPHILIC CATALYSTS

[00167] As decribed herein, the conjugation of water soluble polymers to therapeutic proteins can be catalyzed by aniline. Aniline strongly catalyzes aqueous reactions of aldehydes and ketones with amines to form stable imines such as hydrazones and oximes.

The following diagram compares an uncatalyzed versus the aniline-catalyzed oxime ligation reaction (Kohler JJ, ChemBioChem 2009;10:2147-50):



[00168] However, considering the numerous health risks associated with aniline, alternative catalysts are desirable. The present invention provides aniline derivatives as alternative oxime ligation catalysts. Such aniline derivatives include, but are not limited to, o-amino benzoic acid, m-amino benzoic acid, p-amino benzoic acid, sulfanilic acid, o-aminobenzamide, o-toluidine, m-toluidine, p-toluidine, o-anisidine, m-anisidine, and p-anisidine.

[00169] In one embodiment of the invention, m-toluidine (aka meta-toluidine, m-methylaniline, 3-methylaniline, or 3-amino-1-methylbenzene) is used to catalyze the

conjugation reactions described herein. M-toluidine and aniline have similar physical properties and essentially the same pKa value (m-toluidine: pKa 4.73, aniline: pKa 4.63).

[00170] The nucleophilic catalysts of the invention are useful for oxime ligation (e.g, using aminooxy linkage) or hydrazone formation (e.g., using hydrazide chemistry). In various embodiments of the invention, the nucleophilic catalyst is provided in the conjugation reaction at a concentration of of 0.1, 0.2, 0.3, 0.5, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, or 50 mM. In one embodiment, the nucleophilic catalyst is provided between 1 to 10 mM. In various embodiments of the invention, the pH range of conjugation reaction is 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5. In one embodiment, the pH is between 5.5 to 6.5.

PURIFICATION OF CONJUGATED PROTEINS

[00171] In various embodiments, purification of a protein that has been incubated with an oxidizing agent and/or a therapeutic protein that has been conjugated with a water soluble polymer according to the present disclosure, is desired. Numerous purification techniques are known in the art and include, without limitation, chromatographic methods such as ion-exchange chromatography, hydrophobic interaction chromatography, size exclusion chromatography and affinity chromatography or combinations thereof, filtration methods (e.g., UF/DF), and precipitation methods as well as dialysis procedures and any combinations of the aforementioned techniques (Guide to Protein Purification, Meth. Enzymology Vol 463 (edited by Burgess RR and Deutscher MP), 2nd edition, Academic Press 2009).

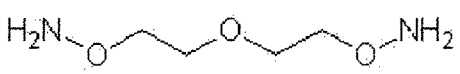
[00172] The following examples are not intended to be limiting but only exemplary of specific embodiments of the invention.

EXAMPLES

Example 1

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

[00173] The homobifunctional linker $NH_2[OCH_2CH_2]_2ONH_2$

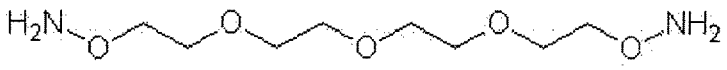


[00174] (3-oxa-pentane-1,5-dioxyamine) containing two active aminooxy groups was synthesized according to Boturyn et al. (Tetrahedron 1997;53:5485-92) in a two step organic reaction employing a modified Gabriel-Synthesis of primary amines (Figure 3). In the first step, one molecule of 2,2-chlorodiethylether was reacted with two molecules of Endo-N-hydroxy-5-norbornene-2,3-dicarboximide in dimethylformamide (DMF). The desired homobifunctional product was prepared from the resulting intermediate by hydrazinolysis in ethanol.

Example 2

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

[00175] The homobifunctional linker $NH_2[OCH_2CH_2]_4ONH_2$

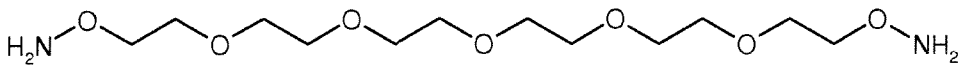


[00176] (3,6,9-trioxa-undecane-1,11-dioxyamine) containing two active aminooxy groups was synthesized according to Boturyn et al. (Tetrahedron 1997;53:5485-92) in a two step organic reaction employing a modified Gabriel-Synthesis of primary amines (Figure 3). In the first step one molecule of Bis-(2-(2-chlorethoxy)-ethyl)-ether was reacted with two molecules of Endo-N-hydroxy-5-norbornene-2,3-dicarboximide in DMF. The desired homobifunctional product was prepared from the resulting intermediate by hydrazinolysis in ethanol.

Example 3

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

[00177] The homobifunctional linker $NH_2[OCH_2CH_2]_6ONH_2$



[00178] (3,6,9,12,15-penatoxa-heptadecane-1,17-dioxyamine) containing two active aminooxy groups was synthesized according to Boturyn et al. (Tetrahedron 1997;53:5485-92) in a two step organic reaction employing a modified Gabriel-Synthesis of primary amines. In the first step one molecule of hexaethylene glycol dichloride was reacted with two molecules of Endo-N-hydroxy-5-norbornene-2,3-dicarboximide in DMF. The desired homobifunctional product was prepared from the resulting intermediate by hydrazinolysis in ethanol.

Example 4

Detailed synthesis of the aminooxy-PSA reagent

[00179] 3-oxa-pentane-1,5 dioxyamine was synthesized according to Botyryn et al (Tetrahedron 1997; 53:5485-92) in a two step organic synthesis as outlined in Example 1.

Step 1:

[00180] To a solution of Endo-N-hydroxy-5-norbonene-2,3- dicarboxiimide (59.0 g; 1.00 eq) in 700 ml anhydrous N,N-dimetylformamide anhydrous K_2CO_3 (45.51 g; 1.00 eq) and 2,2-dichlorodiethylether (15.84 ml; 0.41 eq) were added. The reaction mixture was stirred for 22 h at 50°C. The mixture was evaporated to dryness under reduced pressure. The residue was suspended in 2 L dichloromethane and extracted two times with saturated aqueous NaCl-solution (each 1 L). The Dichloromethane layer was dried over Na_2SO_4 and then evaporated to dryness under reduced pressure and dried in high vacuum to give 64.5 g of 3-oxapentane-1,5-dioxy-endo-2',3'-dicarboxydiimidenorbornene as a white-yellow solid (intermediate 1).

Step2:

[00181] To a solution of intermediate 1 (64.25 g; 1.00 eq) in 800 ml anhydrous Ethanol, 31.0 ml Hydrazine hydrate (4.26 eq) were added. The reaction mixture was then refluxed for 2 hrs. The mixture was concentrated to the half of the starting volume by evaporating the solvent under reduced pressure. The occurring precipitate was filtered off. The remaining ethanol layer was evaporated to dryness under reduced pressure. The residue containing the crude product 3-oxa-pentane -1,5-dioxyamine was dried in vacuum to yield 46.3 g. The crude product was further purified by column chromatography (Silicagel 60; isocratic elution with Dichloromethane/Methanol mixture, 9/1) to yield 11.7 g of the pure final product 3-oxa-pentane -1,5-dioxyamine.

Example 5

Preparation of aminooxy-PSA

[00182] 1000 mg of oxidized PSA (MW = 20 kD) obtained from the Serum Institute of India (Pune, India) was dissolved in 16 ml 50 mM phospate buffer pH 6.0. Then 170 mg 3-oxa-pentane-1,5-dioxyamine was given to the reaction mixture. After shaking for 2 hrs at RT 78.5 mg sodium cyanoborohydride was added and the reaction was performed for 18 hours over night. The reaction mixture was then subjected to an ultrafiltration/diafiltration

procedure (UF/DF) using a membrane with a 5 kD cut-off made of regenerated cellulose (50 cm^2, Millipore).

Example 6

Preparation of aminooxy-PSA employing a chromatographic purification step

[00183] 1290 mg of oxidized PSA (MW = 20 kD) obtained from the Serum Institute of India (Pune, India) was dissolved in 25 ml 50 mM phosphate buffer pH 6.0 (Buffer A). Then 209 mg 3-oxa-pentane-1,5-dioxyamine was given to the reaction mixture. After shaking for 1 h at RT 101 mg sodium cyanoborohydride was added and the reaction was performed for 3 hours. Then the mixture was then subjected to a weak anion exchange chromatography step employing a Fractogel EMD DEAE 650-M chromatography gel (column dimension: XK26/135). The reaction mixture was diluted with 110 ml Buffer A and loaded onto the DEAE column pre-equilibrated with Buffer A at a flow rate of 1 cm/min. Then the column was washed with 20 CV Buffer B (20 mM Hepes, pH 6.0) to remove free 3-oxa-pentane-1,5-dioxyamine and cyanide at a flow rate of 2 cm/min. The aminooxy-PSA reagent was then eluted with a step gradient consisting of 67% Buffer B and 43% Buffer C (20 mM Hepes, 1M NaCl, pH 7.5). The eluate was concentrated by UF/DF using a 5 kD membrane made of polyether sulfone (50 cm^2, Millipore). The final diafiltration step was performed against Buffer D (20mM Hepes, 90mM NaCl, pH 7.4). The preparation was analytically characterized by measuring total PSA (Resorcinol assay) and total aminooxy groups (TNBS assay) to determine the degree of modification. Furthermore the polydispersity as well as free 3-oxa-pentane-1,5-dioxyamine and cyanide was determined.

Example 7

Preparation of aminooxy-PSA without a reduction step

[00184] 573 mg of oxidized PSA (MW = 20 kD) obtained from the Serum Institute of India (Pune, India) was dissolved in 11,3 ml 50mM phosphate buffer pH 6.0 (Buffer A). Then 94 mg 3-oxa-pentane-1,5-dioxyamine was given to the reaction mixture. After shaking for 5 h at RT the mixture was then subjected to a weak anion exchange chromatography step employing a Fractogel EMD DEAE 650-M chromatography gel (column dimension: XK16/105). The reaction mixture was diluted with 50 ml Buffer A and loaded onto the DEAE column pre-equilibrated with Buffer A at a flow rate of 1 cm/min. Then the column was washed with 20 CV Buffer B (20 mM Hepes, pH 6.0) to remove free 3-oxa-pentane-1,5-dioxyamine and cyanide at a flow rate of 2 cm/min. The aminooxy-PSA reagent was the

eluted with a step gradient consisting of 67 % Buffer B and 43 % Buffer C (20 mM Hepes, 1 M NaCl, pH 7.5). The eluate was concentrated by UF/DF using a 5 kD membrane made of polyether sulfone (50 cm^2, Millipore). The final diafiltration step was performed against Buffer D (20 mM Hepes, 90 mM NaCl, pH 7.4). The preparation was analytically characterized by measuring total PSA (Resorcinol assay) and total aminooxy groups (TNBS assay) to determine the degree of modification. Furthermore the polydispersity as well as free 3-oxa-pentane-1,5-dioxyamine was determined.

Example 8

Preparation of aminooxy-PSA without a reduction step in the presence of the nucleophilic catalyst m-toluidine

[00185] 573 mg of oxidized PSA (MW = 20 kD) obtained from the Serum Institute of India (Pune, India) is dissolved in 9 ml 50 mM phosphate buffer pH 6.0 (Buffer A). Then 94 mg 3-oxa-pentane-1,5-dioxyamine is given to this solution. Subsequently 2.3 ml of a 50 mM m-toluidine stock solution are added to this reaction mixture. After shaking for 2 h at RT the mixture is then subjected to a weak anion exchange chromatography step employing a Fractogel EMD DEAE 650-M chromatography gel (column dimension: XK16/105). The reaction mixture is diluted with 50 ml Buffer A and loaded onto the DEAE column pre-equilibrated with Buffer A at a flow rate of 1 cm/min. Then the column is washed with 20CV Buffer B (20 mM Hepes, pH 6.0) to remove free 3-oxa-pentane-1,5-dioxyamine and cyanide at a flow rate of 2 cm/min. The aminooxy-PSA reagent is the eluted with a step gradient consisting of 67 % Buffer B and 43 % Buffer C (20 mM Hepes, 1 M NaCl, pH 7.5). The eluate is concentrated by UF/DF using a 5 kD membrane made of polyether sulfone (50 cm^2, Millipore). The final diafiltration step is performed against Buffer D (20 mM Hepes, 90 mM NaCl, pH 7.4). The preparation is analytically characterized by measuring total PSA (Resorcinol assay) and total aminooxy groups (TNBS assay) to determine the degree of modification. Furthermore the polydispersity as well as free 3-oxa-pentane-1,5-dioxyamine is determined.

Example 9

Preparation of aminooxy-PSA reagent

[00186] An Aminooxy - PSA reagent was prepared according to the Examples 4 - 8. After diafiltration, the product was frozen at -80°C and lyophilized. After lyophilization the reagent was dissolved in the appropriate volume of water and used for preparation of PSA-protein conjugates via carbohydrate modification.

Example 10

Evaluation of the efficacy of different alternative nucleophilic catalysts

[00187] rFIX was incubated with sodium periodate, aminooxy-PSA reagent under standardized conditions (1 mg/ml rFIX in 20 mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$, pH 6.0, 5-fold molar aminooxy-PSA reagent excess, 100 µM $NaIO_4$) using different nucleophilic catalysts (aniline, m-toluidine, o-anisidine, m-anisidine, o-aminobenzoic acid, m-aminobenzoic acid, p-aminobenzoic acid, p-aminobenzamide, sulfanilic acid / standard concentration: 10 mM) The reaction was carried out for 2 hrs in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of aqueous cysteine solution with a final concentration of 1 mM.

[00188] The coupling efficiency was determined by SDS-PAGE using an Invitrogen X-cell mini system. Samples were spiked with lithium dodecyl sulfate (LDS) buffer and denatured for 10 min at 70°C. Then the samples were applied on 3-8 % TRIS-acetate gels and ran at 150 V for 60 min. Subsequently the gels were stained with Coomassie.

[00189] In addition the samples were characterized by use of a SEC-HPLC system using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77).

[00190] 50 µl of samples were injected undiluted and eluted isocratically with a 0.22 µm filtered solution of 20 mM NaH_2PO_4, 50 mM Na_2SO_4, pH 6.1 at a flow rate of 0.5 ml/min. The elution pattern was recorded at 280 nm.

[00191] The results are summarized in Figures 5A-C and 6 (SDS PAGE) and Table 2 (SEC- HPLC results). The catalytic effect of the different preparations is demonstrated. It is shown that the use of m-toluidine leads to equivalent results as obtained with aniline.

Table 2

nucleophilic catalysts	di-PSAylated rFIX	mono-PSAylated rFIX	free rFIX
no catalyst	4.5%	24.9%	70.6%
10mM aniline	47.7%	33.6%	18.7%
10mM m-toluidine	31.4%	40.8%	27.8%
10mM o-aminobenzioc acid	30.9%	38.5%	30.6%
10mM m-aminobenzioc acid	27.6%	38.0%	34.4%
10mM p-aminobenzioc acid	18.1%	39.3%	42.6%
10mM o-aminobenzamide	15.9%	38.4%	45.7%
10mM sulfanilic acid	11.8%	35.8%	52.4%

Example 11

Polysialylation of rFIX using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

Method 1:

[00192] 12.3 mg rFIX was dissolved in 6.1 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). 254 µl of an aqueous sodium periodate solution (5 mM) was then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 6.5 µl of a 1 M aqueous cysteine solution. The mixture was subsequently subjected to UF/DF employing Vivaspin 15R 10 kD centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00193] The retentate (8.8 ml), containing oxidized rFIX was mixed with 2.46 ml of an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Then aminooxy-PSA reagent with a MW of 20 kD (described above) was added to give a 5-fold molar reagent excess. This mixture was incubated for 2.5 h at RT in the dark under gentle stirring.

[00194] The free rFIX was removed by means of anion exchange chromatography (AEC). The reaction mixture was diluted with 15 ml Buffer A (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5) and loaded onto a 20 ml HiPrep QFF 16/10 column (GE Healthcare, Fairfield, CT) pre-

equilibrated with Buffer A. The column was then eluted with Buffer B (50 mM Hepes, 1 M NaCl, 5 mM $CaCl_2$, pH 7.5). Free rFIX elutes at a conductivity between 12-25 mS/cm and the conjugate between 27-45 mS/cm. The conductivity of the conjugate containing fractions was subsequently raised to 190 mS/cm with Buffer C (50 mM Hepes, 5M NaCl, 5 mM $CaCl_2$, pH 6.9) and loaded onto a 20 ml HiPrep Butyl FF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer D (50 mM Hepes, 3 M NaCl, 5 mM $CaCl_2$, pH 6.9). Free aminooxy-PSA reagent was washed out within 5 CV Buffer D. Subsequently the conjugate is eluted with 100 % Buffer E (50 mM Hepes, 5 mM $CaCl_2$, pH 7.4). The conjugate containing fractions were concentrated by UF/DF using Vivaspin 15R 10 kD centrifugal filtrator. The final diafiltration step was performed against histidine buffer, Ph 7.2 containing 150 mM NaCl and 5 mM $CaCl_2$. The preparation was analytically characterized by measuring total protein (Bradford) and FIX chromogenic activity. The PSA-rFIX conjugate showed a specific activity of > 50% in comparison to native rFIX is determined.

Method 2:

[00195] 12.3 mg rFIX is dissolved in in L-histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$) to get a final protein concentration of 1 mg rFIX / ml. A 5 mM aqueous sodium periodate solution is added to get a final concentration of 100 µM and the reaction mixture is incubated for 1 hour in the dark at 4°C under gentle stirring at pH 6.0 and quenched for 15 min at room temperature by the addition of an 1 M aqueous L-cysteine solution (or other quenching reagents) to get a final concentration of 10 mM. The mixture is subsequently subjected to UF/DF employing Vivaspin 15R 10 kD centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00196] The obtained retentate (8.8 ml), containing oxidized rFIX, is mixed with an aqueous m-toluidine solution (50 mM) to give a final concentration of 10 mM and incubated for 30 min at room temperature. Then aminooxy-PSA reagent with a MW of 20 kD (described above) is added to give a 5-fold molar reagent excess. This mixture was incubated at pH 6.0 for 2.5 hours at room temperature; 0.5 hours to 18 hours at +4°C) in the dark under gentle stirring.

[00197] The free rFIX is removed by means of anion exchange chromatography (AEC). The reaction mixture is diluted with appropriate amounts of Buffer A (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5) to correct the solutions conductivity and pH prior to load onto a 20 ml HiPrep QFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with buffer A. Then the column is eluted with Buffer B (50 mM Hepes, 1 M NaCl, 5 mM $CaCl_2$, pH 7.5). Free rFIX is eluted by a step gradient using 25 % of Buffer B, which results in a conductivity between 12-25 mS/cm in the obtained fraction and the conjugate using a step gradient of 50 % Buffer B, which results in a conductivity between between 27-45 mS/cm in the conjugate fraction. The conductivity of the conjugate containing fraction is subsequently raised to 190 mS/cm with Buffer C (50 mM Hepes, 5 M NaCl, 5 mM $CaCl_2$, pH 6.9 or by use of anti-chaotropic salts e.g. ammonium sulphate, ammonium acetate etc.) and loaded onto a 20 ml HiPrep Butyl FF 16/10 column (GE Healthcare, Fairfield, CT or comparable HIC media) pre-equilibrated with Buffer D (50 mM Hepes, 3 M NaCl, 5 mM $CaCl_2$, pH 6.9). Free aminooxy-PSA reagent is washed out within 5 CV Buffer D. Subsequently, the conjugate is eluted with 100 % Buffer E (50 mM Hepes, 5 mM $CaCl_2$, pH 7.4). The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD, Millipore). The final diafiltration step is performed against L-histidine buffer, pH 7.2 containing 150 mM NaCl and 5 mM CaCl2. The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and FIX chromogenic- and clotting activity. For the PSA-rFIX conjugate a specific activity of > 50 % in comparison to native rFIX is determined.

Method 3:

[00198] 25.4 mg rFIX was dissolved in 18.7 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). 531 µl of an aqueous sodium periodate solution (5 mM) and 5.07 ml of an aqueous m-toluidine solution (50 mM) were then added. Subsequently, the aminooxy-PSA reagent with a MW of 20 kD (described above) was added to give a 5-fold molar reagent excess. The mixture was incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 25 µl of 1 M aqueous cysteine solution.

[00199] The free rFIX was removed by means of anion exchange chromatography (AEC). The reaction mixture was diluted with 20 ml Buffer A (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5) and loaded onto a 20 ml HiPrep QFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column was eluted with Buffer B (50 mM Hepes, 1 M

NaCl, 5 mM $CaCl_2$, pH 7.5). Free rFIX eluted at a conductivity between 12-25 mS/cm and the conjugate between 27-45 mS/cm. The conductivity of the conjugate containing fractions was subsequently raised to 190 mS/cm with Buffer C (50 mM Hepes, 5 M NaCl, 5 mM $CaCl_2$, pH 6.9) and loaded onto a 20 ml HiPrep Butyl FF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer D (50 mM Hepes, 3 M NaCl, 5 mM $CaCl_2$, pH 6.9). Free aminooxy-PSA reagent was washed out within 5 CV Buffer D. Subsequently, the conjugate was eluted with 100 % Buffer E (50 mM Hepes, 5 mM $CaCl_2$, pH 7.4). The conjugate containing fractions were concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD, Millipore). The final diafiltration step was performed against histidine buffer, pH 7.2 containing 150 mM NaCl and 5 mM $CaCl_2$. The preparation was analytically characterized by measuring total protein (Bradford) and FIX chromogenic activity. For the PSA-rFIX conjugate a specific activity of > 50 % in comparison to native rFIX was determined. The conjugate was additionally analytically characterized by Size Exclusion HPLC using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77). It was shown that the preparation contains no free FIX. The conjugate consisted of 57 % mono-polysialylated and 31 % di-polysialylated and 12 % tri-polysialyated product.

Method 4:

[00200] 25.4 mg rFIX was dissolved in L-histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$) to get a final protein concentration of 2 mg rFIX / ml. Subsequently an 5 mM aqueous sodium periodate solution was added within 15 minutes to give a final concentration of 100 µM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PSA reagent with a MW of 20 kD (described above) was added to give a 5-fold molar reagent excess. After correction of the pH to 6.0 the mixture was incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of a 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00201] The free rFIX was removed by means of ion exchange chromatography (IEC). The reaction mixture was diluted with appropriate amounts of Buffer A (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5) to correct the solutions conductivity and pH value prior to load onto a 20 ml HiPrep QFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column was eluted with Buffer B (50 mM Hepes, 1 M NaCl, 5 mM $CaCl_2$, pH 7.5).

Free rFIX was eluted by a step gradient using 25 % of Buffer B, which results in a conductivity between 12-25 mS/cm in the obtained fraction and the conjugate using a step gradient of 50 % Buffer B, which results in a conductivity between 27-45 mS/cm in the conjugate fraction. The conductivity of the conjugate containing fraction was subsequently raised to 190 mS/cm with Buffer C (50 mM Hepes, 5 M NaCl, 5 mM $CaCl_2$, pH 6.9; by use of anti-chaotropic salts e.g. ammonium acetate) and loaded onto a 20 ml HiPrep Butyl FF 16/10 column (GE Healthcare, Fairfield, CT; or comparable HIC media) pre-equilibrated with Buffer D (50 mM Hepes, 3 M NaCl, 5 mM $CaCl_2$, pH 6.9). Free aminooxy-PSA reagent was washed out within 5 CV Buffer D. Subsequently the conjugate was eluted with 100 % Buffer E (50 mM Hepes, 5 mM $CaCl_2$, pH 7.4). The conjugate containing fractions were concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD, Millipore). The final diafiltration step was performed against L-histidine buffer, pH 7.2 containing 150 mM NaCl and 5 mM $CaCl_2$. The preparation was analytically characterized by measuring total protein (Bradford and BCA procedure) and FIX chromogenic- and clotting activity. For the PSA-rFIX conjugate a specific activity of > 50 % in comparison to native rFIX was determined. The conjugate was additionally analytically characterized by Size Exclusion HPLC using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77). It was shown that the preparation contains no free FIX. The conjugate consisted of 57 % mono-polysialylated and 31 % di-polysialylated and 12 % tri-polysialyated product.

Example 12

Polysialylation of rFVIII using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

Method 1:

[00202] 50 mg rFVIII was transferred into reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ was added to give a final concentration of 200 µM. The oxidation was carried at RT for 30 min in the dark under gentle shaking. Then the reaction was quenched with cysteine (final concentration: 10 mM) for 60 min at RT. The solution was subjected to an IEX column with a volume of 20 ml (Merck EMD TMAE (M)) which was equilibrated with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 7.0). The column was equilibrated with 5 CV Buffer A. Then the oxidized rFVIII was eluted with Buffer B (20 mM

Hepes, 5 mM $CaCl_2$, 1M NaCl, pH 7.0). The rFVIII containing fractions were collected. The protein content was determined (Coomassie, Bradford) and adjusted to 1 mg/ml with reaction buffer and adjusted to pH 6.0 by dropwise addition of 0.5 M HCl. Then a 50-fold molar excess of a aminooxy-PSA reagent with a MW of 20 kD (described above) was added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction was performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy-PSA reagent was removed by means of HIC. The conductivity of the reaction mixture was raised to 130 mS/cm by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with 80 ml Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.9. Subsequently, the conjugate was eluted with 50 mM Hepes buffer pH 7.5 containing 5 mM $CaCl_2$. Finally, the PSA-rFVIII containing fractions were collected and subjected to UF/DF by use of a 30 kD membrane made of regenerated cellulose (88cm^2, Millipore). The preparation was analytically characterized by measuring total protein (Coomassie, Bradford) and FVIII chromogenic activity. The PSA-rFVIII conjugate showed a specific activity of > 70% in comparison to native rFVIII was determined.

Method 2:

[00203] 58 mg of recombinant factor VIII (rFVIII) derived from the ADVATE process in Hepes buffer (50 mM HEPES, ~350 mM sodium chloride, 5 mM calcium chloride, 0.1 % Polysorbate 80, pH 7.4) is dissolved in reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00204] The oxidized rFVIII is further purified by anion exchange chromatography on EMD TMAE (M) (Merck). The mixture is diluted with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 6.5) to give a conductivity of 5 ms/cm. This solution is loaded onto the IEX column (bed height: 5.4 cm) with a column volume of 10 ml using a flow rate of 1.5 cm/min. This column is subsequently washed (flow rate: 1.5 cm/min) with 5 CV of a 92:8 mixture (w/w) of Buffer A and Buffer B (20 mM Hepes, 5 mM $CaCl_2$, 1.0 M NaCl, pH 7.0). Then the oxidized rFVIII is eluted with a 50:50 (w/w) mixture of Buffer A and Buffer B followed by a postelution step with 5 CV of Buffer B. The elution steps are carried out by use of a flow rate of 1.0 cm/min.

[00205] Subsequently, the aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized rFVIII within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00206] The obtained PSA-rFVIII conjugate is purified by Hydrophobic Interaction Chromatography (HIC) using a Phenyl Sepharose FF low sub resin (GE Healthcare) packed into a column manufactured by GE Healthcare with a bed height (h) of 15 cm and a resulting column volume (CV) of 81 ml.

[00207] The reaction mixture is spiked with ammonium acetate by addition of 50 mM Hepes buffer, containing 350 mM sodium chloride, 8 M ammonium acetate, 5 mM calcium chloride, pH 6.9. Two volumes of the reaction mixture are mixed with 1 volume of the ammonium acetate containing buffer system and the pH value is corrected to pH 6.9 by drop wise addition of a 0.5 N aqueous NaOH solution. This mixture is loaded onto the HIC column at flow rate of 1 cm/min followed by a washing step using > 3 CV equilibration buffer (50 mM Hepes, 350 mM sodium chloride, 2.5 M ammonium acetate, 5 mM calcium chloride, pH 6.9).

[00208] For removal of reaction by-products and anti-chaotropic salt a second washing step is performed with > 5 CV washing buffer 1 (50 mM Hepes, 3 M sodium chloride, 5 mM calcium chloride, pH 6.9) in upflow mode at a flow rate of 2 cm/min. Then elution of purified PSA-rFVIII conjugate is performed in down flow mode using a step gradient of 40 % washing buffer 2 (50 mM Hepes, 1.5 M sodium chloride, 5 mM calcium chloride, pH

6.9) and 60 % elution buffer (20mM Hepes, 5mM calcium chloride, pH 7.5) at a flow rate of 1 cm/min. The elution of the PSA-rFVIII conjugate is monitored at UV 280 nm and the eluate containing the conjugate is collected within < 4 CV. The post elution step is performed with > 3 CV elution buffer under the same conditions to separate minor and/or non modified rFVIII from the main product.

[00209] Finally the purified conjugate is concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with a molecular weight cut off 30kD (88cm^2, Millipore).

[00210] The conjugate prepared by use of this procedure are analytically characterized by measuring total protein, FVIII chromogenic activity and determination of the polysialyation degree by measuring the PSA content (resorcinol assay). For the conjugate obtained a specific activity > 50% and a PSA degree > 5.0 is calculated.

Method 3:

[00211] 50 mg rFVIII was transferred into reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) was added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM) and $NaIO_4$ (final concentration: 400 µM). The coupling reaction was performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction was quenched with cysteine for 60 min at RT (final concentration: 10 mM). Then the conductivity of the reaction mixture was raised to 130 mS/cm by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with 80 ml Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, 0.01 % Tween 80, pH 6.9. Subsequently, the conjugate was eluted with 50 mM Hepes, 5 mM calcium chloride, pH 7.5. Finally, the PSA-rFVIII containing fractions were collected and subjected to UF/DF by use of a 30 kD membrane made of regenerated cellulose (88cm^2, Millipore). The preparation was analytically characterized by measuring total protein (Bradford) and FVIII chromogenic activity. For the PSA-rFVIII conjugate a specific activity of ≥ 70% in comparison to native rFVIII was determined.

Method 4:

[00212] 50 mg recombinant factor VIII (rFVIII) derived from the ADVATE process in 50 mM Hepes buffer (50 mM HEPES, ~350 mM sodium chloride, 5 mM calcium chloride, 0.1 % Polysorbate 80, pH 7.4) was dissolved in reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution was corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution.

[00213] Subsequently, the aminooxy-polysialic acid (PSA-ONH_2) reagent was added in a 50-fold molar excess to this rFVIII solution within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) was added within 15 minutes to get a final concentration of 10 mM. Finally, a 40 mM aqueous sodium periodate solution was added to give a concentration of 400 µM.

[00214] The reaction mixture was incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking. Then the reaction was stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00215] The obtained PSA-rFVIII conjugate was purified by Hydrophobic Interaction Chromatography (HIC) using a Phenyl Sepharose FF low sub resin (GE Healthcare) packed into a column manufactured by GE Healthcare with a bed height (h) of 15 cm and a resulting column volume (CV) of 81 ml.

[00216] The reaction mixture was spiked with ammonium acetate by addition of of 50 mM Hepes buffer, containing 350 mM sodium chloride, 8 M ammonium acetate, 5 mM calcium chloride, pH 6.9. Two volumes of the reaction mixture was mixed with 1 volume of the ammonium acetate containing buffer system and the pH value was corrected to pH 6.9 by drop wise addition of an 0.5 N aqueous NaOH solution. This mixture was loaded onto the HIC column using a flow rate of 1 cm/min followed by a washing step using > 3 CV equilibration buffer (50 mM Hepes, 350 mM sodium chloride, 2.5 M ammonium acetate, 5 mM calcium chloride, pH 6.9).

[00217] For removal of reaction by-products and anti-chaotropic salt a second washing step was performed with > 5CV washing buffer 1 (50 mM Hepes, 3 M sodium chloride, 5 mM calcium chloride, pH 6.9) in upflow mode at a flow rate of 2 cm/min. Then elution of purified rFVIII conjugate was performed in down flow mode using a step gradient of 40 %

washing buffer 2 (50 mM Hepes, 1.5 M sodium chloride, 5 mM calcium chloride, pH 6.9) and 60 % elution buffer (20 mM Hepes, 5 mM calcium chloride, pH 7.5) at a flow rate of 1 cm/min. The elution of the PSA-rFVIII conjugate was monitored at UV 280 nm and the eluate containing the conjugate was collected within < 4 CV. The post elution step was performed with > 3 CV elution buffer under the same conditions to separate minor and/or non modified rFVIII from the main product.

[00218] Finally, the purified conjugate was concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with a molecular weight cut off 30 kD (88 cm^2, Millipore).

[00219] The conjugates prepared by use of this procedure were analytically characterized by measuring total protein, FVIII chromogenic activity and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Analytical data (mean of 6 consecutive batches):

Process yield (Bradford): 58.9%
Process yield (FVIII chrom.): 46.4%
Specific activity: (FVIII chrom. / mg protein): 4148 IU/mg
Specific activity (% of starting material): 79.9 %
PSA degree (mol/mol): 8.1

Example 13

PEGylation of r FVIII using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00220] rFVIII is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). 14.7 mg rFVIII is dissolved in 7.0 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). Then 296 µl of an aqueous sodium periodate solution (5 mM) is added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture was subsequently subjected to UF/DF employing Vivaspin 15R 10 kD centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00221] The retentate (10.9 ml), containing oxidized rFVIII, is mixed with 2.94 ml of an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Then aminooxy-PEG reagent with a MW of 20 kD is added to give a 5-fold molar reagent excess. This mixture was incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00222] Finally, the PEG-rFVIII conjugate is purified by ion-exchange chromatography on Q Sepharose FF. 1.5 mg protein/ml gel is loaded on the column equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using a 30 kD membrane (50cm2, Millipore). The preparation is analytically characterized by measuring total protein (Coomassie, Bradford) and FVIII chromogenic activity. It is expected that the PEG-rFVIII conjugate will demonstrate a specific activity of > 70% in comparison to native rFVIII was determined.

Method 2:

[00223] rFVIII is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). A starting weight or concentration of rFVIII is dissolved in or transferred to a reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/-5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00224] The oxidized rFVIII is further purified by anion exchange chromatography on EMD TMAE (M) (Merck). The mixture is diluted with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 6.5) to give a conductivity of 5 ms/cm. This solution is loaded onto the IEX column (bed height: 5.4 cm) with a column volume of 10 ml using a flow rate of 1.5 cm/min. This column is subsequently washed (flow rate: 1.5 cm/min) with 5 CV of a 92:8 mixture (w/w) of Buffer A and Buffer B (20 mM Hepes, 5 mM CaCl2, 1.0 M NaCl, pH 7.0). Then the oxidized rFVIII is eluted with a 50:50 (w/w) mixture of Buffer A and Buffer B followed

by a postelution step with 5 CV of Buffer B. The elution steps are carried out by use of a flow rate of 1.0 cm/min.

[00225] Subsequently, the aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized rFVIII within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00226] The obtained PEG-rFVIII conjugate is purified by Hydrophobic Interaction Chromatography (HIC) using a Phenyl Sepharose FF low sub resin (GE Healthcare) packed into a column manufactured by GE Healthcare with a bed height (h) of 15 cm and a resulting column volume (CV) of 81 ml.

[00227] The reaction mixture is spiked with ammonium acetate by addition of 50 mM Hepes buffer, containing 350 mM sodium chloride, 8 M ammonium acetate, 5 mM calcium chloride, pH 6.9. Two volumes of the reaction mixture are mixed with 1 volume of the ammonium acetate containing buffer system and the pH value is corrected to pH 6.9 by drop wise addition of a 0.5 N aqueous NaOH solution. This mixture is loaded onto the HIC column using a flow rate of 1 cm/min followed by a washing step using > 3 CV equilibration buffer (50 mM Hepes, 350 mM sodium chloride, 2.5 M ammonium acetate, 5 mM calcium chloride, pH 6.9).

[00228] For removal of reaction by-products and anti-chaotropic salt a second washing step is performed with > 5 CV washing buffer 1 (50 mM Hepes, 3 M sodium chloride, 5 mM calcium chloride, pH 6.9) in upflow mode at a flow rate of 2 cm/min. Then elution of purified rFVIII conjugate is performed in down flow mode using a step gradient of 40 % washing buffer 2 (50 mM Hepes, 1.5 M sodium chloride, 5 mM calcium chloride, pH 6.9) and 60 % elution buffer (20mM Hepes, 5mM calcium chloride, pH 7.5) at a flow rate of 1cm/min. The elution of the PEG-rFVIII conjugate is monitored at UV 280 nm and the eluate containing the conjugate is collected within < 4 CV. The post elution step is performed with > 3 CV elution buffer under the same conditions to separate minor and/or non modified rFVIII from the main product.

[00229] Finally, the purified conjugate is concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with a molecular weight cut off 30kD (Millipore).

[00230] The conjugate prepared by use of this procedure are analytically characterized by measuring total protein and biological activity according to methods known in the art.

Method 3:

[00231] rFVIII is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). 7.84 mg rFVIII, dissolved in 6 ml Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) are mixed with 314 µl of an aqueous sodium periodate solution (10 mM), and 1.57 ml of an aqueous m-toluidine solution (50 mM). Subsequently the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00232] Finally the PEG-rFVIII conjugate is purified by ion-exchange chromatography on Q-Sepharose FF. 1.5 mg protein/ml gel is loaded on the column pre equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using a 30 kD membrane (88cm^2, Millipore). The analytical characterization of the conjugate by FVIII chromogenic assay and determination of total protein (Bradford) shows a specific activity of > 60% compared to the rFVIII starting material.

Method 4:

[00233] rFVIII is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of rFVIII is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 2 mg rFVIII / ml. Subsequently, an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 µM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20-fold molar excess.

After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of a 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00234] The free rFVIII is removed by means of ion exchange chromatography (IEC). The reaction mixture was diluted with appropriate amounts of Buffer A (50 mM Hepes, 5 mM CaCl2, pH 7.5) to correct the solutions conductivity and pH value prior to load onto a 20 ml HiPrep QFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column was eluted with Buffer B (50 mM Hepes, 1 M NaCl, 5 mM $CaCl_2$, pH 7.5). Free rFVIII was eluted by a step gradient using 25 % of Buffer B, which results in a conductivity between 12-25 mS/cm in the obtained fraction and the conjugate using a step gradient of 50 % Buffer B, which results in a conductivity between 27-45 mS/cm in the conjugate fraction. The conductivity of the conjugate containing fraction is subsequently raised with Buffer C (50 mM Hepes, 5 M NaCl, 5 mM $CaCl_2$, pH 6.9; by use of anti-chaotropic salts e.g. ammonium acetate, ammonium sulphate etc.) and loaded onto a 20 ml HiPrep Butyl FF 16/10 column (GE Healthcare, Fairfield, CT; or comparable HIC media) pre-equilibrated with Buffer D (50 mM Hepes, 3 M NaCl, 5 mM $CaCl_2$, pH 6.9). Free PEG-reagent was washed out within 5 CV Buffer D. Subsequently, the conjugate was eluted with 100 % Buffer E (50 mM Hepes, 5 mM $CaCl_2$, pH 7.4). The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD, Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00235] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods.

Example 14

Polysialylation of rFVIIa using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

Method 1:

[00236] A starting concentration or weight of recombinant factor VIIa (rFVIIa) is transferred or dissolved in reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous NaOH solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 50 µM. The oxidation reaction is carried out for 30 +/-

5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T = +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00237] The oxidized rFVIIa is further purified by anion exchange chromatography on EMD TMAE (M) (Merck). The mixture is diluted with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 6.5) to give a conductivity of 5 ms/cm. This solution is loaded onto the IEX column (bed height: 5.4 cm) with a column volume of 10 ml using a flow rate of 1.5 cm/min. This column is subsequently washed (flow rate: 1.5 cm/min) with 5 CV of a 92:8 mixture (w/w) of Buffer A and Buffer B (20 mM Hepes, 5 mM $CaCl_2$, 1.0 M NaCl, pH 7.0). Then the oxidized rFVIIa is eluted with a 50:50 (w/w) mixture of Buffer A and Buffer B followed by a postelution step with 5 CV of Buffer B. The elution steps are carried out by use of a flow rate of 1.0 cm/min.

[00238] Subsequently, the aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized rFVIIa within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00239] The obtained PSA-rFVIIa conjugate is purified by Hydrophobic Interaction Chromatography (HIC) using a Phenyl Sepharose FF low sub resin (GE Healthcare) packed into a column manufactured by GE Healthcare with a bed height (h) of 15 cm and a resulting column volume (CV) of 81 ml.

[00240] The reaction mixture is spiked with ammonium acetate by addition of 50 mM Hepes buffer, containing 350 mM sodium chloride, 8 M ammonium acetate, 5 mM calcium chloride, pH 6.9. Two volumes of the reaction mixture are mixed with 1 volume of the ammonium acetate containing buffer system and the pH value is corrected to pH 6.9 by drop wise addition of a 0.5 N aqueous NaOH solution. This mixture is loaded onto the HIC column using a flow rate of 1 cm/min followed by a washing step using > 3 CV equilibration buffer (50 mM Hepes, 350 mM sodium chloride, 2.5 M ammonium acetate, 5 mM calcium chloride, pH 6.9).

[00241] For removal of reaction by-products and anti-chaotropic salt a second washing step is performed with > 5CV washing buffer 1 (50 mM Hepes, 3 M sodium chloride, 5 mM calcium chloride, pH 6.9) in upflow mode at a flow rate of 2 cm/min. Then elution of purified rFVIIa conjugate is performed in down flow mode using a step gradient of 40 % washing buffer 2 (50 mM Hepes, 1.5 M sodium chloride, 5 mM calcium chloride, pH 6.9) and 60 % elution buffer (20mM Hepes, 5mM calcium chloride, pH 7.5) at a flow rate of 1 cm/min. The elution of the PSA-rFVIIa conjugate is monitored at UV 280 nm and the eluate containing the conjugate is collected within < 4 CV. The post elution step is performed with > 3 CV elution buffer under the same conditions to separate minor and/or non modified rFVIIa from the main product.

[00242] Finally, the purified conjugate is concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (e.g. 10 kD MWCO, 88cm^2, Millipore).

[00243] The conjugate prepared by use of this procedure is analytically characterized by measuring total protein, biological activity, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Method 2:

[00244] A starting weight or concentration of rFVIIa is dissolved in or transferred to a reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous NaOH solution.

[00245] Subsequently, the aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to this rFVIIa solution within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. Finally a 40 mM aqueous sodium periodate solution is added to give a concentration of 150 µM.

[00246] The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking. Then the reaction is stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00247] The obtained PSA-rFVIIa conjugate is purified by Hydrophobic Interaction Chromatography (HIC) using a Phenyl Sepharose FF low sub resin (GE Healthcare) packed into a column manufactured by GE Healthcare with a bed height (h) of 15 cm and a resulting column volume (CV) of 81 ml.

[00248] The reaction mixture is spiked with ammonium acetate by addition of of 50 mM Hepes buffer, containing 350 mM sodium chloride, 8 M ammonium acetate, 5 mM calcium chloride, pH 6.9. Two volumes of the reaction mixture is mixed with 1 volume of the ammonium acetate containing buffer system and the pH value is corrected to pH 6.9 by drop wise addition of an 0.5 N aqueous NaOH solution. This mixture is loaded onto the HIC column using a flow rate of 1 cm/min followed by a washing step using > 3CV equilibration buffer (50 mM Hepes, 350 mM sodium chloride, 2.5 M ammonium acetate, 5 mM calcium chloride, pH 6.9).

[00249] For removal of reaction by-products and anti-chaotropic salt a second washing step is performed with > 5CV washing buffer 1 (50 mM Hepes, 3 M sodium chloride, 5 mM calcium chloride, pH 6.9) in upflow mode at a flow rate of 2 cm/min. Then elution of purified rFVIIa conjugate is performed in down flow mode using a step gradient of 40 % washing buffer 2 (50 mM Hepes, 1.5 M sodium chloride, 5 mM calcium chloride, pH 6.9) and 60 % elution buffer (20mM Hepes, 5mM calcium chloride, pH 7.5) at a flow rate of 1 cm/min. The elution of the PSA-rFVIIa conjugate is monitored at UV 280 nm and the eluate containing the conjugate was collected within < 4 CV. The post elution step is performed with > 3 CV elution buffer under the same conditions to separate minor and/or non modified rFVIII from the main product.

[00250] Finally, the purified conjugate is concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose (Millipore).

[00251] The conjugates prepared by use of this procedure are analytically characterized by measuring total protein, biological activity according to methods known in the art, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Example 15

PEGylation of rFIX using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00252] rFIX is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). A starting weight or concentration of rFIX is dissolved in or transferred to a reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T = +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00253] The oxidized rFVIII is further purified by anion exchange chromatography on EMD TMAE (M) (Merck). The mixture is diluted with Buffer A (20 mM Hepes, 5 mM CaCl2, pH 6.5) to give a conductivity of 5 mS/cm. This solution is loaded onto the IEX column (bed height: 5.4 cm) with a column volume of 10 ml using a flow rate of 1.5 cm/min. This column is subsequently washed (flow rate: 1.5 cm/min) with 5 CV of a 92:8 mixture (w/w) of Buffer A and Buffer B (20 mM Hepes, 5 mM CaCl2, 1.0 M NaCl, pH 7.0). Then the oxidized rFIX is eluted with a 50:50 (w/w) mixture of Buffer A and Buffer B followed by a postelution step with 5 CV of Buffer B. The elution steps are carried out by use of a flow rate of 1.0 cm/min.

[00254] Subsequently, the aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized rFIX within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00255] The obtained PEG-rFIX conjugate is purified by Hydrophobic Interaction Chromatography (HIC) using a Phenyl Sepharose FF low sub resin (GE Healthcare) packed into a column manufactured by GE Healthcare with a bed height (h) of 15 cm and a resulting column volume (CV) of 81 ml.

[00256] The reaction mixture is spiked with ammonium acetate by addition of 50 mM Hepes buffer, containing 350 mM sodium chloride, 8 M ammonium acetate, 5 mM calcium chloride, pH 6.9. Two volumes of the reaction mixture are mixed with 1 volume of the ammonium acetate containing buffer system and the pH value is corrected to pH 6.9 by drop wise addition of a 0.5 N aqueous NaOH solution. This mixture is loaded onto the HIC column using a flow rate of 1 cm/min followed by a washing step using > 3 CV equilibration buffer (50 mM Hepes, 350 mM sodium chloride, 2.5 M ammonium acetate, 5 mM calcium chloride, pH 6.9).

[00257] For removal of reaction by-products and anti-chaotropic salt a second washing step is performed with > 5CV washing buffer 1 (50 mM Hepes, 3 M sodium chloride, 5 mM calcium chloride, pH 6.9) in upflow mode at a flow rate of 2 cm/min. Then elution of purified rFIX conjugate is performed in down flow mode using a step gradient of 40 % washing buffer 2 (50 mM Hepes, 1.5 M sodium chloride, 5 mM calcium chloride, pH 6.9) and 60 % elution buffer (20 mM Hepes, 5 mM calcium chloride, pH 7.5) at a flow rate of 1 cm/min. The elution of the PEG-rFIX conjugate is monitored at UV 280 nm and the eluate containing the conjugate is collected within < 4 CV. The post elution step is performed with > 3 CV elution buffer under the same conditions to separate minor and/or non modified rFIX from the main product.

[00258] Finally, the purified conjugate is concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with a molecular weight cut off 10kD (88cm2, Millipore).

[00259] The conjugate prepared by use of this procedure are analytically characterized by measuring total protein and biological activity according to methods known in the art.

Method 2:

[00260] rFIX is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of rFIX is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride,

pH 6.0) to get a final protein concentration of 2 mg rFIX / ml. Subsequently, an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 µM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20-fold molar reagent excess. After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of a 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00261] The free rFIX is removed by means of ion exchange chromatography (IEC). The reaction mixture was diluted with appropriate amounts of Buffer A (50 mM Hepes, 5 mM CaCl2, pH 7.5) to correct the solutions conductivity and pH value prior to load onto a 20 ml HiPrep QFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column was eluted with Buffer B (50 mM Hepes, 1 M NaCl, 5 mM $CaCl_2$, pH 7.5). Free rFIX was eluted by a step gradient using 25 % of Buffer B, which results in a conductivity between 12- 25 mS/cm in the obtained fraction and the conjugate using a step gradient of 50 % Buffer B, which results in a conductivity between 27-45 mS/cm in the conjugate fraction. The conductivity of the conjugate containing fraction is subsequently raised with Buffer C (50 mM Hepes, 5 M NaCl, 5 mM $CaCl_2$, pH 6.9; by use of anti-chaotropic salts e.g. ammonium acetate, etc) and loaded onto a 20 ml HiPrep Butyl FF 16/10 column (GE Healthcare, Fairfield, CT; or comparable HIC media) pre-equilibrated with Buffer D (50 mM Hepes, 3 M NaCl, 5 mM $CaCl_2$, pH 6.9). Free aminooxy-PEG reagent was washed out within 5 CV Buffer D. Subsequently, the conjugate was eluted with 100 % Buffer E (50 mM Hepes, 5 mM CaCl2, pH 7.4). The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD, Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00262] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods.

Example 16

PEGylation of rFVIIa using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00263] rFVIIa is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). A starting weight or concentration of rFVIIa is dissolved in or transferred to a reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous NaOH solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 50 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00264] The oxidized rFVIIa is further purified by anion exchange chromatography on EMD TMAE (M) (Merck). The mixture is diluted with Buffer A (20 mM Hepes, 5 mM CaCl2, pH 6.5) to give a conductivity of 5 mS/cm. This solution is loaded onto the IEX column (bed height: 5.4 cm) with a column volume of 10 ml using a flow rate of 1.5 cm/min. This column is subsequently washed (flow rate: 1.5 cm/min) with 5 CV of a 92:8 mixture (w/w) of Buffer A and Buffer B (20 mM Hepes, 5 mM $CaCl_2$, 1.0 M NaCl, pH 7.0). Then the oxidized rFVIIa is eluted with a 50:50 (w/w) mixture of Buffer A and Buffer B followed by a postelution step with 5 CV of Buffer B. The elution steps are carried out by use of a flow rate of 1.0 cm/min.

[00265] Subsequently, the aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized rFVIIa within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00266] The obtained PEG-rFVIIa conjugate is purified by Hydrophobic Interaction Chromatography (HIC) using a Phenyl Sepharose FF low sub resin (GE Healthcare) packed into a column manufactured by GE Healthcare with a bed height (h) of 15 cm and a resulting column volume (CV) of 81 ml.

[00267] The reaction mixture is spiked with ammonium acetate by addition of 50 mM Hepes buffer, containing 350 mM sodium chloride, 8 M ammonium acetate, 5 mM calcium chloride, pH 6.9. Two volumes of the reaction mixture are mixed with 1 volume of the ammonium acetate containing buffer system and the pH value is corrected to pH 6.9 by drop wise addition of a 0.5 N aqueous NaOH solution. This mixture is loaded onto the HIC column using a flow rate of 1 cm/min followed by a washing step using > 3 CV equilibration buffer (50 mM Hepes, 350 mM sodium chloride, 2.5 M ammonium acetate, 5 mM calcium chloride, pH 6.9).

[00268] For removal of reaction by-products and anti-chaotropic salt a second washing step is performed with > 5CV washing buffer 1 (50 mM Hepes, 3 M sodium chloride, 5 mM calcium chloride, pH 6.9) in upflow mode at a flow rate of 2 cm/min. Then elution of purified rFVIIa conjugate is performed in down flow mode using a step gradient of 40 % washing buffer 2 (50 mM Hepes, 1.5 M sodium chloride, 5 mM calcium chloride, pH 6.9) and 60 % elution buffer (20 mM Hepes, 5 mM calcium chloride, pH 7.5) at a flow rate of 1 cm/min. The elution of the PEG-rFVIIa conjugate is monitored at UV 280 nm and the eluate containing the conjugate is collected within < 4 CV. The post elution step is performed with > 3 CV elution buffer under the same conditions to separate minor and/or non modified rFVIIa from the main product.

[00269] Finally, the purified conjugate is concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with a molecular weight cut off 10kD (Millipore).

[00270] The conjugate prepared by use of this procedure are analytically characterized by measuring total protein and biological activity according to methods known in the art.

Method 2:

[00271] rFVIIa is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of rFVIIa is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride,

pH 6.0) to get a final protein concentration of 2 mg rFVIIa / ml. Subsequently an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 µM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20-fold molar reagent excess. After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of a 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00272] The free rFVIIa is removed by means of ion exchange chromatography (IEC). The reaction mixture was diluted with appropriate amounts of Buffer A (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5) to correct the solutions conductivity and pH value prior to load onto a 20 ml HiPrep QFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column was eluted with Buffer B (50 mM Hepes, 1 M NaCl, 5 mM CaCl2, pH 7.5). Free rFVIIa was eluted by a step gradient using 25 % of Buffer B, which results in a conductivity between 12-25 mS/cm in the obtained fraction and the conjugate using a step gradient of 50 % Buffer B, which results in a conductivity between 27-45 mS/cm in the conjugate fraction. The conductivity of the conjugate containing fraction is subsequently raised with Buffer C (50 mM Hepes, 5 M NaCl, 5 mM $CaCl_2$, pH 6.9; by use of anti-chaotropic salts e.g. ammonium acetate) and loaded onto a 20 ml HiPrep Butyl FF 16/10 column (GE Healthcare, Fairfield, CT; or comparable HIC media) pre-equilibrated with Buffer D (50 mM Hepes, 3 M NaCl, 5 mM $CaCl_2$, pH 6.9). Free PEG-reagent was washed out within 5 CV Buffer D. Subsequently the conjugate was eluted with 100% Buffer E (50 mM Hepes, 5 mM $CaCl_2$, pH 7.4). The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD, Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00273] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods.

Example 17

Polysialylation of rFIX in the presence of o-amino benzoic acid

Method 1:

[00274] 8.2 mg rFIX is dissolved in 4.0 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). Then 82 µl of an aqueous sodium periodate solution (5 mM) is added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 4 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin 6 10 kD centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00275] The retentate (6.5 ml), containing oxidized rFIX, is mixed with 1.64 ml of an aqueous o-amino benzoic acid (50 mM) and incubated for 30 min at room temperature. Then aminooxy-PSA reagent with a MW of 20 kD (described above) is added to give a 5-fold molar reagent excess. This mixture was incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00276] The further purification of the conjugate is carried out as described herein.

Method 2:

[00277] A solution of 1 mg rFIX in 0.65 ml sodium phosphate buffer, pH 6.0 containing a 5-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) was prepared. Then 333 µl of an aqueous o-amino benzoic acid solution (30 mM) was added as nucleophilic catalyst to give a final concentration of 10 mM. Subsequently 20 µl of an aqueous solution of $NaIO_4$ (5 mM) was added yielding in a final concentration of 100 µM. The coupling process was performed for 2 hours in the dark under gentle shaking at room temperature and quenched for 15 min at room temperature by the addition of 1 µl of aqueous cysteine solution (1 M). The further purification of the conjugate is carried out as described herein.

Example 18

Polysialylation of EPO using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

Method 1:

[00278] A starting concentration of erythropoietin (EPO) is transferred into a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and

diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 µM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00279] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof or, in the alternative, to an IEX column with a volume of 20 ml (Merck EMD TMAE (M)) which is equilibrated with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 7.0). The column is equilibrated with 5 CV Buffer A. The oxidized EPO is eluted with Buffer B (20 mM Hepes, 5 mM $CaCl_2$, 1M NaCl, pH 7.0). The EPO containing fractions are collected. The protein content is determined (Coomassie, Bradford) and adjusted to 1 mg/ml with reaction buffer and adjusted to pH 6.0 by dropwise addition of 0.5M HCl.

[00280] A 50-fold molar excess of a aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy-PSA reagent is removed by means of HIC. The conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with 80 ml Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.9. Subsequently, the conjugate is eluted with 50 mM Hepes buffer pH 7.5 containing 5 mM $CaCl_2$. Finally the PSA-EPO containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (MWCO 10kD, 50cm^2, Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00281] In an alternative embodiment, Method 1 is carried out as follows.

[00282] 10 mg EPO is dissolved in 5 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl). 100 µl of an aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 50 µl of a 1 M aqueous cysteine solution. The

mixture is subsequently subjected to UF/DF employing Vivaspin 15R 10 kD centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00283] The retentate (approx. 7 ml), containing oxidized EPO, is mixed with 2 ml of an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Then aminooxy-PSA reagent with a MW of 20 kD (described above) is added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at RT in the dark under gentle stirring.

[00284] The free EPO is removed by means of anion exchange chromatography (AEC). The reaction mixture is diluted with 20 ml Buffer A (50 mM Hepes, pH 7.5) and loaded onto a 20 ml HiPrep QFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column is eluted with Buffer B (50 mM Hepes, 1 M NaCl, pH 7.5). Free EPO is eluted by washing the column with 25 % Buffer B and the conjugate at 50 % Buffer B. The conductivity of the conjugate containing fractions is subsequently raised to ~190 mS/cm with Buffer C (50 mM Hepes, 5 M NaCl, pH 6.9) and loaded onto a 20 ml HiPrep Butyl FF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer D (50 mM Hepes, 3 M NaCl, pH 6.9). Free PSA-reagent is washed out within 5 CV Buffer D. Subsequently, the conjugate is eluted with 100 % Buffer E (50 mM Hepes, pH 7.4). The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against histidine buffer, pH 7.2 containing 150 mM NaCl. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art. For the PSA-EPO conjugate a specific activity of > 50 % in comparison to native EPO is determined. The conjugate is additionally analytically characterized by Size Exclusion HPLC using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77). It is shown that the preparation contains no free EPO.

Method 2:

[00285] EPO is transferred or dissolved in reaction buffer (e.g. 50mM Hepes, 350mM sodium chloride, 5mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T = +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00286] The oxidized EPO is further purified by ion exchange chromatography. The oxidized EPO containing fractions of the eluate are collected and used for the conjugation reaction.

[00287] The aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized EPO within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. at pH 6.0 in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking (protein concentration: 1 mg/ml).

[00288] The obtained PSA-EPO conjugate is further purified by ion exchange chromatography. The PSA-EPO conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00289] The conjugate prepared by use of this procedureis analytically characterized by measuring total protein, biological activity, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Method 3:

[00290] Erythropoietin (EPO) is transferred into reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50 fold molar excess of a aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and NaIO4 (final concentration: 400 µM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently,

the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM). Then the conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, 0.01% Tween 80, pH 6.9. Subsequently, the conjugate is eluted with 50 mM Hepes, 5 mM calcium chloride, pH 7.5. Finally, the PSA-EPO containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (MWCO 10 kD, 88 cm^2, Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00291] In an alternative embodiment, Method 3 is carried out as follows. 10 mg EPO is dissolved in 8 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl). 200 µl of an aqueous sodium periodate solution (5 mM) and 2 ml of an aqueous m-toluidine solution (50 mM) are then added. Subsequently, the aminooxy-PSA reagent with a MW of 20 kD (described above) is added to give a 5-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 100 µl of 1 M aqueous cysteine solution.

[00292] The free EPO is removed by means of anion exchange chromatography (AEC). The reaction mixture is diluted with 20 ml Buffer A (50 mM Hepes, pH 7.5) and loaded onto a 20 ml HiPrep QFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column is eluted with Buffer B (50 mM Hepes, 1 M NaCl, pH 7.5). Free EPO is eluted by washing the column with 25 % Buffer B and the conjugate at 50 % Buffer B. The conductivity of the conjugate containing fractions is subsequently raised to ~190 mS/cm with Buffer C (50 mM Hepes, 5 M NaCl, pH 6.9) and loaded onto a 20 ml HiPrep Butyl FF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer D (50 mM Hepes, 3 M NaCl, pH 6.9). Free PSA-reagent is washed out within 5 CV Buffer D. Subsequently, the conjugate is eluted with 100% Buffer E (50 mM Hepes, pH 7.4). The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD, Millipore). The final diafiltration step is performed against histidine buffer, pH 7.2 containing 150 mM NaCl. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art. For the PSA-EPO conjugate a specific activity of >

50 % in comparison to native EPO is determined. The conjugate is additionally analytically characterized by Size Exclusion HPLC using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77). It is shown that the preparation contains no free EPO.

Method 4:

[00293] EPO is dissolved in or transferred to a reaction buffer (e.g. 50mM Hepes, 350mM sodium chloride, 5mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution.

[00294] Subsequently, the aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to this EPO solution within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. Finally a 40 mM aqueous sodium periodate solution is added to give a concentration of 400 µM.

[00295] The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking. Then the reaction is stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00296] The obtained PSA-EPO conjugate is purified by ion-exchange chromatography. The PSA-EPO containing fractions of the eluate are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose (MWCO 10 kD, 88 cm^2, Millipore).

[00297] The conjugates prepared by use of this procedure are analytically characterized by measuring total protein, biological activity according to methods known in the art, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Example 19

Polysialylation of Ang-2 using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

Method 1:

[00298] A starting concentration of angiopoietin-2 (Ang-2) is transferred into a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give

a final concentration of 200 μM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00299] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts, or, in the alternative, subjected to an IEX column with a volume of 20 ml (Merck EMD TMAE (M)) which is equilibrated with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 7.0). The column is equilibrated with 5 CV Buffer A. The oxidized Ang-2 is eluted with Buffer B (20 mM Hepes, 5 mM $CaCl_2$, 1 M NaCl, pH 7.0). The Ang-2 containing fractions are collected. The protein content is determined (Coomassie, Bradford) and adjusted to 1 mg/ml with reaction buffer and adjusted to pH 6.0 by dropwise addition of 0.5 M HCl.

[00300] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of HIC. The conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with 80 ml Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.9. Subsequently, the conjugate is eluted with 50 mM Hepes buffer pH 7.5 containing 5 mM $CaCl_2$. Finally, the PSA - Ang-2-containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00301] In an alternative embodiment, Method 1 is carried out as follows. Angiopoietin-2 (Ang-2) is transferred into a reaction buffer (e.g., 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 μM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at R.T.

[00302] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00303] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of ion exchange chromatography. The PSA-Ang-2 conjugate-containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00304] Ang-2 is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00305] The oxidized Ang-2 is further purified by ion exchange chromatography. The oxidized Ang-2 containing fractions of the eluate are collected and used for the conjugation reaction.

[00306] The aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized Ang-2 within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. at pH 6.0 in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking (protein concentration: 1 mg/ml).

[00307] The obtained PSA- Ang-2 conjugate is further purified by ion-exchange chromatographyn

[00308] The PSA-Ang-2 conjugate containing fractions are collected and concentrated by ultra- / diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00309] The conjugate prepared by use of this procedureis analytically characterized by measuring total protein, biological activity, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Method 3:

[00310] Angiopoietin-2 (Ang-2) is transferred into reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50 fold molar excess of a PSA aminooxy reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 μM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM). Then the conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, 0.01% Tween 80, pH 6.9. Subsequently, the conjugate is eluted with 50 mM Hepes, 5 mM calcium chloride, pH 7.5. Finally, the PSA Ang-2-containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00311] In an alternative embodiment, Method 3 is carried out as follows. Angiopoietin-2 (Ang-2) is transferred into reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of a PSA aminooxy reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 μM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM) and the conjugate is purified by

ion exchange chromatography. PSA Ang-2-containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00312] Ang-2 is dissolved in or transferred to a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution.

[00313] Subsequently, the aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to this Ang-2 solution within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. Finally a 40 mM aqueous sodium periodate solution is added to give a concentration of 400 µM.

[00314] The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking. Then the reaction is stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00315] The obtained PSA-Ang-2 conjugate is purified by ion-exchange chromatography. The PSA-Ang-2 containing fractions of the eluate are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose (Millipore).

[00316] The conjugates prepared by use of this procedure are analytically characterized by measuring total protein, biological activity according to methods known in the art, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Example 20

Polysialylation of VEGF using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

Method 1:

[00317] A starting concentration of vascular endothelial growth factor (VEGF) is transferred into a reaction buffer (e.g., 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 µM. The oxidation is carried at

RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00318] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof or, in the alternative, to an IEX column with a volume of 20 ml (Merck EMD TMAE (M)) which is equilibrated with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 7.0). The column is equilibrated with 5 CV Buffer A. The oxidized VEGF is eluted with Buffer B (20 mM Hepes, 5 mM $CaCl_2$, 1 M NaCl, pH 7.0). The VEGF containing fractions are collected. The protein content is determined (Coomassie, Bradford) and adjusted to 1 mg/ml with reaction buffer and adjusted to pH 6.0 by dropwise addition of 0.5M NaOH.

[00319] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of HIC. The conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with 80 ml Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.9. Subsequently, the conjugate is eluted with 50 mM Hepes buffer pH 7.5 containing 5 mM $CaCl_2$. Finally the PSA - VEGF - containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00320] In an alternative embodiment, Method 1 is carried out as follows. Vascular endothelial growth factor (VEGF) is transferred into a reaction buffer (e.g., 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 μM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00321] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00322] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of ion exchange chromatography. The PSA - VEGF -containing fractions of the eluate are collected and subjected to UF/DF by use of a a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00323] VEGF is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00324] The oxidized VEGF is further purified by ion exchange chromatography. The oxidized VEGF containing fractions of the eluate are collected and used for the conjugation reaction.

[00325] The aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized VEGF within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. at pH 6.0 in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking (protein concentration: 1 mg/ml).

[00326] The obtained PSA-VEGF conjugate is further purified by ion exchange chromatography. The PSA-VEGF conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00327] The conjugate prepared by use of this procedureis analytically characterized by measuring total protein, biological activity, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Method 3:

[00328] Vascular endothelial growth factor (VEGF) is transferred into reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of a PSA aminooxy reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 μM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM). Then the conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, 0.01% Tween 80, pH 6.9. Subsequently the conjugate is eluted with 50 mM Hepes, 5 mM calcium chloride, pH 7.5. Finally, the PSA-VEGF containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00329] In an alternative embodiment, Method 3 is carried out as follows. Vascular endothelial growth factor (VEGF) is transferred into reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 μM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM) and the conjugate is purified by ion exchange chromatography. The PSA-VEGF containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00330] VEGF is dissolved in or transferred to a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution.

[00331] Subsequently, the aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to this VEGF solution within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. Finally a 40 mM aqueous sodium periodate solution is added to give a concentration of 400 µM.

[00332] The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking. Then the reaction is stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00333] The obtained VEGF- conjugate is purified by ion-exchange chromatography. The PSA-VEGF containing fractions of the eluate are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose (Millipore).

[00334] The conjugates prepared by use of this procedure are analytically characterized by measuring total protein, biological activity according to methods known in the art, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Example 21

Polysialylation of EGF using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

Method 1:

[00335] A starting concentration of epidermal growth factor (EGF) is transferred into a reaction buffer (e.g., 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 µM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at R.T.

[00336] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof or, in the alternative, to an IEX column with a volume of 20 ml (Merck EMD TMAE (M)) which is equilibrated with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 7.0). The column is equilibrated with 5 CV Buffer A. The oxidized EGF is eluted with Buffer B (20 mM Hepes, 5 mM $CaCl_2$, 1M NaCl, pH 7.0). The EGF containing fractions are collected. The protein content is determined (Coomassie, Bradford) and adjusted to 1 mg/ml with reaction buffer and adjusted to pH 6.0 by dropwise addition of 0.5M HCl.

[00337] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of HIC. The conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with 80 ml Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.9. Subsequently, the conjugate is eluted with 50 mM Hepes buffer pH 7.5 containing 5 mM $CaCl_2$. Finally, the PSA-EGF containing fractions are collected and subjected to UF/DF by use of a a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00338] In an alternative embodiment, Method 1 is carried out as follows. Epidermal growth factor (EGF) is transferred into a reaction buffer (e.g., 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 µM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at R.T.

[00339] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00340] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of ion exchange chromatography. The PSA-EGF containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00341] EGF is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00342] The oxidized EGF is further purified by ion exchange chromatography. The oxidized EGF containing fractions of the eluate are collected and used for the conjugation reaction.

[00343] The aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized EGF within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. at pH 6.0 in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking (protein concentration: 1 mg/ml).

[00344] The obtained PSA-EGF conjugate is further purified by ion exchange chromatography. The PSA-EGF conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00345] The conjugate prepared by use of this procedureis analytically characterized by measuring total protein, biological activity, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Method 3:

[00346] Epidermal growth factor (EGF) is transferred into reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of a PSA aminooxy reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 μM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM). Then the conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, 0.01% Tween 80, pH 6.9. Subsequently the conjugate is eluted with 50 mM Hepes, 5 mM calcium chloride, pH 7.5. Finally the PSA-EGF containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00347] In an alternative embodiment, Method 3 is carried out as follows. Epidermal growth factor (EGF) is transferred into reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of a PSA aminooxy reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 μM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM) and the conjugate is purified by ion exchange chromatography. The conjugate containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00348] EGF is dissolved in or transferred to a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution.

[00349] Subsequently the aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to this EGF-solution within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. Finally a 40 mM aqueous sodium periodate solution is added to give a concentration of 400 µM.

[00350] The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking. Then the reaction is stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00351] The obtained EGF-conjugate is purified by ion-exchange chromatography. The PSA-EGF containing fractions of the eluate are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose (Millipore).

[00352] The conjugates prepared by use of this procedure are analytically characterized by measuring total protein, biological activity according to methods known in the art, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Example 22

Polysialylation of NGF using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

Method 1:

[00353] A starting concentration of nerve growth factor (NGF) is transferred into a reaction buffer (e.g., 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 µM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00354] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof or, in the alternative, to an IEX column with a volume of 20 ml (Merck EMD TMAE (M)) which is equilibrated with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 7.0). The column is equilibrated with 5 CV Buffer A. The oxidized NGF is eluted with Buffer B (20 mM Hepes, 5 mM $CaCl_2$, 1M NaCl, pH 7.0). The NGF containing fractions are collected. The protein content is determined (Coomassie, Bradford) and adjusted to 1 mg/ml with reaction buffer and adjusted to pH 6.0 by dropwise addition of 0.5M HCl.

[00355] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of HIC. The conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with 80 ml Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.9. Subsequently, the conjugate is eluted with 50 mM Hepes buffer pH 7.5 containing 5 mM $CaCl_2$. Finally, the PSA-NGF containing fractions are collected and subjected to UF/DF by use of a a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00356] In an alternative embodiment, Method 1 is carried out as follows. Nerve growth factor (NGF) is transferred into a reaction buffer (e.g., 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 μM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00357] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00358] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of ion exchange chromatography. The PSA-NGF containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00359] NGF is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00360] The oxidized NGF is further purified by ion exchange chromatography. The oxidized NGFcontaining fractions of the eluate are collected and used for the conjugation reaction.

[00361] The aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized NGF within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. at pH 6.0 in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking (protein concentration: 1 mg/ml).

[00362] The obtained PSA-NGF conjugate is further purified by ion exchange chromatography. The PSA-NGF conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00363] The conjugate prepared by use of this procedureis analytically characterized by measuring total protein, biological activity, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Method 3:

[00364] Nerve growth factor (NGF) is transferred into reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 µM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM). Then the conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, 0.01 % Tween 80, pH 6.9. Subsequently the conjugate is eluted with 50 mM Hepes, 5 mM calcium chloride, pH 7.5. Finally, the PSA NGF -containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00365] In an alternative embodiment, Method 3 is carried out as follows. Nerve growth factor (NGF) is transferred into reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 µM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM) and the conjugate is purified by ion exchange chromatography. Then the PSA-NGF containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00366] NGF is dissolved in or transferred to a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution.

[00367] Subsequently, the aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to this NGF-solution within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. Finally a 40 mM aqueous sodium periodate solution is added to give a concentration of 400 µM.

[00368] The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking. Then the reaction is stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00369] The obtained NGF- conjugate is purified by ion-exchange chromatography. The PSA-NGF containing fractions of the eluate are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose (Millipore).

[00370] The conjugates prepared by use of this procedure are analytically characterized by measuring total protein, biological activity according to methods known in the art, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Example 23

Polysialylation of HGH using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

Method 1:

[00371] As described herein, the amino acid sequence of human growth hormone (HGH) is first modified to incorporate at least one glycosylation site. Following purification, HGH is glycosylated in vitro according to methods known in the art.

[00372] A starting concentration of human growth hormone (HGH) is transferred into a reaction buffer (e.g., 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 µM. The oxidation is carried at RT for 30 min in

the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00373] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof or, in the alternative, to an IEX column with a volume of 20 ml (Merck EMD TMAE (M)) which is equilibrated with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 7.0). The column is equilibrated with 5 CV Buffer A. The oxidized HGH is eluted with Buffer B (20 mM Hepes, 5 mM $CaCl_2$, 1 M NaCl, pH 7.0). The HGH containing fractions are collected. The protein content is determined (Coomassie, Bradford) and adjusted to 1 mg/ml with reaction buffer and adjusted to pH 6.0 by dropwise addition of 0.5 M HCl.

[00374] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of HIC. The conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with 80 ml Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.9. Subsequently the conjugate is eluted with 50 mM Hepes buffer pH 7.5 containing 5 mM $CaCl_2$. Finally, the PSA-HGH containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00375] In an alternative embodiment, Method 1 is carried out as follows. As described herein, the amino acid sequence of human growth hormone (HGH) is first modified to incorporate at least one glycosylation site. Following purification, HGH is glycosylated in vitro according to methods known in the art. HGH is transferred into a reaction buffer (e.g., 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 μM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00376] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00377] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of ion exchange chromatography. The PSA-HGH containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00378] As described herein, the amino acid sequence of human growth hormone (HGH) is first modified to incorporate at least one glycosylation site. Following purification, HGH is glycosylated in vitro according to methods known in the art.

[00379] HGH is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00380] The oxidized HGH is further purified by ion exchange chromatography. The oxidized HGH containing fractions of the eluate are collected and used for the conjugation reaction.

[00381] The aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized HGH within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. at pH 6.0 in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking (protein concentration: 1 mg/ml).

[00382] The obtained PSA-HGH conjugate is further purified by ion exchange chromatography. The PSA-HGH conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00383] The conjugate prepared by use of this procedureis analytically characterized by measuring total protein, biological activity, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Method 3:

[00384] As described herein, the amino acid sequence of human growth hormone (HGH) is first modified to incorporate at least one glycosylation site. Following purification, HGH is glycosylated in vitro according to methods known in the art.

[00385] Human growth hormone (HGH) is transferred into reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50 fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 μM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM). Then the conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, 0.01% Tween 80, pH 6.9. Subsequently the conjugate is eluted with 50 mM Hepes, 5 mM calcium chloride, pH 7.5. Finally, the PSA HGH -containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00386] In an alternative embodiment, Method 3 is carried out as follows. As described herein, the amino acid sequence of human growth hormone (HGH) is first modified to incorporate at least one glycosylation site. Following purification, HGH is glycosylated in vitro according to methods known in the art. HGH is transferred into reaction buffer (e.g. 50

mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50 fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 µM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM) and the conjugate is purified by ion exchange chromatography. Then the PSA -HGH -containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00387] As described herein, the amino acid sequence of human growth hormone (HGH) is first modified to incorporate at least one glycosylation site. Following purification, HGH is glycosylated in vitro according to methods known in the art.

[00388] HGH is dissolved in or transferred to a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution.

[00389] Subsequently, the aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to this HGH- solution within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. Finally a 40 mM aqueous sodium periodate solution is added to give a concentration of 400 µM.

[00390] The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking. Then the reaction is stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00391] The obtained HGH- conjugate is purified by ion-exchange chromatography. The PSA-HGH containing fractions of the eluate are collected and concentrated by ultra- / diafiltration (UF/DF) using a membrane made of regenerated cellulose (Millipore).

[00392] The conjugates prepared by use of this procedure are analytically characterized by measuring total protein, biological activity according to methods known in the art, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Example 24

Polysialylation of TNF-alpha using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

[00393] A starting concentration of tumor necrosis factor-alpha (TNF-alpha) is transferred into a reaction buffer (e.g., 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 μM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00394] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof or, in the alternative, to an IEX column with a volume of 20 ml (Merck EMD TMAE (M)) which is equilibrated with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 7.0). The column is equilibrated with 5 CV Buffer A. The oxidized TNF-alpha is eluted with Buffer B (20 mM Hepes, 5 mM $CaCl_2$, 1M NaCl, pH 7.0). The TNF-alpha containing fractions are collected. The protein content is determined (Coomassie, Bradford) and adjusted to 1 mg/ml with reaction buffer and adjusted to pH 6.0 by dropwise addition of 0.5 M HCl.

[00395] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of HIC. The conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with 80 ml Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.9. Subsequently, the conjugate is eluted with 50 mM Hepes buffer pH 7.5 containing 5 mM $CaCl_2$. Finally the PSA- TNF-alpha -containing fractions are collected and subjected to UF/DF by use of a a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized

by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00396] In an alternative embodiment, Method 1 is carried out as follows. Tumor necrosis factor-alpha (TNF-alpha) is transferred into a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 µM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00397] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of ion exchange chromatography. The PSA-TNF-alpha containing fractions of the eluate are collected and subjected to UF/DF by use of a a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00398] TNF-alpha is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00399] The oxidized TNF-alpha is further purified by ion exchange chromatography. The oxidized TNF-alpha containing fractions of the eluate are collected and used for the conjugation reaction.

[00400] The aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized TNF-alpha within a maximum time

period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. at pH 6.0 in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking (protein concentration: 1 mg/ml).

[00401] The obtained PSA-TNF-alpha conjugate is further purified by ion exchange chromatography. The PSA-TNF-alpha conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00402] The conjugate prepared by use of this procedureis analytically characterized by measuring total protein, biological activity, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Method 3:

[00403] Tumor necrosis factor-alpha (TNF-alpha) is transferred into reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 µM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM). Then the conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, 0.01 % Tween 80, pH 6.9. Subsequently the conjugate is eluted with 50 mM Hepes, 5 mM calcium chloride, pH 7.5. Finally the PSA-TNF-alpha -containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00404] In an alternative embodiment, Method 3 is carried out as follows. Tumor necrosis factor-alpha (TNF-alpha) is transferred into reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein

concentration of 1 mg/ml. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 μM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM) .and the conjugate is purified by ion exchange chromatography. The PSA-TNF-alpha containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00405] TNF-alpha is dissolved in or transferred to a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution.

[00406] Subsequently the aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to this TNF-alpha-solution within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. Finally a 40 mM aqueous sodium periodate solution is added to give a concentration of 400 μM.

[00407] The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking. Then the reaction is stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00408] The obtained TNF-alpha conjugate is purified by ion-exchange chromatography. The PSA-TNF-alpha containing fractions of the eluate are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose (Millipore).

[00409] The conjugates prepared by use of this procedure are analytically characterized by measuring total protein, biological activity according to methods known in the art, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Example 25

Polysialylation of insulin using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

Method 1:

[00410] As described herein, the amino acid sequence of insulin is first modified to incorporate at least one glycosylation site. Following purification, insulin is glycosylated in vitro according to methods known in the art. A starting concentration of insulin is transferred into a reaction buffer (e.g., 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 μM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00411] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof or, in the alternative, to an IEX column with a volume of 20 ml (Merck EMD TMAE (M)) which is equilibrated with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 7.0). The column is equilibrated with 5 CV Buffer A. The oxidized insulin is eluted with Buffer B (20 mM Hepes, 5 mM $CaCl_2$, 1 M NaCl, pH 7.0). The insulin containing fractions are collected. The protein content is determined (Coomassie, Bradford) and adjusted to 1 mg/ml with reaction buffer and adjusted to pH 6.0 by dropwise addition of 0.5 M HCl.

[00412] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of HIC. The conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with 80 ml Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.9. Subsequently the conjugate is eluted with 50 mM Hepes buffer pH 7.5 containing 5 mM $CaCl_2$. Finally the PSA-insulin containing fractions are collected and subjected to UF/DF by use of a a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00413] In an alternative embodiment, Method 1 is carried out as follows. As described herein, the amino acid sequence of insulin is first modified to incorporate at least one glycosylation site. Following purification, insulin is glycosylated in vitro according to methods known in the art. Insulin is transferred into a reaction buffer (e.g., 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 μM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00414] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00415] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of ion exchange chromatography. The PSA-insulin containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00416] As described herein, the amino acid sequence of insulin is first modified to incorporate at least one glycosylation site. Following purification, insulin is glycosylated in vitro according to methods known in the art.

[00417] Insulin is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350mM sodium chloride, 5mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 μM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00418] The oxidized insulin is further purified by ion exchange chromatography. The oxidized insulin containing fractions of the eluate are collected and used for the conjugation reaction.

[00419] The aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized insulin within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. at pH 6.0 in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking (protein concentration: 1 mg/ml).

[00420] The obtained PSA-insulin conjugate is further purified by ion exchange chromatography. The PSA-insulin conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00421] The conjugate prepared by use of this procedureis analytically characterized by measuring total protein, biological activity, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Method 3:

[00422] As described herein, the amino acid sequence of insulin is first modified to incorporate at least one glycosylation site. Following purification, insulin is glycosylated in vitro according to methods known in the art.

[00423] Insulin is transferred into reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 µM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM). Then the conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, 0.01 % Tween 80, pH 6.9. Subsequently the

conjugate is eluted with 50 mM Hepes, 5 mM calcium chloride, pH 7.5. Finally, the PSA-insulin containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00424] In an alternative embodiment, Method 3 is carried out as follows. As described herein, the amino acid sequence of insulin is first modified to incorporate at least one glycosylation site. Following purification, insulin is glycosylated in vitro according to methods known in the art.

[00425] Insulin is transferred into reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 µM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM) and the conjugate is purified by ion exchange chromatography. PSA-insulin containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00426] As described herein, the amino acid sequence of insulin is first modified to incorporate at least one glycosylation site. Following purification, insulin is glycosylated in vitro according to methods known in the art.

[00427] Insulin is dissolved in or transferred to a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution.

[00428] Subsequently, the aminooxy-polysialic acid (PSA-ONH2) reagent is added in a 50-fold molar excess to this insulin-solution within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. Finally a 40 mM aqueous sodium periodate solution is added to give a concentration of 400 µM.

[00429] The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking. Then the reaction is stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00430] The obtained insulin conjugate is purified by ion-exchange chromatography. The PSA- insulin containing fractions of the eluate are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose (Millipore).

[00431] The conjugates prepared by use of this procedure are analytically characterized by measuring total protein, biological activity according to methods known in the art, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Example 26

Polysialylation of interferon-alpha using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

Method 1:

[00432] A starting concentration of interferon-alpha is transferred into a reaction buffer (e.g., 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 µM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00433] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof or, in the alternative, to an IEX column with a volume of 20 ml (Merck EMD TMAE (M)) which is equilibrated with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 7.0). The column is equilibrated with 5 CV Buffer A. The oxidized interferon-alpha is eluted with Buffer B (20 mM Hepes, 5 mM $CaCl_2$, 1M NaCl, pH 7.0). The interferon-alpha containing fractions are collected. The

protein content is determined (Coomassie, Bradford) and adjusted to 1 mg/ml with reaction buffer and adjusted to pH 6.0 by dropwise addition of 0.5 M HCl.

[00434] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of HIC. The conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with 80 ml Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.9. Subsequently, the conjugate is eluted with 50 mM Hepes buffer pH 7.5 containing 5 mM $CaCl_2$. Finally the PSA-interferon-alpha containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00435] In an alternative embodiment, Method 1 is carried out as follows. Interferon-alpha is transferred into a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 μM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00436] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00437] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of ion-exchange chromatography. The PSA-interferon-alpha containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00438] Interferon-alpha is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 μM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00439] The oxidized interferon-alpha is further purified by ion exchange chromatography. The oxidized interferon-alpha containing fractions of the eluate are collected and used for the conjugation reaction.

[00440] The aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized interferon-gamma within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. at pH 6.0 in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking (protein concentration: 1 mg/ml).

[00441] The obtained PSA-interferon-alpha conjugate is further purified by ion exchange chromatography. The PSA-interferon-alpha conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

Method 3:

[00442] Interferon-alpha is transferred into reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of a PSA aminooxy reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 μM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM). Then the conductivity of the reaction mixture is adjusted by adding a buffer containing

ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, 0.01 % Tween 80, pH 6.9. Subsequently the conjugate is eluted with 50 mM Hepes, 5 mM calcium chloride, pH 7.5. Finally, the PSA-interferon-alpha containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00443] In an alternative embodiment, Method 3 is carried out as follows. Interferon-alpha is transferred into reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 µM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM) and the conjugate is purified by ion exchange chromatography. The PSA-interferon-alpha containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00444] Interferon-alpha is dissolved in or transferred to a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution.

[00445] Subsequently, the aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to this interferon-alpha solution within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. Finally, a 40 mM aqueous sodium periodate solution is added to give a concentration of 400 µM.

[00446] The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking. Then the reaction is stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00447] The obtained interferon-alpha conjugate is purified by ion-exchange chromatography. The PSA-interferon-alpha containing fractions of the eluate are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose (Millipore).

[00448] The conjugates prepared by use of this procedure are analytically characterized by measuring total protein, biological activity according to methods known in the art, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Example 27

Polysialylation of Interferon-gamma using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

Method 1:

[00449] 10 mg interferon-gamma is dissolved in 5 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl). 100 µl of an aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 50 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin 15R 10 kD centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00450] The retentate (approx. 7 ml), containing oxidized interferon-gamma, is mixed with 2 ml of an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Then aminooxy-PSA reagent with a MW of 20 kD (described above) is added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at RT in the dark under gentle stirring.

[00451] The free Interferon-gamma is removed by means of cation exchange chromatography (CEC). The reaction mixture is diluted with 20 ml Buffer A (50 mM Hepes, pH 6.5) and loaded onto a 20 ml HiPrep SPFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column is eluted with Buffer B (50 mM Hepes, 1 M NaCl, pH 6.5). Free interferon-gamma is eluted by washing the column with 25 % Buffer B

and the conjugate at 50 % Buffer B. The conductivity of the conjugate containing fractions is subsequently raised to ~190 mS/cm with Buffer C (50 mM Hepes, 5 M NaCl, pH 6.9) and loaded onto a 20 ml HiPrep Butyl FF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer D (50 mM Hepes, 3 M NaCl, pH 6.9). Free PSA-reagent is washed out within 5 CV Buffer D. Subsequently, the conjugate is eluted with 100 % Buffer E (50 mM Hepes, pH 6.9). The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD, Millipore). The final diafiltration step is performed against histidine buffer, pH 6.9 containing 150 mM NaCl. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art. For the PSA-Interferon-gamma conjugate a specific activity of > 50 % in comparison to native Interferon-gamma is determined. The conjugate is additionally analytically characterized by Size Exclusion HPLC using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77). It is shown that the preparation contains no free Interferon gamma.

Method 2:

[00452] 10 mg interferon-gamma is dissolved in 8 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl). 200 µl of an aqueous sodium periodate solution (5 mM) and 2 ml of an aqueous m-toluidine solution (50 mM) are then added. Subsequently the aminooxy-PSA reagent with a MW of 20 kD (described above) is added to give a 5-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 100 µl of 1 M aqueous cysteine solution.

[00453] The free interferon gamma is removed by means of cation exchange chromatography (CEC). The reaction mixture is diluted with 20 ml Buffer A (50 mM Hepes, pH 6.5) and loaded onto a 20 ml HiPrep SPFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column is eluted with Buffer B (50 mM Hepes, 1 M NaCl, pH 6.5). Free interferon-gamma is eluted by washing the column with 25 % Buffer B and the conjugate at 50 % Buffer B. The conductivity of the conjugate containing fractions is subsequently raised to ~190 mS/cm with Buffer C (50 mM Hepes, 5 M NaCl, pH 6.9) and loaded onto a 20 ml HiPrep Butyl FF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer D (50 mM Hepes, 3 M NaCl, pH 6.9). Free PSA-reagent is washed out within 5 CV Buffer D. Subsequently, the conjugate is eluted with 100 % Buffer E (50

mM Hepes, pH 6.9). The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against histidine buffer, pH 6.9 containing 150 mM NaCl. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art. For the PSAinterferon-gamma conjugate a specific activity of > 50 % in comparison to native interferon-gamma is determined. The conjugate is additionally analytically characterized by Size Exclusion HPLC using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77). It is shown that the preparation contains no free interferon-gamma.

Method 3:

[00454] 10 mg interferon-gamma is dissolved in 8 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl). 200 µl of an aqueous sodium periodate solution (5 mM) and 2 ml of an aqueous m-toluidine solution (50 mM) are then added. Subsequently the aminooxy-PSA reagent with a MW of 20 kD (described above) is added to give a 5-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 100 µl of 1 M aqueous cysteine solution.

[00455] The free interferon gamma is removed by means of cation exchange chromatography (CEC). The reaction mixture is diluted with 20 ml Buffer A (50 mM Hepes, pH 6.5) and loaded onto a 20 ml HiPrep SPFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column is eluted with Buffer B (50 mM Hepes, 1 M NaCl, pH 6.5). Free interferon-gamma is eluted by washing the column with 25 % Buffer B and the conjugate at 50 % Buffer B. The conductivity of the conjugate containing fractions is subsequently raised to ~190 mS/cm with Buffer C (50 mM Hepes, 5 M NaCl, pH 6.9) and loaded onto a 20 ml HiPrep Butyl FF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer D (50 mM Hepes, 3 M NaCl, pH 6.9). Free PSA-reagent is washed out within 5 CV Buffer D. Subsequently the conjugate is eluted with 100% Buffer E (50 mM Hepes, pH 6.9). The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against histidine buffer, pH 6.9 containing 150 mM NaCl. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art. For the PSAinterferon-gamma conjugate a

specific activity of > 50 % in comparison to native interferon-gamma is determined. The conjugate is additionally analytically characterized by Size Exclusion HPLC using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77). It is shown that the preparation contains no free interferon-gamma.

Method 4:

[00456] Interferon-gamma is dissolved in or transferred to a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution.

[00457] Subsequently, the aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to this interferon-gamma solution within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. Finally, a 40 mM aqueous sodium periodate solution is added to give a concentration of 400 µM.

[00458] The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking. Then the reaction is stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00459] The obtained interferon-gamma conjugate is purified by ion-exchange chromatography. The PSA-interferon-gamma containing fractions of the eluate are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose (Millipore).

[00460] The conjugates prepared by use of this procedure are analytically characterized by measuring total protein, biological activity according to methods known in the art, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Example 28

Polysialylation of G-CSF using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

Method 1:

[00461] A starting concentration of granulocyte-colony stimulating factor (G-CSF) is transferred into a reaction buffer (e.g., 50 mM Hepes, 350 mM sodium chloride, 5 mM

calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 µM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00462] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof or, in the alternative, to an IEX column with a volume of 20 ml (Merck EMD TMAE (M)) which is equilibrated with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 7.0). The column is equilibrated with 5 CV Buffer A. The oxidized G-CSF is eluted with Buffer B (20 mM Hepes, 5 mM $CaCl_2$, 1 M NaCl, pH 7.0). The G-CSF containing fractions are collected. The protein content is determined (Coomassie, Bradford) and adjusted to 1 mg/ml with reaction buffer and adjusted to pH 6.0 by dropwise addition of 0.5 M HCl.

[00463] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of HIC. The conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with 80 ml Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.9. Subsequently, the conjugate is eluted with 50 mM Hepes buffer pH 7.5 containing 5 mM $CaCl_2$. Finally the PSA- G-CSF - containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00464] In an alternative embodiment, Method 1 is carried out as follows. Granulocyte-colony stimulating factor (G-CSF) is transferred into a reaction buffer (e.g., 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 µM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00465] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of ion exchange chromatography. The PSA-G-CSF containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00466] G-CSF is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00467] The oxidized G-CSF is further purified by ion exchange chromatography. The oxidized G-CSF containing fractions of the eluate are collected and used for the conjugation reaction.

[00468] The aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized G-CSF within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. at pH 6.0 in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking (protein concentration: 1 mg/ml).

[00469] The obtained PSA-G-CSF conjugate is further purified by ion exchange chromatography. The PSA-G-CSF conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00470] The conjugate prepared by use of this procedureis analytically characterized by measuring total protein, biological activity, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Method 3:

[00471] Granulocyte-colony stimulating factor (G-CSF) is transferred into reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 μM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM). Then the conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, 0.01 % Tween 80, pH 6.9. Subsequently the conjugate is eluted with 50 mM Hepes, 5 mM calcium chloride, pH 7.5. Finally, the PSA-G-CSF -containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00472] In an alternative embodiment, Method 3 is carried out as follows. Granulocyte-colony stimulating factor (G-CSF) is transferred into reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 μM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM) and the conjugate is purified by ion exchange chromatography. The PSA-G-CSF containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00473] G-CSF is dissolved in or transferred to a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution.

[00474] Subsequently, the aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to this G-CSF solution within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. Finally, a 40 mM aqueous sodium periodate solution is added to give a concentration of 400 µM.

[00475] The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking. Then the reaction is stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00476] The obtained G-CSF conjugate is purified by ion-exchange chromatography. The PSA-G-CSF containing fractions of the eluate are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose (Millipore).

[00477] The conjugates prepared by use of this procedure are analytically characterized by measuring total protein, biological activity according to methods known in the art, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Example 29

Polysialylation of Humira using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

Method 1:

[00478] A starting concentration of Humira is transferred into a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 µM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00479] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof or, in the alternative, to an

IEX column with a volume of 20 ml (Merck EMD TMAE (M)) which is equilibrated with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 7.0). The column is equilibrated with 5 CV Buffer A. The oxidized Humira is eluted with Buffer B (20 mM Hepes, 5 mM $CaCl_2$, 1M NaCl, pH 7.0). The Humira containing fractions are collected. The protein content is determined (Coomassie, Bradford) and adjusted to 1 mg/ml with reaction buffer and adjusted to pH 6.0 by dropwise addition of 0.5M HCl.

[00480] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of HIC. The conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with 80 ml Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.9. Subsequently the conjugate is eluted with 50 mM Hepes buffer pH 7.5 containing 5 mM $CaCl_2$. Finally, the PSA-Humira containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00481] In an alternative embodiment, Method 1 is carried out as follows. Humira is transferred into a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 µM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00482] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of ion exchange chromatographyThe PSA-Humira containing fractions of the elutae are collected and subjected to UF/DF by use of a a

membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00483] Humira is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00484] The oxidized Humira is further purified by ion exchange chromatography. The oxidized Humira containing fractions of the eluate are collected and used for the conjugation reaction.

[00485] The aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized Humira within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. at pH 6.0 in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking (protein concentration: 1 mg/ml).

[00486] The obtained PSA-Humira conjugate is further purified by ion exchange chromatography. The PSA-Humira conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00487] The conjugate prepared by use of this procedureis analytically characterized by measuring total protein, biological activity, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Method 3:

[00488] Humira is transferred into reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 µM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM). Then the conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, 0.01 % Tween 80, pH 6.9. Subsequently the conjugate is eluted with 50 mM Hepes, 5 mM calcium chloride, pH 7.5. Finally the PSA-Humira containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00489] In an alternative embodiment, Method 3 is carried out as follows. Humira is transferred into reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 µM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM) and the conjugate is purified by ion exchange chromatography. The PSA-Humira containing fractions of the eluate are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose (Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00490] Humira is dissolved in or transferred to a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution.

[00491] Subsequently, the aminooxy-polysialic acid (PSA- ONH_2) reagent is added in a 50-fold molar excess to this Humira solution within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. Finally a 40 mM aqueous sodium periodate solution is added to give a concentration of 400 µM.

[00492] The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking. Then the reaction is stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00493] The obtained Humira-conjugate is purified by ion-exchange chromatography. The PSA- Humira containing fractions of the eluate are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose (Millipore).

[00494] The conjugates prepared by use of this procedure are analytically characterized by measuring total protein, biological activity according to methods known in the art, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Example 30

Polysialylation of Prolia using aminooxy-PSA and m-toluidine as a nucleophilic catalyst

Method 1:

[00495] A starting concentration of Prolia is transferred into a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. To this solution, $NaIO_4$ is added to give a final concentration of 200 µM. The oxidation is carried at RT for 30 min in the dark under gentle shaking. The reaction is then quenched with cysteine (final concentration: 10 mM) for 60 min at RT.

[00496] The solution is next subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof or, in the alternative, to an IEX column with a volume of 20 ml (Merck EMD TMAE (M)) which is equilibrated with Buffer A (20 mM Hepes, 5 mM $CaCl_2$, pH 7.0). The column is equilibrated with 5 CV Buffer A. The oxidized Prolia is eluted with Buffer B (20 mM Hepes, 5 mM $CaCl_2$, 1M NaCl, pH 7.0). The Prolia containing fractions are collected. The protein content is determined (Coomassie, Bradford) and adjusted to 1 mg/ml with reaction buffer and adjusted to pH 6.0 by dropwise addition of 0.5 M HCl.

[00497] A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (final concentration: 10 mM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. The excess of aminooxy reagent is removed by means of HIC. The conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with 80 ml Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.9. Subsequently the conjugate is eluted with 50 mM Hepes buffer pH 7.5 containing 5 mM $CaCl_2$. Finally, the PSA-Prolia containing fractions are collected and subjected to UF/DF by use of a a membrane made of regenerated cellulose (Millipore). The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00498] In an alternative embodiment, Method 1 is carried out as follows. 10 mg Prolia is dissolved in 5 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl). 100 µl of an aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 50 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin 15R 10 kD centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00499] The retentate (approx. 7 ml), containing oxidized Prolia, is mixed with 2 ml of an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Then aminooxy-PSA reagent with a MW of 20 kD (described above) is added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at RT in the dark under gentle stirring.

[00500] The free Prolia is removed by means of cation exchange chromatography (CEC). The reaction mixture is diluted with 20 ml Buffer A (50 mM Hepes, pH 6.5) and loaded onto a 20 ml HiPrep SPFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column is eluted with Buffer B (50 mM Hepes, 1 M NaCl, pH 6.5). Free Prolia is eluted by washing the column with 25 % Buffer B and the conjugate at 50 % Buffer B. The conductivity of the conjugate containing fractions is subsequently raised to ~190 mS/cm with Buffer C (50 mM Hepes, 5 M NaCl, pH 6.9) and loaded onto a 20 ml HiPrep Butyl FF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer D (50 mM Hepes, 3 M NaCl, pH 6.9). Free PSA-reagent is washed out within 5 CV Buffer D. Subsequently, the conjugate is eluted with 100 % Buffer E (50 mM Hepes, pH 6.9). The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD, Millipore). The final diafiltration step is performed against histidine buffer, pH 6.9 containing 150 mM NaCl. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art. For the PSA-Prolia conjugate a specific activity of > 50 % in comparison to native Prolia is determined. The conjugate is additionally analytically characterized by Size Exclusion HPLC using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77). It is shown that the preparation contains no free Prolia.

Method 2:

[00501] Prolia is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00502] The oxidized Prolia is further purified by ion exchange chromatography. The oxidized Prolia containing fractions of the eluate are collected and used for the conjugation reaction.

[00503] The aminooxy-polysialic acid (PSA- ONH_2) reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized Prolia within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. at pH 6.0 in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking (protein concentration: 1 mg/ml).

[00504] The obtained Prolia conjugate is further purified by ion exchange chromatography. The Prolia conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00505] The conjugate prepared by use of this procedureis analytically characterized by measuring total protein, biological activity, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Method 3:

[00506] Prolia is transferred into reaction buffer (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and diluted to obtain a protein concentration of 1 mg/ml. A 50-fold molar excess of aminooxy-PSA reagent with a MW of 20 kD (described above) is added followed by m-toluidine as a nucleophilic catalyst (10 mM final concentration) and $NaIO_4$ (final concentration: 400 µM). The coupling reaction is performed for 2 hours in the dark under gentle shaking at room temperature. Subsequently, the reaction is quenched with cysteine for 60 min at RT (cysteine concentration: 10 mM). Then the conductivity of the reaction mixture is adjusted by adding a buffer containing ammonium acetate (50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, 8 M ammonium acetate, pH 6.9) and loaded onto a column filled with Phenyl Sepharose FF (GE Healthcare, Fairfield, CT) pre-equilibrated with 50 mM Hepes, 2.5 M ammonium acetate, 350 mM sodium chloride, 5 mM calcium chloride, 0.01 % Tween 80, pH 6.9. Subsequently the conjugate is eluted with 50 mM Hepes, 5 mM calcium chloride, pH 7.5. Finally the PSA Prolia -containing fractions are collected and subjected to UF/DF by use of a membrane made of regenerated cellulose

(Millipore). The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00507] In an alternative embodiment, Method 3 is carried out as follows. 10 mg Prolia is dissolved in 8 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl). 200 µl of an aqueous sodium periodate solution (5 mM) and 2 ml of an aqueous m-toluidine solution (50 mM) are then added. Subsequently the aminooxy-PSA reagent with a MW of 20 kD (described above) is added to give a 5 fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 100 µl of 1 M aqueous cysteine solution.

[00508] The free Prolia is removed by means of cation exchange chromatography (CEC). The reaction mixture is diluted with 20 ml Buffer A (50 mM Hepes, pH 6.5) and loaded onto a 20 ml HiPrep SPFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column is eluted with Buffer B (50 mM Hepes, 1 M NaCl, pH 6.5). Free Prolia is eluted by washing the column with 25 % Buffer B and the conjugate at 5 0% Buffer B. The conductivity of the conjugate containing fractions is subsequently raised to ~190 mS/cm with Buffer C (50 mM Hepes, 5 M NaCl, pH 6.9) and loaded onto a 20 ml HiPrep Butyl FF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer D (50 mM Hepes, 3 M NaCl, pH 6.9). Free PSA-reagent is washed out within 5 CV Buffer D. Subsequently the conjugate is eluted with 100% Buffer E (50 mM Hepes, pH 6.9). The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm^2, cut-off 10 kD / Millipore). The final diafiltration step is performed against histidine buffer, pH 6.9 containing 150 mM NaCl. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art. For the PSA-Prolia conjugate a specific activity of > 50 % in comparison to native Prolia is determined. The conjugate is additionally analytically characterized by Size Exclusion HPLC using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77). It is shown that the preparation contains no free Prolia.

Method 4:

[00509] **Prolia** is dissolved in or transferred to a reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution.

[00510] Subsequently the aminooxy-polysialic acid (PSA-ONH_2) reagent is added in a 50-fold molar excess to this Prolia - solution within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. Finally a 40 mM aqueous sodium periodate solution is added to give a concentration of 400 µM.

[00511] The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking. Then the reaction is stopped by the addition of an aqueous L-cysteine solution (1 M) to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00512] The obtained Prolia conjugate is purified by ion-exchange chromatography. The PSA- Prolia containing fractions of the eluate are collected and concentrated by ultra- / diafiltration (UF/DF) using a membrane made of regenerated cellulose (Millipore).

[00513] The conjugates prepared by use of this procedure are analytically characterized by measuring total protein, biological activity according to methods known in the art, and determination of the polysialyation degree by measuring the PSA content (resorcinol assay).

Example 31

Polysialylation of other therapeutic proteins

[00514] Polysialylation reactions performed in the presence of alternative nucleophilic catalysts like m-toluidine or o-aminobenzoic acid as described herein may be extended to other therapeutic proteins. For example, in various aspects of the invention, the above polysialylation or PEGylation reactions as described herein with PSA aminooxy or PEG aminooxy reagents is repeated with therapeutic proteins such as those proteins described herein.

Example 32

PEGylation of EPO using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00515] Erythropoietin (EPO) is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). EPO is dissolved in 7.0 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00516] The retentate containing oxidized EPO is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00517] Finally, the PEG-EPO conjugate is purified by ion-exchange chromatography (e.g. on Q Sepharose FF). For example, 1.5 mg protein/ml gel is loaded on the column equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00518] In an alternative embodiment, Method 1 is carried out as follows. EPO is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). 10 mg EPO is dissolved in 5 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl). 100 µl of an aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 50 µl of a 1 M aqueous cysteine solution. The

mixture is subsequently subjected to UF/DF employing Vivaspin 15R 10 kD centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00519] The retentate (approx. 7 ml), containing oxidized EPO, is mixed with 2 ml of an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Then aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at RT in the dark under gentle stirring.

[00520] Finally, the PEG-EPO conjugate is purified by ion-exchange chromatography on Q Sepharose FF. The reaction mixture is diluted with 20 ml Buffer A (50 mM Hepes, pH 7.5) and loaded onto a 20 ml HiPrep QFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column is eluted with Buffer B (50 mM Hepes, 1 M NaCl, pH 7.5). Free EPO is eluted by washing the column with 25 % Buffer B and the conjugate at 50 % Buffer B. The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against histidine buffer, pH 7.2 containing 150 mM NaCl. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity biological activity according to methods known in the art.. For the PEG-EPO conjugate a specific activity of > 50 % in comparison to native EPO is determined. The conjugate is additionally analytically characterized by Size Exclusion HPLC using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77). It is shown that the preparation contains no free EPO.

Method 2:

[00521] EPO is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan).

[00522] EPO is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by

addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00523] The oxidized EPO is further purified by ion exchange chromatography. The oxidized EPO containing fractions of the eluate are collected and used for the conjugation reaction.

[00524] The aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized EPO within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00525] The obtained PEG- EPO conjugate is further purified by ion exchange chromatography. The PEG-EPO conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00526] The conjugate prepared by use of this procedure are analytically characterized by measuring total protein and biological activity according to methods known in the art.

Method 3:

[00527] EPO is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). EPO is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently, the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00528] Finally, the PEG-EPO conjugate is purified by ion-exchange chromatography on Q Sepharose FF. 1.5 mg protein/ml gel is loaded on the column pre equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using a membrane. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00529] In an alternative embodiment, Method 3 is carried out as follows. EPO is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). 10 mg EPO is dissolved in ~8 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl). 200 µl of an aqueous sodium periodate solution (5 mM) and 2 ml of an aqueous m-toluidine solution (50 mM) are then added. Subsequently, the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 5-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 100 µl of 1 M aqueous cysteine solution.

[00530] Finally, the PEG-EPO conjugate is purified by ion-exchange chromatography on Q Sepharose FF. The reaction mixture is diluted with 20 ml Buffer A (50 mM Hepes, pH 7.5) and loaded onto a 20 ml HiPrep QFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column is eluted with Buffer B (50 mM Hepes, 1 M NaCl, pH 7.5). Free EPO is eluted by washing the column with 25 % Buffer B and the conjugate at 50 % Buffer B. The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against histidine buffer, pH 7.2 containing 150 mM NaCl. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art. For the PEG-EPO conjugate a specific activity of > 50 % in comparison to native EPO is determined. The conjugate is additionally analytically characterized by Size Exclusion HPLC using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77). It is shown that the preparation contains no free EPO.

Method 4:

[00531] EPO is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of EPO is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 2 mg EPO / ml. Subsequently an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 µM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final

concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20-fold molar reagent excess. After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of a 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00532] The PEG-EPO conjugate is purified by means of ion exchange chromatography (IEC). The conjugate containing fractions of the eluate are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm^2, cut-off 10 kD / Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00533] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods.

Example 33

PEGylation of Ang-2 using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00534] Ang-2 is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Ang-2 is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00535] The retentate containing oxidized Ang-2 is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00536] Finally, the PEG-Ang-2 conjugate is purified by ion-exchange chromatography (e.g. on Q Sepharose FF). For example, 1.5 mg protein/ml gel is loaded on the column equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00537] In an alternative embodiment, Method 1 is carried out as follows. Ang-2 is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Ang-2 is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00538] The retentate containing oxidized Ang-2 is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00539] Finally, the PEG- Ang-2 conjugate is purified by ion-exchange chromatography. The conjugate containing fraction of the eluate are collected and then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00540] Ang-2 is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan).

[00541] Ang-2 is transferred or dissolved in reaction buffer (e.g. 50mM Hepes, 350mM sodium chloride, 5mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a

0.5N aqueous HCl solution. Subsequently a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00542] The oxidized Ang-2 is further purified by ion exchange chromatography. The oxidized Ang-2 containing fractions of the eluate are collected and used for the conjugation reaction.

[00543] The aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized Ang-2 within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00544] The obtained PEG- Ang-2 conjugate is further purified by ion exchange chromatography. The PEG-Ang-2 conjugate containing fractions are collected and concentrated by ultra- / diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00545] The conjugate prepared by use of this procedure are analytically characterized by measuring total protein and biological activity according to methods known in the art.

Method 3:

[00546] Ang-2 is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Ang-2 is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00547] Finally, the PEG- Ang-2 conjugate is purified by ion-exchange chromatography on Q Sepharose FF. 1.5 mg protein/ml gel is loaded on the column pre equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM

Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using a membrane. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00548] In an alternative embodiment, Method 3 is carried out as follows. Ang-2 is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Ang-2 is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00549] Finally the PEG-Ang-2 conjugate is purified by ion-exchange chromatography The conjugatecontaing freactions of the eluate are collected and then subjected to UF/DF.. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00550] Ang-2 is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of Ang-2 is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 2 mg Ang-2 / ml. Subsequently an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 µM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20- fold molar reagent excess. After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of an 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00551] The PEG-Ang-2 conjugate is purified by means of ion exchange chromatography (IEC). The conjugate containing fractions of the eluate are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00552] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods..

[00553] Subsequently, the free Ang-2 is removed by means of ion exchange chromatography (IEC). The conjugate containing fractions of the eluate are concentrated by UF/DF.

Example 34

PEGylation of VEGF using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00554] VEGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). VEGF is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00555] The retentate containing oxidized VEGF is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00556] Finally, the PEG- VEGF conjugate is purified by ion-exchange chromatography (e.g., on Q Sepharose FF). For example, 1.5 mg protein/ml gel is loaded on the column equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH

7.4 and is then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00557] In an alternative embodiment, Method 1 is carried out as follows. VEGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). VEGF is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00558] The retentate containing oxidized VEGF is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00559] Finally, the PEG- VEGF conjugate is purified by ion-exchange chromatography The conjugate containg fractions of the eluate are collected and then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00560] VEGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan).VEGF is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15

minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00561] The oxidized VEGF is further purified by ion exchange chromatography. The oxidized VEGF containing fractions of the eluate are collected and used for the conjugation reaction.

[00562] The aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized VEGF within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00563] The obtained PEG-VEGF conjugate is further purified by ion exchange chromatography. The PEG-VEGF conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00564] The conjugate prepared by use of this procedure are analytically characterized by measuring total protein and biological activity according to methods known in the art.

Method 3:

[00565] VEGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). VEGF is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently, the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00566] Finally, the PEG- VEGF conjugate is purified by ion-exchange chromatography on Q Sepharose FF. 1.5 mg protein/ml gel is loaded on the column pre equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using a membrane. The preparation is analytically characterized by

measuring total protein (Bradford) and biological activity according to methods known in the art.

[00567] In an alternative embodiment, Method 3 is carried out as follows. VEGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). VEGF is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently, the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00568] Finally, the PEG-VEGF conjugate is purified by ion-exchange chromatography. The conjugate conjugate fractions of the eluate are collected and then subjected to UF/DF. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00569] VEGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of VEGF is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 2 mg VEGF / ml. Subsequently, an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 µM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20-fold molar reagent excess. After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of an 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00570] The PEG-VEGF conjugate is purified by means of ion exchange chromatography (IEC). The conjugate containing fractions of the eluate are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00571] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods.

Example 35

PEGylation of EGF using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00572] EGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). EGF is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00573] The retentate containing oxidized EGF is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00574] Finally, the PEG-EGF conjugate is purified by ion-exchange chromatography (e.g., on Q Sepharose FF). For example, 1.5 mg protein/ml gel is loaded on the column equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00575] In an alternative embodiment, Method 1 is carried out as follows. EGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). EGF is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00576] The retentate containing oxidized EGF is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00577] Finally, the PEG-EGF conjugate is purified by ion-exchange chromatography. The conjugate containg fractions of the eluate are collected and then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00578] EGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). EGF is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00579] The oxidized EGF is further purified by ion exchange chromatography. The oxidized EGF containing fractions of the eluate are collected and used for the conjugation reaction.

[00580] The aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized NGF within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00581] The obtained PEG- EGF conjugate is further purified by ion exchange chromatography. The PEG-EGF conjugate containing fractions are collected and concentrated by ultra- / diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00582] The conjugate prepared by use of this procedure are analytically characterized by measuring total protein and biological activity according to methods known in the art.

Method 3:

[00583] EGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). EGF is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00584] Finally, the PEG-EGF conjugate is purified by ion-exchange chromatography on Q-Sepharose FF. 1.5 mg protein/ml gel is loaded on the column pre equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using a membrane. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00585] In an alternative embodiment, Method 3 is carried out as follows. EGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An

example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). EGF is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00586] Finally, the PEG-EGF conjugate is purified by ion-exchange chromatography. The conjugate containing fractions of the eluate are collected and then subjected to UF/DF. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00587] EGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of EGF is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 2 mg EGF / ml. Subsequently an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 µM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20-fold molar reagent excess. After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of an 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00588] The PEG-EGF conjugate is purified by means of ion exchange chromatography (IEC). The conjugate containing fractions of the eluate are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00589] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods.

Example 36

PEGylation of NGF using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00590] NGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). NGF is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00591] The retentate containing oxidized NGF is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00592] Finally, the PEG-NGF conjugate is purified by ion-exchange chromatography (e.g., on Q-Sepharose FF). For example, 1.5 mg protein/ml gel is loaded on the column equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00593] In an alternative embodiment, Method 1 is carried out as follows. NGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). NGF is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00594] The retentate containing oxidized NGF is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00595] Finally, the PEG-NGF conjugate is purified by ion-exchange chromatography (The conjugate containing fractions of the eluate are collected and then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00596] NGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). NGF is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5N aqueous HCl solution. Subsequently a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00597] The oxidized NGF is further purified by ion exchange chromatography. The oxidized NGF containing fractions of the eluate are collected and used for the conjugation reaction.

[00598] The aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized NGF within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00599] The obtained PEG- NGF conjugate is further purified by ion exchange chromatography. The PEG-NGF conjugate containing fractions are collected and

concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00600] The conjugate prepared by use of this procedure are analytically characterized by measuring total protein and biological activity according to methods known in the art.

Method 3:

[00601] NGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). NGF is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00602] Finally, the PEG-NGF conjugate is purified by ion-exchange chromatography on Q Sepharose FF. 1.5 mg protein/ml gel is loaded on the column pre equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using a membrane. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00603] In an alternative embodiment, Method 3 is carried out as follows. NGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). NGF is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00604] Finally, the PEG-NGF conjugate is purified by ion-exchange chromatography. The conjugate containg fractions are collected and then subjected to UF/DF. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00605] NGF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of NGF is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 2 mg NGF / ml. Subsequently an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 μM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20- fold molar reagent excess. After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of an 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00606] The PEG- NGF conjugate is purified by means of ion exchange chromatography (IEC). The conjugate containing fractions of the eluate are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00607] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods.

Example 37

PEGylation of HGH using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00608] As described herein, the amino acid sequence of human growth hormone (HGH) is first modified to incorporate at least one glycosylation site. Following purification, HGH is glycosylated in vitro according to methods known in the art.

[00609] HGH is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). HGH is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00610] The retentate containing oxidized HGH is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00611] Finally, the PEG- HGH conjugate is purified by ion-exchange chromatography (e.g., on Q Sepharose FF). For example, 1.5 mg protein/ml gel is loaded on the column equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00612] In n alternative embodiment, Method 1 is carried out as follows. As described herein, the amino acid sequence of human growth hormone (HGH) is first modified to incorporate at least one glycosylation site. Following purification, HGH is glycosylated in vitro according to methods known in the art.

[00613] HGH is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). HGH is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing

Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00614] The retentate containing oxidized HGH is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00615] Finally, the PEG-HGH conjugate is purified by ion-exchange chromatography (The conjugate containg fractions of the eluate are collected and then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00616] As described herein, the amino acid sequence of human growth hormone (HGH) is first modified to incorporate at least one glycosylation site. Following purification, HGH is glycosylated in vitro according to methods known in the art.

[00617] HGH is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). HGH is transferred or dissolved in reaction buffer (e.g. 50mM Hepes, 350mM sodium chloride, 5mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00618] The oxidized HGH is further purified by ion exchange chromatography. The oxidized HGH containing fractions of the eluate are collected and used for the conjugation reaction.

[00619] The aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized HGH within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM)

is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00620] The obtained PEG- HGH conjugate is further purified by ion exchange chromatography. The PEG-NGF conjugate containing fractions are collected and concentrated by ultra- / diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00621] The conjugate prepared by use of this procedure are analytically characterized by measuring total protein and biological activity according to methods known in the art.

Method 3:

[00622] As described herein, the amino acid sequence of human growth hormone (HGH) is first modified to incorporate at least one glycosylation site. Following purification, HGH is glycosylated in vitro according to methods known in the art.

[00623] HGH is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). HGH is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00624] Finally, the PEG-HGH conjugate is purified by ion-exchange chromatography on Q-Sepharose FF. 1.5 mg protein/ml gel is loaded on the column pre equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using a membrane. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00625] In an alternative embodiment, Method 3 is carried out as follows. As described herein, the amino acid sequence of human growth hormone (HGH) is first modified to incorporate at least one glycosylation site. Following purification, HGH is glycosylated in vitro according to methods known in the art. HGH is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the

Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). HGH is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00626] Finally, the PEG-HGH conjugate is purified by ion-exchange chromatography. The conjugate containg fractions are collected and then subjected to UF/DF. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00627] As described herein, the amino acid sequence of human growth hormone (HGH) is first modified to incorporate at least one glycosylation site. Following purification, HGH is glycosylated in vitro according to methods known in the art.

[00628] HGH is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of HGH is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 2 mg HGH / ml. Subsequently an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 µM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20-fold molar reagent excess. After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of a 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00629] The PEG - HGH conjugate is purified by means of ion exchange chromatography (IEC). The conjugate containing fractions of the eluate are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00630] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods.

Example 38

PEGylation of TNF-alpha using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00631] TNF-alpha is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). TNF-alpha is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00632] The retentate containing oxidized TNF-alpha is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00633] Finally, the PEG-TNF-alpha conjugate is purified by ion-exchange chromatography (e.g., on Q-Sepharose FF). For example, 1.5 mg protein/ml gel is loaded on the column equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00634] In an alternative embodiment, Method 1 is carried out as follows. TNF-alpha is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). TNF-alpha is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched

for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00635] The retentate containing oxidized TNF-alpha is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00636] Finally, the PEG-TNF-alpha conjugate is purified by ion-exchange chromatography. The conjugate containg fractions of the eluate are collected and then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00637] TNF-alpha is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). TNF-alpha is transferred or dissolved in reaction buffer (e.g. 50mM Hepes, 350mM sodium chloride, 5mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5N aqueous HCl solution. Subsequently a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00638] The oxidized TNF-alpha is further purified by ion exchange chromatography. The oxidized TNF-alpha containing fractions of the eluate are collected and used for the conjugation reaction.

[00639] The aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized TNF alpha within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture

is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00640] The obtained PEG- TNF-alpha conjugate is further purified by ion exchange chromatography. The PEG- TNF-alpha conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00641] The conjugate prepared by use of this procedure are analytically characterized by measuring total protein and biological activity according to methods known in the art.

Method 3:

[00642] TNF-alpha is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). TNF-alpha is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00643] Finally, the PEG-TNF-alpha conjugate is purified by ion-exchange chromatography on Q-Sepharose FF. 1.5 mg protein/ml gel is loaded on the column pre equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using a membrane. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00644] In an alternative embodiment, Method 3 is carried out as follows. TNF-alpha is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). TNF-alpha is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently, the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at

room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00645] Finally, the PEG- TNF-alpha conjugate is purified by ion-exchange chromatography. The conjugate containing fractions are collected and then subjected to UF/DF. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00646] TNF-alpha is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of TNF-alpha is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 2 mg TNF-alpha / ml. Subsequently, an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 µM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20- fold molar reagent excess. After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of an 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00647] The PEG- TNF-alpha conjugate is purified by means of ion exchange chromatography (IEC). The conjugate containing fractions of the eluate are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00648] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods.

Example 39

PEGylation of insulin using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00649] As described herein, the amino acid sequence of insulin is first modified to incorporate at least one glycosylation site. Following purification, insulin is glycosylated in vitro according to methods known in the art. Insulin is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Insulin is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00650] The retentate containing oxidized insulin is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00651] Finally, the PEG-insulin conjugate is purified by ion-exchange chromatography (e.g., on Q-Sepharose FF). For example, 1.5 mg protein/ml gel is loaded on the column equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00652] In an alternative embodiment, Method 1 is carried out as follows. As described herein, the amino acid sequence of insulin is first modified to incorporate at least one glycosylation site. Following purification, insulin is glycosylated in vitro according to methods known in the art. Insulin is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Insulin is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF

employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00653] The retentate containing oxidized insulin is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00654] Finally, the PEG-insulin conjugate is purified by ion-exchange chromatography. The conjugate containg fractions of the eluate are collected and then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00655] As described herein, the amino acid sequence of insulin is first modified to incorporate at least one glycosylation site. Following purification, insulin is glycosylated in vitro according to methods known in the art.

[00656] Insulin is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Insulin is transferred or dissolved in reaction buffer (e.g. 50mM Hepes, 350mM sodium chloride, 5mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00657] The oxidized insulin is further purified by ion exchange chromatography. The oxidized insulin containing fractions of the eluate are collected and used for the conjugation reaction.

[00658] The aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized insulin within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM)

is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00659] The obtained PEG - insulin conjugate is further purified by ion exchange chromatography. The PEG - insulin conjugate containing fractions are collected and concentrated by ultra- / diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00660] The conjugate prepared by use of this procedure are analytically characterized by measuring total protein and biological activity according to methods known in the art.

Method 3:

[00661] As described herein, the amino acid sequence of insulin is first modified to incorporate at least one glycosylation site. Following purification, insulin is glycosylated in vitro according to methods known in the art.

[00662] Insulin is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Insulin is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently, the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00663] Finally, the PEG-insulin conjugate is purified by ion-exchange chromatography on Q Sepharose FF. 1.5 mg protein/ml gel is loaded on the column pre equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using a membrane. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00664] In an alternative embodiment, Method 3 is carried out as follows. As described herein, the amino acid sequence of insulin is first modified to incorporate at least one glycosylation site. Following purification, insulin is glycosylated in vitro according to methods known in the art. Insulin is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA

series from NOF (NOF Corp., Tokyo, Japan). Insulin is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00665] Finally, the insulin-conjugate is purified by ion-exchange chromatography. The conjugate containing fractions are collected and then subjected to UF/DF. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00666] As described herein, the amino acid sequence of insulin is first modified to incorporate at least one glycosylation site. Following purification, insulin is glycosylated in vitro according to methods known in the art.

[00667] Insulin is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of insulin is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 2 mg insulin / ml. Subsequently an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 µM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20- fold molar reagent excess. After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of a 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00668] The PEG - insulin conjugate is purified by means of ion exchange chromatography (IEC). The conjugate containing fractions of the eluate are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00669] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods.

Example 40

PEGylation of interferon-alpha using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00670] Interferon-alpha is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Interferon-alpha is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00671] The retentate containing oxidized interferon-alpha is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00672] Finally, the PEG-interferon-alpha conjugate is purified by ion-exchange chromatography (e.g., on Q-Sepharose FF). For example, 1.5 mg protein/ml gel is loaded on the column equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00673] In an alternative embodiment, Method 1 is carried out as follows. Interferon-alpha is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Interferon-alpha is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle

stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00674] The retentate containing oxidized interferon-alpha is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00675] Finally, the PEG-interferon-alpha conjugate is purified by ion-exchange chromatography. The conjugate containing freactions are collected and then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00676] Interferon-alpha is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Interferon-alpha is transferred or dissolved in reaction buffer (e.g. 50 mM Hepes, 350 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00677] The oxidized interferon-alpha is further purified by ion exchange chromatography. The oxidized interferon-alpha containing fractions of the eluate are collected and used for the conjugation reaction.

[00678] The aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized interferon-alpha within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The

reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00679] The obtained PEG- interferon-alpha conjugate is further purified by ion exchange chromatography. The PEG- interferon alpha conjugate containing fractions are collected and concentrated by ultra- / diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00680] The conjugate prepared by use of this procedure are analytically characterized by measuring total protein and biological activity according to methods known in the art.

Method 3:

[00681] Interferon-alpha is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Interferon-alpha is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00682] Finally, the PEG-interferon-alpha conjugate is purified by ion-exchange chromatography on Q-Sepharose FF. 1.5 mg protein/ml gel is loaded on the column pre equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using a membrane. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00683] In an alternative embodiment, Method 3 is carried out as follows. Interferon-alpha is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Interferon-alpha is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated

for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00684] Finally, the PEG-interferon-alpha conjugate is purified by ion-exchange chromatography. The conjugate containg fractions are collected and then subjected to UF/DF using a membrane. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00685] Interferon-alpha is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of interferon-alpha is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 2 mg interferon -alpha / ml. Subsequently, an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 µM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20-fold molar reagent excess. After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of an 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00686] The PEG- interferon -alpha conjugate is purified by means of ion exchange chromatography (IEC). The conjugate containing fractions of the eluate are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00687] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods.

Example 41

PEGylation of interferon-gamma using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00688] Interferon-gamma is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). 10 mg Interferon-gamma is dissolved in 5 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl). 100 µl of an aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 50 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin 15R 10 kD centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00689] The retentate (approx. 7 ml), containing oxidized interferon-gamma, is mixed with 2 ml of an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Then aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at RT in the dark under gentle stirring.

[00690] Finally, the PEG-interferon-gamma conjugate is purified by ion-exchange chromatography on SP Sepharose FF. The reaction mixture is diluted with 20 ml Buffer A (50 mM Hepes, pH 6.5) and loaded onto a 20 ml HiPrep SPFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column is eluted with Buffer B (50 mM Hepes, 1 M NaCl, pH 6.5). Free interferon-gamma is eluted by washing the column with 25 % Buffer B and the conjugate at 50 % Buffer B. The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against histidine buffer, pH 6.9 containing 150 mM NaCl. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art. For the PEG-interferon-gamma conjugate a specific activity of > 50 % in comparison to native Interferon gamma is determined. The conjugate is additionally analytically characterized by Size Exclusion HPLC using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77). It is shown that the preparation contains no free Interferon-gamma.

Method 2:

[00691] Interferon-gamma is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA

series from NOF (NOF Corp., Tokyo, Japan). Interferon-gamma is transferred or dissolved in reaction buffer (e.g. 50mM Hepes, 350mM sodium chloride, 5mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5 N aqueous HCl solution. Subsequently a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00692] The oxidized interferon-gamma is further purified by ion exchange chromatography. The oxidized interferon-gamma containing fractions of the eluate are collected and used for the conjugation reaction.

[00693] The aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized interferon-gamma within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00694] The obtained PEG-interferon-gamma conjugate is further purified by ion exchange chromatography. The PEG-interferon-gamma conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

[00695] The conjugate prepared by use of this procedure are analytically characterized by measuring total protein and biological activity according to methods known in the art.

Method 3:

[00696] Interferon-gamma is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). 10 mg interferon-gamma is dissolved in ~8 ml histidine - buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl). 200 µl of an aqueous sodium periodate solution (5 mM) and 2 ml of an aqueous m-toluidine solution (50 mM) are then added. Subsequently the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 5-fold molar reagent excess. The mixture is incubated for 2 h in the dark at

room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 100 µl of 1 M aqueous cysteine solution.

[00697] Finally the PEG-interferon-gamma conjugate is purified by ion-exchange chromatography on SP-Sepharose FF. The reaction mixture is diluted with 20 ml Buffer A (50 mM Hepes, pH 6.5) and loaded onto a 20 ml HiPrep SP FF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column is eluted with Buffer B (50 mM Hepes, 1 M NaCl, pH 6.5). Free intergferon-gamma is eluted by washing the column with 25 % Buffer B and the conjugate at 50 % Buffer B. The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against histidine buffer, pH 6.9 containing 150 mM NaCl. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according according to methods known in the art. For the PEG-interferon-gamma conjugate a specific activity of > 50 % in comparison to native interferon-gamma is determined. The conjugate is additionally analytically characterized by Size Exclusion HPLC using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77). It is shown that the preparation contains no free interferon-gamma.

Method 4:

[00698] Interferon-gamma is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of interferon-gamma is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 2 mg interferon-gamma / ml. Subsequently an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 µM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20-fold molar reagent excess. After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of an 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00699] The PEG-interferon-gamma conjugate is purified by means of ion exchange chromatography (IEC). The conjugate containing fractions of the eluate are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00700] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods.

Example 42

PEGylation of G-CSF using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00701] G-CSF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). G-CSF is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00702] The retentate containing oxidized G-CSF is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00703] Finally, the PEG- G-CSF conjugate is purified by ion-exchange chromatography (e.g., on Q-Sepharose FF). For example, 1.5 mg protein/ml gel is loaded on the column equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00704] In an alternative embodiment, Method 1 is carried out as follows. G-CSF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). G-CSF is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00705] The retentate containing oxidized G-CSF is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00706] Finally, the PEG- G-CSF conjugate is purified by ion-exchange chromatography (The conjugate containg fractions of the eluate are collected and then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00707] G-CSF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). G-CSF is transferred or dissolved in reaction buffer (e.g. 50mM Hepes, 350mM sodium chloride, 5mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5N aqueous HCl solution. Subsequently a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00708] The oxidized G-CSF is further purified by ion exchange chromatography. The oxidized G-CSF containing fractions of the eluate are collected and used for the conjugation reaction.

[00709] The aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized G-CSF within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00710] The obtained PEG- G-CSF conjugate is further purified by ion exchange chromatography. The PEG- G-CSF conjugate containing fractions are collected and concentrated by ultra-/diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

Method 3:

[00711] G-CSF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). G-CSF is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently, the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00712] Finally, the PEG- G-CSF conjugate is purified by ion-exchange chromatography on Q-Sepharose FF. 1.5 mg protein/ml gel is loaded on the column pre equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using a membrane. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00713] In an alternative embodiment, Method 3 is carried out as follows. G-CSF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo,

Japan). G-CSF is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently, the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00714] Finally, the PEG- G-CSF conjugate is purified by ion-exchange chromatography. The conjugate containing fractions of the eluate are collected and then subjected to UF/DF using a membrane. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00715] G-CSF is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of G-CSF is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 2 mg G-CSF / ml. Subsequently, an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 µM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20-fold molar reagent excess. After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of an 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00716] The G-CSF conjugate is purified by means of ion exchange chromatography (IEC). The conjugate containing fractions of the eluate are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00717] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods.

Example 43

PEGylation of Humira using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00718] Humira is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Humira is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00719] The retentate containing oxidized Humira is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00720] Finally, the PEG-Humira conjugate is purified by ion-exchange chromatography (e.g., on Q-Sepharose FF). For example, 1.5 mg protein/ml gel is loaded on the column equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00721] In an alternative embodiment, Method 1 is carried out as follows. Humira is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Humira is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00722] The retentate containing oxidized Humira is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00723] Finally, the PEG- Humira conjugate is purified by ion-exchange chromatography. The conjugate containg fractions of the eluate are collected and then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

Method 2:

[00724] Humira is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Humira is transferred or dissolved in reaction buffer (e.g. 50mM Hepes, 350mM sodium chloride, 5mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5N aqueous HCl solution. Subsequently a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00725] The oxidized Humira is further purified by ion exchange chromatography. The oxidized Humira containing fractions of the eluate are collected and used for the conjugation reaction.

[00726] The aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized Humira within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00727] The obtained PEG-Humira conjugate is further purified by ion exchange chromatography. The PEG-Humira conjugate containing fractions are collected and

concentrated by ultra- / diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

Method 3:

[00728] Humira is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Humira is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently, the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00729] Finally, the PEG- Humira conjugate is purified by ion-exchange chromatography on Q-Sepharose FF. 1.5 mg protein/ml gel is loaded on the column pre equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using a membrane. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00730] In an alternative embodiment, Method 3 is carried out as follows. Humira is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Humira is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00731] Finally, the PEG-Humira conjugate is purified by ion-exchange chromatography. The conjugate containing fractions are collected and then subjected to UF/DF using a membrane. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

Method 4:

[00732] Humira is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of HJumira is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 2 mg Humira / ml. Subsequently an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 μM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20- fold molar reagent excess. After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of a 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00733] The Humira conjugate is purified by means of ion exchange chromatography (IEC). The conjugate containing fractions of the eluate are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00734] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods.

Example 44

PEGylation of Prolia using an aminooxy-PEG reagent and m-toluidine as a nucleophilic catalyst

Method 1:

[00735] Prolia is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Prolia is dissolved in 7.0 ml histidine buffer, pH 6.0 (20mM L-histidine, 150 mM NaCl, 5 mM $CaCl_2$). An aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 7.5 μl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00736] The retentate containing oxidized Prolia is next mixed with an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Aminooxy-PEG reagent with a MW of 20 kD is then added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at room temperature in the dark under gentle stirring.

[00737] Finally, the PEG- Prolia conjugate is purified by ion-exchange chromatography (e.g., on Q-Sepharose FF). For example, 1.5 mg protein/ml gel is loaded on the column equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using an appropriate MW cutoff membrane. The preparation is next analytically characterized by measuring total protein (Coomassie, Bradford) and biological activity according to methods known in the art.

[00738] In an alternative embodiment, Method 1 is carried out as follows. Prolia is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). 10 mg rFIX is dissolved in 5 ml histidine - buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl). 100 µl of an aqueous sodium periodate solution (5 mM) is then added and the reaction mixture is incubated for 1 h in the dark at 4°C under gentle stirring and quenched for 15 min at room temperature by the addition of 50 µl of a 1 M aqueous cysteine solution. The mixture is subsequently subjected to UF/DF employing Vivaspin 15R 10 kD centrifugal filtrators to remove excess periodate, quencher and the byproducts thereof.

[00739] The retentate (approx. 7 ml), containing oxidized Prolia, is mixed with 2 ml of an aqueous m-toluidine solution (50 mM) and incubated for 30 min at room temperature. Then aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 5-fold molar reagent excess. This mixture is incubated for 2.5 h at RT in the dark under gentle stirring.

[00740] Finally the PEG-Prolia conjugate is purified by ion-exchange chromatography on SP Sepharose FF. The reaction mixture is diluted with 20 ml Buffer A (50 mM Hepes, pH 6.5) and loaded onto a 20 ml HiPrep SP FF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column is eluted with Buffer B (50 mM Hepes, 1 M NaCl, pH 6.5). Free Prolia is eluted by washing the column with 25 % Buffer B and the conjugate at 50 % Buffer B. The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore).

The final diafiltration step is performed against histidine buffer, pH 6.9 containing 150 mM NaCl. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art. For the PEG-Prolia conjugate a specific activity of > 50 % in comparison to native Prolia is determined. The conjugate is additionally analytically characterized by Size Exclusion HPLC using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77). It is shown that the preparation contains no free Prolia.

Method 2:

[00741] Prolia is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). Prolia is transferred or dissolved in reaction buffer (e.g. 50mM Hepes, 350mM sodium chloride, 5mM calcium chloride, pH 6.0) to get a final protein concentration of 1.0 +/- 0.25 mg/ml. Then the pH of the solution is corrected to 6.0 by drop wise addition of a 0.5N aqueous HCl solution. Subsequently, a 40 mM aqueous sodium periodate solution is added within 10 minutes to give a concentration of 200 µM. The oxidation reaction is carried out for 30 +/- 5 min at a temperature (T) of T= +22 +/- 2°C. Then the reaction is stopped by addition of an aqueous L-cysteine solution (1 M) within 15 minutes at T= +22 +/- 2°C to give a final concentration of 10 mM in the reaction mixture and incubation for 60 +/- 5 min.

[00742] The oxidized Prolia is further purified by ion exchange chromatography. The oxidized Humira containing fractions of the eluate are collected and used for the conjugation reaction.

[00743] The aminooxy-PEG reagent with a MW of 20 kD reagent is added in a 50-fold molar excess to the eluate containing the purified oxidized Prolia within a maximum time period (t) of 15 minutes under gentle stirring. Then an aqueous m-toluidine solution (50 mM) is added within 15 minutes to get a final concentration of 10 mM. The reaction mixture is incubated for 120 +/- 10 min. in the dark at a temperature (T) of T= +22 +/- 2°C under gentle shaking.

[00744] The obtained PEG- Prolia conjugate is further purified by ion exchange chromatography. The PEG- Prolia conjugate containing fractions are collected and

concentrated by ultra- / diafiltration (UF/DF) using a membrane made of regenerated cellulose with an appropriate molecular weight cut off (Millipore).

Method 3:

[00745] Prolia is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). EPO is dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) and mixed with an aqueous sodium periodate solution (10 mM), and an aqueous m-toluidine solution (50 mM). Subsequently the aminooxy reagent is added to give a 20-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 8 µl of aqueous cysteine solution (1 M).

[00746] Finally, the PEG- Prolia conjugate is purified by ion-exchange chromatography on Q-Sepharose FF. 1.5 mg protein/ml gel is loaded on the column pre equilibrated with 50 mM Hepes buffer, pH 7.4 containing 5 mM $CaCl_2$. The conjugate is eluted with 50 mM Hepes buffer containing 5 mM $CaCl_2$ and 500 mM sodium chloride, pH 7.4 and is then subjected to UF/DF using a membrane. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art.

[00747] In an alternative embodiment, Method 3 is carried out as follows.

[00748] Prolia is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). 10 mg Prolia is dissolved in ~8 ml histidine buffer, pH 6.0 (20 mM L-histidine, 150 mM NaCl). 200 µl of an aqueous sodium periodate solution (5 mM) and 2 ml of an aqueous m-toluidine solution (50 mM) are then added. Subsequently, the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 5-fold molar reagent excess. The mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of 100 µl of 1 M aqueous cysteine solution.

[00749] Finally the PEG-Prolia conjugate is purified by ion-exchange chromatography on SP-Sepharose FF. The reaction mixture is diluted with 20 ml Buffer A (50 mM Hepes, pH 6.5) and loaded onto a 20 ml HiPrep SPFF 16/10 column (GE Healthcare, Fairfield, CT) pre-equilibrated with Buffer A. Then the column is eluted with Buffer B (50 mM Hepes, 1 M NaCl, pH 6.5). Free Prolia is eluted by washing the column with 25% Buffer B and the

conjugate at 50% Buffer B. The conjugate containing fractions are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against histidine buffer, pH 6.9 containing 150 mM NaCl. The preparation is analytically characterized by measuring total protein (Bradford) and biological activity according to methods known in the art. For the PEG-Prolia conjugate a specific activity of > 50 % in comparison to native Prolia is determined. The conjugate is additionally analytically characterized by Size Exclusion HPLC using a Agilent 1200 HPLC system equipped with a Shodex KW 803 column under conditions as previously described (Kolarich et al, Transfusion 2006;46:1959-77). It is shown that the preparation contains no free Prolia.

Method 4:

[00750] Prolia is PEGylated by use of a linear 20 kD PEGylation reagent containing an aminooxy group. An example of this type of reagent is the Sunbright ® CA series from NOF (NOF Corp., Tokyo, Japan). An intital concentration or weight of HJumira is transferred or dissolved in Hepes buffer (50 mM Hepes, 150 mM sodium chloride, 5 mM calcium chloride, pH 6.0) to get a final protein concentration of 2 mg Prolia / ml. Subsequently an 5 mM aqueous sodium periodate solution is added within 15 minutes to give a final concentration of 100 µM, followed by addition of an 50 mM aqueous m-toluidine solution to get a final concentration of 10 mM within a time period of 30 minutes. Then the aminooxy-PEG reagent with a MW of 20 kD (described above) is added to give a 20- fold molar reagent excess. After correction of the pH to 6.0 the mixture is incubated for 2 h in the dark at room temperature under gentle stirring and quenched for 15 min at room temperature by the addition of an 1 M aqueous L-cysteine solution to give a final concentration of 10 mM.

[00751] The Prolia conjugate is purified by means of ion exchange chromatography (IEC). The conjugate containing fractions of the eluate are concentrated by UF/DF using a 10 kD membrane made of regenerated cellulose (88 cm2, cut-off 10 kD / Millipore). The final diafiltration step is performed against Hepes buffer (50 mM Hepes, 5 mM $CaCl_2$, pH 7.5).

[00752] The preparation is analytically characterized by measuring total protein (Bradford and BCA procedure) and biological activity according to known methods.

Example 45

PEGylation of a therapeutic protein using branched PEG

[00753] PEGylation of a therapeutic protein of the invention may be extended to a branched or linear PEGylation reagent, which is made of an aldehyde and a suitable linker containing an active aminooxy group.

Example 46

Coupling of a diamonooxy linker to native PSA

[00754] This Example describes procedures to prepare aminooxy-PSA reagents using native PSA (i.e. without prior oxidation), which can be used for chemical modification of therapeutic proteins.

[00755] a) Coupling at ambient temperature

[00756] 52.2 mg of native PSA (MW = 20 kD) obtained from the Serum Institute of India (Pune, India) was dissolved in 1.05 ml 50mM phosphate buffer pH 6.0. Then 10.3 mg 3-oxa-pentane-1,5-dioxyamine (linker molecule) was added drop wise to the reaction mixture. The reaction was incubated for 2 hrs at room temperature under gentle agitation in the dark.

[00757] b) Coupling at increased temperature

[00758] 52.2 mg of native PSA (MW = 20 kD) obtained from the Serum Institute of India (Pune, India) was dissolved in 1.05 ml 50mM phosphate buffer pH 6.0. Then 10.3 mg 3-oxa-pentane-1,5-dioxyamine (linker molecule) was added drop wise to the reaction mixture. The reaction was incubated for 2 hrs at room temperature under gentle agitation in the dark. Then the temperature was increased to 32-37°C and the reaction mixture was incubated for another 14 hrs.

[00759] c) Coupling at increased temperature and increased linker excess

[00760] 52.2 mg of native PSA (MW = 20 kD) obtained from the Serum Institute of India (Pune, India) was dissolved in 1.05 ml 50 mM phosphate buffer pH 6.0. Then 10.3 mg 3-oxa-pentane-1,5-dioxyamine (linker molecule) was added drop wise to the reaction mixture. The reaction was incubated for 2 hrs at room temperature under gentle agitation in the dark. Then 26.3 mg 3-oxa-pentane-1,5-dioxyamine were added drop wise to the reaction, the temperature was increased to 32-37°C and the reaction mixture was incubated for another 14 hrs.

[00761] d) Purification of PSA derivatives

[00762] After the incubation was completed, the reaction mixtures generated under points a-c were purified by extensive dialysis. Therefore samples of the reaction mixtures were loaded into Slide-A-Lyzer dialysis cassettes (0-5-3ml, MWCO 3.5kD, reg. cellulose, Pierce) and dialyzed against 10 mM phosphate buffer pH 8.0 according to the following pattern:

[00763] 2 hrs against 500ml buffer at room temperature

[00764] 2 hrs against 500ml buffer at room temperature

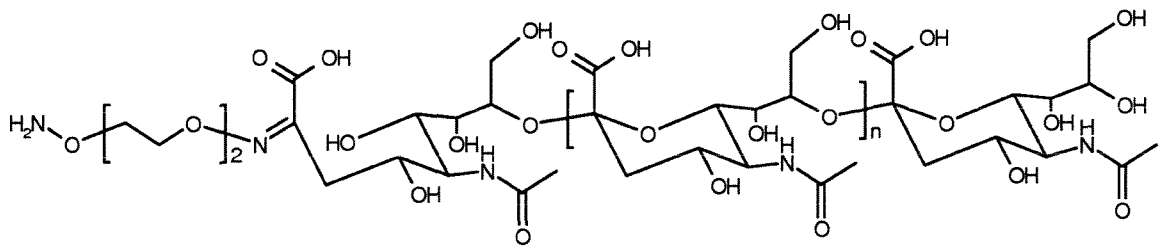
[00765] 12 hrs against 500ml buffer at 4°C

[00766] 1h against 50ml 'Slide-A-Lyzer Concentrating Solution for Dialysis' at room temperature for concentration to initial sample volume.

[00767] The purified aminooxy-PSA is thus ready to be used in a protein conjugation reaction according to, for example, Examples 11, 12, 14, and 17-31, above. Likewise, any of the water-soluble polymers described herein can be coupled to an aminooxy linker as described in this Example and then conjugated to a protein as set out in the above Examples.

[00768] The preparation was analytically characterized by measuring total PSA (Resorcinol assay) and total aminooxy groups (TNBS assay) to determine the degree of modification. For preparation (a) a modification degree (MD) of 0.35, for (b) MD=0.54 and for (c) MD = 0.58 was determined. Furthermore the polydispersity as well as free 3-oxa-pentane-1,5-dioxyamine was measured. The polydispersity was lower than 1.15 for all preparations and the content of free linker was lower than 0.15 mol % of the PSA concentration.

[00769] For the PSA modified at the reducing end the following structure was determined by ^{13}C NMR spectroscopy.



Example 47

Preparation of aminooxy-PSA at 4°C employing a chromatographic purification step

[00770] During a detailed analytical characterization of the aminooxy-PSA reagent prepared at room temperature, NMR studies (See, e.g., US Provisional Application No. 61/647,814, incorporated by reference in it's entirety) revealed that the derivatization of oxidized PSA with the diaminooxy linker consists of two distinct reactions: a quick reaction of the aldehyde group at the non-reducing end of PSA and a slow reaction of the aldehyde group (in the form of a hemiketal) at the reducing end of PSA. The latter reaction could be considered an unwanted side reaction that is to be avoided for reagent production.

[00771] Therefore, the process for the preparation of the aminooxy-PSA reagent has been optimized as described in the instant Example. The reducing end only occurs to a significant degree if the process is performed at room temperature. Hence, the process was adjusted and is conducted at 2-8°C. By performing the whole process (chemical reaction and purification of the PSA reagent by IEX) at 2-8°C, the side reaction at the reducing end of PSA was substantially reduced. This process change thus leads to a reagent of higher quality.

[00772] Procedure

[00773] 1290 mg of oxidized PSA (MW = 20 kD) obtained from the Serum Institute of India (Pune, India) was dissolved in 25 ml 50 mM phosphate buffer pH 6.0 (Buffer A). Then 209 mg 3-oxa-pentane-1,5-dioxyamine was added to the reaction mixture and incubated for 1 h at 2-8°C under gentle agitation in the dark.

[00774] After incubation, the mixture was subjected to a weak anion exchange chromatography step employing a Fractogel EMD DEAE 650-M chromatography gel (column dimension: XK26/135) carried out in a cold room at temperature of 2-8°C. The reaction mixture was diluted with pre-cooled 110 ml Buffer A and loaded onto the DEAE column pre-equilibrated with Buffer A at a flow rate of 1 cm/min. Then the column was washed with 20 CV Buffer B (20 mM Hepes, pH 6.0) at a flow rate of 2 cm/min to remove free 3-oxa-pentane-1,5-dioxyamine. The aminooxy-PSA reagent was then eluted with a step gradient consisting of 67% Buffer B and 43% Buffer C (20 mM Hepes, 1M NaCl, pH 7.5). The eluate was concentrated by UF/DF using a 5 kD membrane made of polyether sulfone (50 cm2, Millipore). The preparation was analytically characterized by measuring total PSA (Resorcinol assay) and total aminooxy groups (TNBS assay) to determine the degree of modification. The PSA concentration in the final preparation was 46.0 mg/ml and the

modification degree was 83.5 %. Furthermore a polydispersity value of 1.131 was deterrmined. In addition a concentration of 0.22 µg/ml (0.07 mol % of PSA) was measured for free 3-oxa-pentane-1,5-dioxyamine..

[00775] The purified aminooxy-PSA is thus ready to be used in a conjugation reaction according to Examples 11, 12, 14, and 17-31, above.

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, 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.

2. The method according t oclaim 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 3 wherein the 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 3 wherein the oxidized water soluble polymer is PSA and is oxidized by incubation with $NaIO_4$.

5. 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, 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.

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 about 32°C and about 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 5-9 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 or chromatography at 4°C.

11. The method according to any one of claims 1-10 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, 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.

12. The method according to claim 11 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.

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

14. The method of claim 11 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.

15. The method of claim 14 wherein the excess concentration is about 50-fold molar excess.

16. The method of claim 14 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.

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

18. The method according to claim 11 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.

19. The method of claim 18 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.

20. The method according to claim 11 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.

21. The method of claim 20 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.

22. The method of claim 11 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.

23. The method of claim 22 wherein the quenching agent is L-cysteine.

24. The method of claim 23 wherein 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.

25. The method of claim 11 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.

26. The method of claim 25 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.

27. The method according to claim 11 wherein the water soluble polymer is PSA.

28. The method according to claim 11 wherein the water soluble polymer is PEG.

29. The method according to claim 11 wherein the water soluble polymer is HES.

30. The method according to claim 11 wherein the water soluble polymer is HAS.

31. The method according to claim 27 wherein the PSA is comprised of about 10 – 300 sialic acid units.

32. The method according to any one of claims 11-31 wherein the therapeutic protein is FIX.

33. The method according to any one of claims 11-31 wherein the therapeutic protein is FVIIa.

34. The method according to any one of claims 11-31 wherein the therapeutic protein is FVIII.

35. The method according to any one of claims 11-34 wherein the oxidizing agent is sodium periodate ($NaIO_4$).

36. The method according to any one of claims 32-34 wherein the oxidized carbohydrate moiety of the therapeutic protein is located in the activation peptide of the blood coagulation protein.

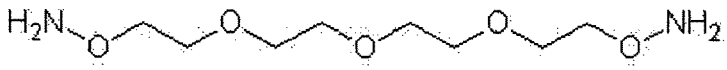
37. The method according to claim 27 wherein the PSA comprises an activated aminooxy linker selected from the group consisting of:

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



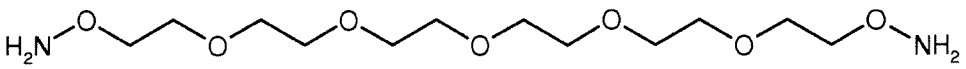
,

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 an oxidizing agent to form a terminal aldehyde group at the non-reducing end of the PSA.

38. The method according to claim 37 wherein the aminooxy linker is 3-oxa-pentane-1,5-dioxyamine.

39. The method according to any one of claims 11-38 wherein the nucleophilic catalyst is provided at a concentration between about 1 mM and about 50 mM.

40. The method according to claim 39 wherein the nucleophilic catalyst is m-toluidine.

41. The method according to claim 40 wherein the m-toluidine is present in the conjugation reaction at a concentration of about 10 mM.

42. The method according to any one of claims 11-41 further comprising the step of purifying the conjugated therapeutic protein.

43. The method according to claim 42 wherein the conjugated therapeutic protein is purified by a method selected from the group consisting of chromatography, filtration and precipitation.

44. The method according to claim 43 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.

45. The method of claim 44 wherein an anti-chaotropic salt is used in a chromotagraphy loading step and in a chromatography washing step.

46. The method of claim 44 wherein the chromatography takes place in a column.

47. The method of claim 46 wherein the column comprises a chromatography resin selected from the group consisting of Phenyl-Sepharose FF and Butyl-Sepharose FF.

48. The method of claim 47 wherein the resin is present in the column at a bed height of between about 5 cm and about 20 cm.

49. The method according to claim 48 wherein the bed height is about 10 cm.

50. The method of claim 46 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.

51. The method according to claim 50 wherein the flow rate is about 2 cm/min.

52. The method of any one of claims 47-52 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.

53. The method according to claim 52 wherein the flow rate is about 1 cm/min.

54. The method of any one of claims 42-53 further comprising concentrating the conjugated therapeutic protein by ultra-/diafiltration (UF/DF).

55. The method of any one of claims 42-54 wherein the final concentration of therapeutic protein is between about 0.5 and about 3 mg/ml.

56. The method according to any one of claims 42-55 wherein the therapeutic protein comprises between about 5 and about 11 water soluble polymer moieties.

57. The method of claim 11 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).

58. 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.

59. A modified therapeutic protein produced by the method according to any one of claims 1-58.

60. A method of preparing a water-soluble polymer containing an active aminooxy group 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), 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.

61. The method according to claim 60 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.

62. The method according to claim 60 wherein the water soluble polymer is PSA.

63. The method according to claim 60 wherein the water-soluble polymer is PEG.

64. The method according to claim 60 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.

65. The method according to claim 64 wherein the water soluble polymer containing an active aminooxy group is purified by anion exchange chromatography at a temperature of 4°C.

66. A method of preparing a water soluble polymer containing an active aminooxy group 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.

67. The method according to claim 66 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.

68. The method according to claim 66 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.

69. The method according to claim 66 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.

70. The method according to claim 69 comprising the additional step of adding an additional amount of aminooxy linker comprising an active aminooxy group immediately prior to increasing the temperature.

71. The method according to claim 66 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.

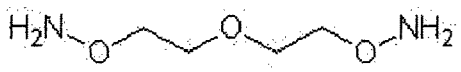
72. The method according to claim 71 further comprising the step of purifying the water soluble polymer containing an active aminooxy group by a method selected from the group consisting of dialysis, UF/DF or chromatography at 4°C.

73. A method of preparing a PSA-aminooxy reagent aminooxy group 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:



;

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

74. A method of preparing a PSA-aminooxy reagent aminooxy group 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:



;

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

75. A method of conjugating a water soluble polymer to an oxidized carbohydrate moiety of a blood coagulation protein comprising contacting the oxidized carbohydrate moiety with an activated water soluble polymer under conditions that allow conjugation;

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, 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.

SEQUENCE LISTING

<110> BAXTER INTERNATIONAL INC.
BAXTER HEALTHCARE SA

<120> NUCLEOPHILIC CATALYSTS FOR OXIME LINKAGE

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<210> 1
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<213> Homo sapiens

<400> 1

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35 40 45

Lys Gln Tyr Val Asp Gly Asp Gln Cys Glu Ser Asn Pro Cys Leu Asn
50 55 60

Gly Gly Ser Cys Lys Asp Asp Ile Asn Ser Tyr Glu Cys Trp Cys Pro
65 70 75 80

Phe Gly Phe Glu Gly Lys Asn Cys Glu Leu Asp Val Thr Cys Asn Ile
85 90 95

Lys Asn Gly Arg Cys Glu Gln Phe Cys Lys Asn Ser Ala Asp Asn Lys
100 105 110

Val Val Cys Ser Cys Thr Glu Gly Tyr Arg Leu Ala Glu Asn Gln Lys
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Ser Cys Glu Pro Ala Val Pro Phe Pro Cys Gly Arg Val Ser Val Ser
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Gln Thr Ser Lys Leu Thr Arg Ala Glu Ala Val Phe Pro Asp Val Asp
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Ala Lys Pro Gly Gln Phe Pro Trp Gln Val Val Leu Asn Gly Lys Val
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Asp Ala Phe Cys Gly Gly Ser Ile Val Asn Glu Lys Trp Ile Val Thr
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Ala Ala His Cys Val Glu Thr Gly Val Lys Ile Thr Val Val Ala Gly
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Glu His Asn Ile Glu Glu Thr Glu His Thr Glu Gln Lys Arg Asn Val
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Ile Arg Ala Ile Ile Pro His His Asn Tyr Asn Ala Ala Ile Asn Lys
            260                 265                 270

Tyr Asn His Asp Ile Ala Leu Leu Glu Leu Asp Glu Pro Leu Val Leu
        275                 280                 285

Asn Ser Tyr Val Thr Pro Ile Cys Ile Ala Asp Lys Glu Tyr Thr Asn
    290                 295                 300

Ile Phe Leu Lys Phe Gly Ser Gly Tyr Val Ser Gly Trp Ala Arg Val
305                 310                 315                 320

Phe His Lys Gly Arg Ser Ala Leu Val Leu Gln Tyr Leu Arg Val Pro
                325                 330                 335

Leu Val Asp Arg Ala Thr Cys Leu Arg Ser Thr Lys Phe Thr Ile Tyr
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Asn Asn Met Phe Cys Ala Gly Phe His Glu Gly Gly Arg Asp Ser Cys
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Gln Gly Asp Ser Gly Gly Pro His Val Thr Glu Val Glu Gly Thr Ser
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Phe Leu Thr Gly Ile Ile Ser Trp Gly Glu Glu Cys Ala Met Lys Gly
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Lys Tyr Gly Ile Tyr Thr Lys Val Ser Arg Tyr Val Asn Trp Ile Lys
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Glu Lys Thr Lys Leu Thr
420