

FACTOR VIIa GLYCOFORMS

REFERENCE TO SEQUENCE LISTING

[0001] This application contains a sequence listing submitted by EFS-web, thereby satisfying the requirements of 37 C.F.R. §1.821-1.825.

FIELD OF THE INVENTION

[0002] This disclosure is in the field of biotechnology and concerns the human coagulation protein Factor VII. More particularly, it concerns Factor VII and Factor VIIa glycoforms that have high clearance from a mammalian body

BACKGROUND OF THE INVENTION

[0003] Blood coagulation is a process consisting of a complex interaction of various blood components (or factors) that eventually gives rise to a fibrin clot. Generally, the blood components, which participate in what has been referred to as the coagulation “cascade,” are enzymatically inactive proteins (proenzymes or zymogens) that are converted to proteolytic enzymes by the action of an activator (which itself is an activated clotting factor). Coagulation factors that have undergone such a conversion are generally referred to as “active factors” and are designated by the addition of the letter “a” to the name of the coagulation factor (e.g., Factor VIIa).

[0004] Initiation of the haemostatic process is mediated by the formation of a complex between tissue factor, which is exposed to the circulating blood following injury to the vessel wall, and Factor VIIa, which is present in the circulation in an amount corresponding to about 1% of the total Factor VII protein mass. This complex is anchored to the tissue factor-bearing cell and converts Factors IX and X to their active forms Factor IXa and Factor Xa on the cell surface. Factor Xa converts prothrombin to thrombin on the tissue factor-bearing cell, which activates Factor VIII, Factor V, Factor XI, and Factor XIII. Furthermore, the limited amount of thrombin formed in this initial step of haemostasis also activates the platelets. Following the action of thrombin on the platelets, the platelets change shape and expose charged phospholipids on their surface. This activated platelet surface forms the template for further Factor X activation and the full thrombin generation. The further Factor X activation on the activated platelet surface occurs via a Factor IXa and

Factor VIIIa complex formed on the surface of the activated platelet, and Factor Xa then converts prothrombin into thrombin while still on the surface. Thrombin then converts fibrinogen into fibrin, which is insoluble and which stabilizes the initial platelet plug. This process is localized to the site of the tissue factor exposure thereby minimizing the risk of a systemic activation of the coagulation system. Factor VII and tissue factor have been found to be the main initiators of blood coagulation.

[0005] Factor VIIa is also known to activate Factor X directly, without Factor VIIIa or IXa, if given at sufficiently high levels where it acts independent of tissue factor. Therefore, Factor VIIa is used therapeutically, at higher than physiologic levels, to promote coagulation in patients with hemophilia A and B, particularly for patients having inhibitory antibodies to recombinant Factor VIII or Factor IX that limits the effectiveness of replacement therapy with these factors.

[0006] Factor VIIa is produced from its precursor, Factor VII, which is synthesized in the liver and secreted into the blood where it circulates as a single-chain glycoprotein (molecular weight of about 50,000 Da). Wild-type human Factor VII as used herein has the nucleotide sequence and amino acid sequence disclosed in FIGs. 1 and 2 (SEQ ID NOs. 1 and 2, respectively). Wild type Factor VII is cleaved typically between residues 152 and 153 to produce Factor VIIa. A recombinantly produced Factor VIIa polypeptide is commercially sold in the US under the trade name NOVOSEVEN by Novo Nordisk.

[0007] The term “Factor VII” means a Factor VII polypeptide in its uncleaved form (the zymogen form). The term “Factor VIIa” means a Factor VIIa polypeptide in its activated two-chain form. When the amino acid sequence of SEQ ID NO: 2 is used herein to describe the amino acid sequence of Factor VIIa it will be understood that one of the chains comprises amino acid residues 1-152, and the other chain amino acid residues 153-406.

[0008] Wild type Factor VII is a glycoprotein with N-linked glycosylation sites where sugar residues, called glycans, are attached. Wild type human Factor VII has two N-linked glycans, one at N145 and the other at N322.

[0009] In general, the glycans of glycoproteins contribute to the folding of nascent polypeptide chains, e.g. in the endoplasmic reticulum, and serve to protect the protein moieties from the action of proteases, and serve to modulate biologic activities of a glycoprotein. In contrast to the synthesis of the polypeptide chain of a glycoprotein, which is

genetically regulated, the oligosaccharide units or glycans are attached and processed by a series of enzyme reactions. A glycoprotein thus generally appears as a mixture of different glycoforms resulting from varying enzymatic activity.

[0010] The oligosaccharide component of the N-glycan structures of glycoproteins can influence therapeutic efficacy, physical stability, resistance to protease attack, pharmacokinetics, interaction with the immune system, and specific biological activity. See, for example, Jenkins et al. (1996) *Nature Biotechnol.* 14:975-981.

[0011] U.S. Patent App. Pub. No. 20140363419 (Bauzon et al.) discloses short acting Factor VII polypeptides useful for treatment of acute bleeding and similar disorders. Bauzon et al. discloses Factor VII variants in which the terminal sialic acid molecules found on the oligosaccharides of wild type or mutant Factor VII are missing, which is referred to as desialylated Factor VII. Bauzon et al. discloses that such desialylated variants have enhanced clearance from the blood and a decrease in the duration of efficacy. Bauzon discloses that desialylated wildtype Factor VIIa is efficacious in coagulation assays but shows no observable systemic coagulation. Due to the great medical need for treatment of hemorrhage, particularly acute hemorrhage, improvements and alternative short-acting Factor VIIa glycoproteins is desired.

[0012] U.S. Patent App. Pub. No. 20080058255 (Bolt et al.) discloses glycosylation-disrupted Factor VII variants, such as those having a glycosylation-disrupting substitution at either N145 or N322, or at both N145 and N322. Appa et al, *Thrombosis and Haemostasis* 104.2/2010, pp. 243-251, reports investigations into clearance mechanisms for recombinant Factor VIIa in which asialo-rFactor VIIa is prepared via enzymatic hydrolysis of recombinant Factor VIIa using neuraminidase agarose. Toso et. al. *Blood* (abstract), Nov 16, 2000 discloses Factor VIIa having mutations leading to altered glycosylation including desialylation as compared to wild type human Factor VIIa.

[0013] Desialylation of glycoproteins is known. For example, U.S. Patent App. No. 20080226681 (Glycotope GmbH) relates to using mutant cells (with a mutation in synthesis of sialic acid) to cause desialylation of proteins. The medium is supplemented with sialic acid precursor. Through the described method, a user may determine the proper degree of sialylation of specific protein to cause the highest activity of the specific protein.

[0014] Therapeutic uses for recombinant Factor VIIa are known. However, there is a strong need for a fast acting treatment for acute bleeding conditions, such as postpartum bleed. A fast acting, efficacious Factor VIIa that clears the body rapidly and has few negative coagulation effects on areas outside of the area of trauma is desirable. In particular, there is a need for a fast-acting procoagulant therapeutic with little or no systemic coagulation effects. Improved Factor VIIa glycoforms with Factor VIIa procoagulation activity and rapid and/or high clearance rates are desirable for treatment of acute bleeds.

[0015] Thus, it is an object of the present invention to have Factor VIIa glycoforms and compositions thereof that display pharmacokinetic properties favorable to use for acute bleeding in respect to coagulation activity, half-life, and clearance rate, and have low or no observable systemic coagulation activity.

SUMMARY

[0016] This disclosure concerns a Factor VII or Factor FVIIa glycoprotein having an N-linked glycosylation pattern comprising glycans; wherein the glycosylation pattern comprises:

- (1) substantially no glycans with terminal N-acetylneuraminic acid and
- (2) a substantial glycan that is a glycan of formula I: $(\text{GlcNAc})_4(\text{Man})_3(\text{Gal})_2(\text{Fuc})$ and
- (3) trace amounts or less of one or more glycans selected from the group consisting of a glycan of formula II: $(\text{GlcNAc})_5(\text{Man})_3(\text{Gal})(\text{Fuc})$ and a glycan of formula III: $(\text{GlcNAc})_6(\text{Man})_3(\text{Fuc})$.

[0017] The description comprises the above Factor VII or Factor VIIa glycoproteins that further comprise (1) a glycan of formula IV: $(\text{GlcNAc})_5(\text{Man})_3(\text{Gal})_3(\text{Fuc})$ and/or (2) a glycan of formula V: $(\text{GlcNAc})_6(\text{Gal})_4(\text{Man})_3(\text{Fuc})$. In another embodiment, the Factor VII or Factor VIIa glycoprotein described herein has trace amounts or less of the glycan of formula II. In one further embodiment, the Factor VII or Factor VIIa glycoprotein described herein has trace amounts or less of the glycan of formula III. In a further embodiment, the Factor VII or Factor VIIa glycoprotein described herein comprises an amino acid sequence having three or less mutations relative to SEQ ID NO: 2. In a further embodiment, the Factor VII or

Factor VIIa is a Factor VIIa glycoprotein and comprises a heavy chain; wherein the heavy chain has an N-linked glycosylation pattern comprising glycans; and further wherein the glycosylation pattern comprises: (1) substantially no glycans with terminal N-acetylneuraminic acid and (2) a substantial glycan that is a glycan of formula I: $(\text{GlcNAc})_4(\text{Man})_3(\text{Gal})_2(\text{Fuc})$; (3) trace amounts or less of one or more glycans selected from the group consisting of a glycan of formula II: $(\text{GlcNAc})_5(\text{Man})_3(\text{Gal})(\text{Fuc})$ and a glycan of formula III: $(\text{GlcNAc})_6(\text{Man})_3(\text{Fuc})$; in a further embodiment having trace amounts or less of the glycan of formula III and the glycan of formula I.

[0018] In one embodiment, the Factor VII or Factor VIIa glycoprotein as described above and further herein has trace amounts or less of the glycan of formula II and/or the glycan of formula III. The present disclosure also includes methods of making the Factor VII or Factor VIIa glycoproteins described herein, methods for treating a mammal having a disease or a disorder wherein blood clot formation is desirable by administering therapeutically effective amounts of the Factor VII or Factor VIIa glycoproteins described herein, and pharmaceutical formulations of the Factor VII and Factor VIIa glycoproteins described herein in combination with a pharmaceutically acceptable excipient.

BRIEF DESCRIPTION OF THE DRAWING FIGURES

[0019] Figure 1 sets forth the cDNA sequence for human wild type Factor VII (SEQ ID NO: 1) and the human wild type Factor VII amino acid sequence (SEQ ID NO: 2).

[0020] Figure 2 sets forth a DNA sequence encoding human wild type Factor VII (SEQ ID NO: 3). The region encoding the signal peptide is underlined.

[0021] Figure 3 sets forth a table correlating Roman numerals with glycosylation structures. “Formula” refers to a chemical formula that may be determined by overall glycan mass such as is measured by mass spectroscopy. “Exemplary Structure” shows an example of possible glycan linkages within the scope of the Formula identified by mass. The Exemplary Structures are likely in view of previous published studies of glycans that provided experimentally determined order of the glycan sugars and how they are linked.

[0022] Figure 4 sets forth a codon-optimized DNA sequence of *Artherobacter urefaciens* sialidase catalytic domain (SEQ ID NO: 4).

[0023] Figure 5 sets forth the amino acid sequence of *Artherobacter urefaciens* sialidase catalytic domain (SEQ ID NO: 5). A secretion signal sequence from human growth hormone gene (matgsrtslllafgllclpwlqegsa) was added and is underlined.

[0024] Figure 6 is a plasmid map for the sialidase expression plasmid pMB279. The base expression vector is the 3 Kb UCOE vector (Millipore) that utilizes a human CMV promoter. The puromycin resistance gene was changed to blasticidin resistance (amino acid sequence of GenBank D83710.1) that was codon optimized for mammalian expression.

[0025] Figure 7 is the MALDI (Matrix-assisted laser desorption/ionization mass spectroscopy data from neutral N-glycans for Factor VIIa clones produced in CHO K-1 cells transfected with nucleic acids encoding sialidase and Factor VIIa (subclones 9 and 11), and the MALDI data of the reference preparation, which is NOVOSEVEN recombinant Factor VIIa, referred to as “NOVO 7” herein, desialylated by contact with a sialidase.

[0026] Figure 8 is HPLC data analyzing N-glycans for Factor VIIa produced in CHO K-1 cells (subclones 9, 11 and 9 DS) and for the desialylated reference product NOVO 7, after labeling with 2-AA (2-aminobenzoic acid). Subclone 9 DS refers to subclone 9 that has been further purified.

[0027] Figure 9 shows mass spectrum for Factor VIIa heavy chain that resolves the glycans present on the molecule. Upper panel shows the N-glycan distribution for Factor VIIa heavy chain in Factor VIIa of the present invention that was produced in CHO cells also expressing sialidase. Figure 9 (lower panel) shows the N-glycan distribution for Factor VIIa heavy chain for NOVOSEVEN recombinant Factor VIIa desialylated by contact with a sialidase. The downward arrows mark certain differences between the glycans on desialylated Factor VIIa according to the present disclosure and glycans on desialylated NOVOSEVEN Factor VIIa.

DETAILED DESCRIPTION OF THE INVENTION

[0028] Factor VII and Factor VIIa glycoproteins having specific N-linked glycosylation patterns, methods of their manufacture and use, pharmaceutical compositions comprising them, methods of treatment of disorders where blood clotting is desired, and cell lines to produce them are described herein. The Factor VII and Factor VIIa glycoproteins

have reduced sialic acid content with reference to wild type human Factor VII, and rapid clearance.

[0029] Applicants have discovered recombinant Factor VII and Factor VIIa glycoproteins that are rapidly cleared from the human body and whose pharmacokinetic properties make them suitable for therapeutic use in acute bleeding disorders. The glycoproteins' rapid clearing reduces unnecessary exposure to a pro-thrombotic molecule after the bleeding is resolved. The glycoprotein of the present disclosure has a distinct glycosylation profile when compared to NOVOSEVEN recombinant Factor VIIa (Novo Nordisk) and when compared to NOVOSEVEN recombinant Factor VIIa that has been desialylated by contact with sialidase according to prior teachings.

Definitions

[0030] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry, and nucleic acid chemistry and hybridization are those well-known and commonly employed in the art. Standard techniques are used for nucleic acid and polypeptide synthesis. The nomenclature used herein and the laboratory procedures in analytical chemistry and organic synthesis described below are those well-known and commonly employed in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses. Procedures used for genetic engineering are well known and can be found, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N.Y. All U.S. patents and U.S. patent applications disclosed herein are incorporated herein by reference in their entireties.

[0031] The term "sialic acid" or "sialyl" refers to any member of a family of nine-carbon carboxylated sugars. The most common member of the sialic acid family is N-acetylneuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranos-1-onic acid (often abbreviated as Neu5Ac, NeuAc, or NANA)).

[0032] The terms "polypeptide" and "protein" are used interchangeably herein and refer to a polymer in which the monomers are amino acids and are joined together through

amide bonds. As used herein, "polypeptide" and "protein" refer to both glycosylated and unglycosylated polypeptides and proteins, respectively.

[0033] The term "glycoprotein" refers to a protein having at least one glycan attached. It is understood that glycans are created and formed through enzymatic processes in the cell.

[0034] The term "glycoform" as used herein refers to a glycoprotein containing a particular carbohydrate structure or structures. A glycoprotein having more than one glycosylation site can have the same glycan species attached to each glycosylation site, or can have different glycan species attached to different glycosylation sites. In this manner, different patterns of glycan attachment yield different glycoforms of a glycoprotein.

[0035] The terms "glycosylation pattern" and "glycosylation profile" are used interchangeably herein and mean the characteristic "fingerprint" of the representative N-glycan species that have been released from a glycoprotein composition or glycoprotein product, either enzymatically or chemically, and then analyzed for their carbohydrate structure, for example, using LC-HPLC, or MALDI-TOF MS, and the like. See, for example, the review in *Current Analytical Chemistry*, Vol. 1, No. 1 (2005), pp. 28-57; herein incorporated by reference in its entirety.

[0036] The term "trace amount" of a glycan means that very low levels of that glycan are present in comparison with the amount of other glycans in the glycoprotein. Without limitation, a "trace amount or less" of a glycan means that a MALDI-TOF MS, LC-HPLC or similar analytical test shows a glycosylation profile in which the glycan is present at less than 10%, preferably less than 5%, preferably less than 4%, less than 3%, less than 2%, less than 1%, and less than 0.5% or less than 0.1% of the total amount of N-glycan species appearing in the profile, or that is not detectable in the analytical test.

[0037] The term "significant glycan" or "substantial glycan" refers to a glycan that is observed under MALDI-TOF MS, LC-HPLC or similar analytical tests to be present in the glycosylation profile as being more than 10%, more than 20%, more than 25%, or more than 30% of the total amount of N-glycan species appearing in the profile. A quantitative determination of glycans may be made, for example, by LC-MS analysis in which the area under the curve (AUC) of the peaks is compared to determine the relative quantity of each glycan in a protein batch. Alternatively, the "significant glycan" or "substantial glycan" may

be present in the glycosylation profile as 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35% or more of the total amount of N-glycan species appearing in the glycosylation profile for the product.

[0038] "N-glycan," "N-linked glycan," and "glycan" are used interchangeably and refer to an N-linked oligosaccharide, e.g., one that is or was attached by an N-acetylglucosamine (GlcNAc) residue linked to the amide nitrogen of an asparagine residue in a protein. The predominant sugars found on glycoproteins are glucose, galactose (Gal), mannose (Man), fucose (Fuc), N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), and sialic acid (e.g., N-acetyl-neuraminic acid (NeuAc)). A "*" at the end of a glycan represents the reducing end of the sugar chain that binds to asparagine. An N-linked glycan can be shown by symbolic depiction such as those in Figure 3. The symbols disclosed in Figure 3 are used in other figures and have the same meaning throughout their use herein.

[0039] By "oligomannosidic core structure" or "trimannose core structure" of a complex N-glycan is intended the core structure that comprises three mannose (Man) and two N-acetylglucosamine (GlcNAc) monosaccharide residues that are attached to the asparagine residue of the glycoprotein. Subsequent glycosylation steps yield the final complex N-glycan structure.

[0040] The N-glycans attached to glycoproteins differ with respect to the number of branches (antennae) comprising peripheral sugars (e.g., GlcNAc, galactose, fucose, and sialic acid) that are added to the trimannose core structure. N-glycans are commonly classified according to their branched constituents (e.g., complex, high mannose, or hybrid). A "complex" type N-glycan typically has at least one GlcNAc attached to the 1,3 mannose arm and at least one GlcNAc attached to the 1,6 mannose arm of a "trimannose" core. Where one GlcNAc is attached to each mannose arm, the species of N-linked glycan is denoted as "GlcNAc₂Man₃GlcNAc₂." Where only one GlcNAc is attached, the N-glycan species is denoted as "GlcNAc₁Man₃GlcNAc₂", wherein the GlcNAc is attached to either the 1,3 mannose arm (denoted "MGn") or the 1,6 mannose arm (denoted "GnM"). Complex N-glycans may also have galactose ("Gal") or N-acetylgalactosamine ("GalNAc") sugar residues that are optionally modified with sialic acid or derivatives (e.g., "NeuAc," where "Neu" refers to neuraminic acid and "Ac" refers to acetyl). Where a galactose sugar residue is attached to each GlcNAc on each mannose arm, the species of N-linked glycan is denoted as

"Gal₂GlcNAc₂Man₃GlcNAc₂." Complex N-glycans may also have intrachain substitutions comprising "bisecting" GlcNAc and core fucose ("Fuc"). Complex N-glycans may also have multiple antennae on the "trimannose core," often referred to as "multiple antennary glycans." A "high mannose" type N-glycan has five or more mannose residues. A "hybrid" N-glycan has at least one GlcNAc on the terminal of the 1,3 mannose arm of the trimannose core and zero or more mannoses on the 1,6 mannose arm of the trimannose core.

[0041] Many molecules of the present disclosure have very high clearance from circulation in a human. The clearance may be measured by means known to those of ordinary skill in the art, such as by measuring the terminal half-life, which is measured after any initial distribution of the molecule occurs.

[0042] Half-life can be determined in test animals, for example, by administering a dose of about 25-250 microgram/kg of the preparation; obtaining plasma samples at predetermined times after administration; and determining the content of the Factor VIIa polypeptide in the samples using one or more of a clotting assay (or any bioassay), an immunoassay, or an equivalent. The data can be displayed graphically and then the bioavailability will be determined as the area under the curve. In certain examples, rat or murine models are used for half-life measurements. Relative bioavailability of a Factor VIIa polypeptide or composition thereof refers to the ratio of the area under the curve of the short-acting Factor VIIa polypeptide to that of wild-type Factor VIIa or another appropriate comparator polypeptide or protein.

[0043] The glycoproteins of the present disclosure may be produced in host cells. A cell may be "derived from" a host cell when it is obtained from the host cell by any of the normal growth and culture techniques known to those of ordinary skill in the art, such as normal mitotic cell division, cloning such as single cell dilution cloning, etc. "Derived from" a host cell also includes recombinant modifications made to the host cell that do not affect the primary function of expressing Factor VII and sialidase. A heterologous protein is a protein that a cell has been engineered to produce.

Preferred Embodiments

[0044] In one embodiment, the Factor VII or VIIa glycoprotein comprises one or two, preferably two, N-linked glycans and has a glycosylation pattern characterized by reduced sialylation compared to wild type human Factor VII or VIIa. Preferably the moles of

conjugated sialic acid per mole of N-linked glycan in the glycoprotein is less than 0.05, less than 0.1, less than 1.0, less than 2.0, less than 3.0, less than 4.0, less than 5.0 or less than 6.0, or the ratio of moles of conjugated sialic acid per mole of N-linked glycan is within a range selected from the group consisting of (1) from 0 to 8; (2) from 0 to 7; (3) from 0 to 6; (4) from 0 to 5; (5) from 0 to 4; (6) from 0 to 3; (7) from 0 to 2; (8) from 0 to 1 and (9) from 0 to 0.5, or ratios of from 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, 1 to 2, 2 to 8, 2 to 7, 2 to 6, 2 to 5, 2 to 4, 2 to 3, 3 to 8, 3 to 7, 3 to 6, 3 to 5, 3 to 4, 4 to 8, 4 to 7, 4 to 6, 4 to 5, and 0.1 to 1. The ratio is a measurement of the moles of sialic acid bound to a glycoprotein relative to the number of glycans on the glycoprotein. Most preferably, the glycoprotein has one or two N-linked glycans and trace amounts or less of sialic acid covalently attached to these glycans. Still more preferably, the glycoprotein has substantially no glycans with terminal N-acetylneuraminic acid, and has two N-linked glycans. The number of glycans refers to the number of sugar moieties attached to an N-linked glycan in the glycoprotein, where one N-linked glycosylation site can support only one glycan as defined herein for purposes of this ratio.

[0045] The ratio is determined using a sialic acid fluorescence labeling kit such as that sold by Takara Bio Inc. (cat. #4400). Such a sialic acid fluorescence labeling kit includes a step for the release of sialic acid from the bound glycoprotein, such as by partial acid hydrolysis or by use of sialidase, such as *Arthrobacter ureafaciens* sialidase. The free sialic acids are then labeled with a fluorophore such as 1,2-diamino-4,5-methyleneoxybenzene ("DMB"). The labeled sialic acids are then quantitatively measured using HPLC and comparing peak heights to a calibration curve. Thus, the ratio measured is a ratio of moles of sialic acid per mole of glycan released from all the Factor VII or Factor VIIa glycoproteins of the composition.

[0046] By having reduced sialylation, the Factor VII and VIIa glycoproteins have a shorter terminal half-life than wild type Factor VII or VIIa.

[0047] In one series of embodiments, the compositions of Factor VII or Factor VIIa glycoproteins or the isolated polypeptides themselves have a terminal half-life as measured in human or mammalian plasma, for example murine or rat plasma, of less than 2 hours, less than 1.5 hours, less than 1 hour, less than 0.75 hour, less than 0.5 hour, less than 0.25 hour, less than 0.1 hour, or so short that it cannot reasonably be measured.

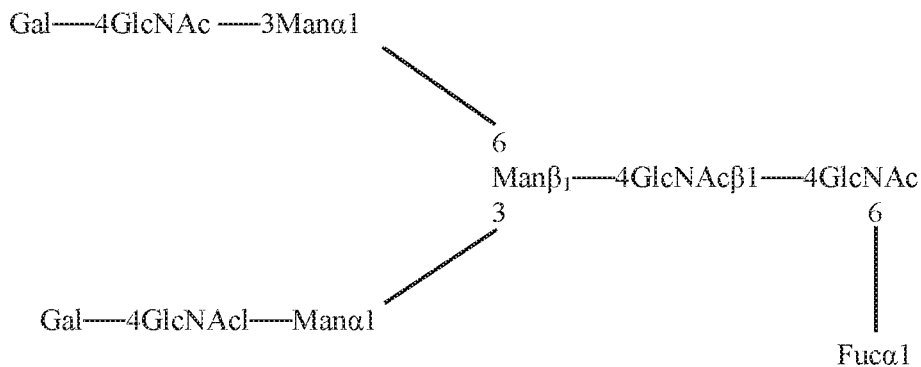
[0048] The Factor VII or Factor VIIa glycoform preferably has an N-linked glycosylation pattern that has substantially reduced sialylation as disclosed above, such as substantially no glycans with a terminal N-acetylneuraminic acid, and has a substantial glycan that is a glycan of formula I and trace amounts or less of the glycan of formula II and/or of the glycan of formula III. In another embodiment, the Factor VII or Factor VIIa glycoform has trace amounts or less of glycans of formula III, in another embodiment has trace amounts or less of glycans of formulae II and III, and in another embodiment has glycans of formula I and trace amounts or less of glycans of formulae II and III. In another embodiment, the Factor VII or Factor VIIa glycoform has an N-linked glycosylation pattern that has substantially reduced sialylation as disclosed above, such as substantially no glycans with a terminal N-acetylneuraminic acid, and has glycans of formula IV, in another embodiment has glycans of formula IV and formula V, in another embodiment has glycans of formulae IV, V and XI, and in another embodiment has glycans of formulae IV, V, XI, and I. In one embodiment, the Factor VII or Factor VIIa glycoform has an N-linked glycosylation pattern that has substantially reduced sialylation as disclosed above, such as substantially no glycans with a terminal N-acetylneuraminic acid, and has trace amounts or less of glycans of formula II and/or of formula III.

[0049] The glycan patterns as described herein are described by mass-derived formulae that may be generic to encompass more than one structure having the same mass but different linkages between the glycan. The “Exemplary Structure” column in Figure 3 shows possible glycan linkages for the structures of each of formulae I-XI.

[0050] Using nomenclature that identifies specific glycan linkages, and is consistent with the glycan mass data described herein, one embodiment of the invention is a Factor VII or Factor VIIa glycoprotein having an N-linked glycosylation pattern comprising glycans; wherein the glycosylation pattern comprises:

- (1) substantially no glycans with terminal N-acetylneuraminic acid and

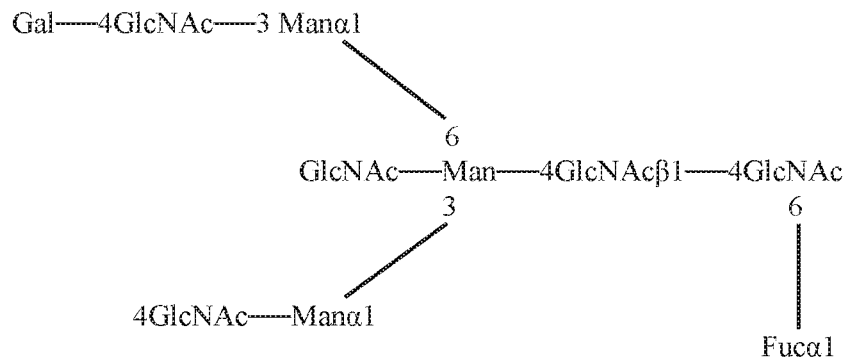
- (2) a substantial glycan that is a glycan of the following formula Ia



formula Ia

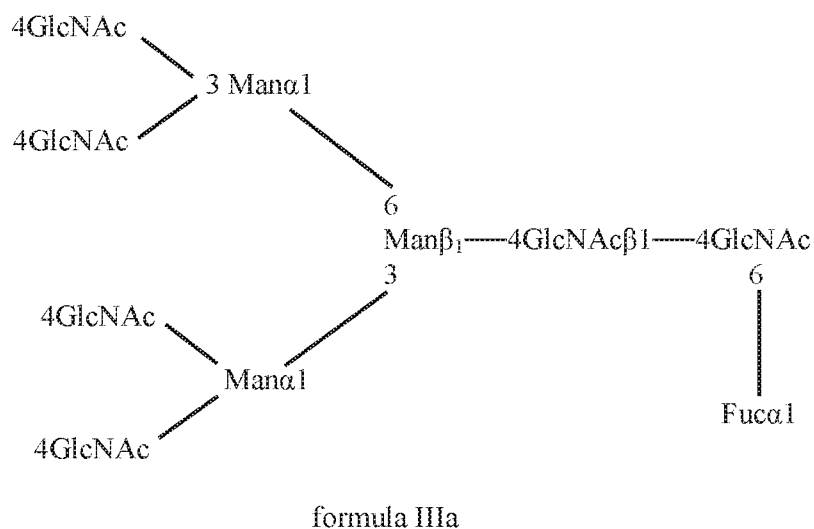
and

- (3) trace amounts or less of one or more glycans selected from the group consisting of a glycan of formula IIa:



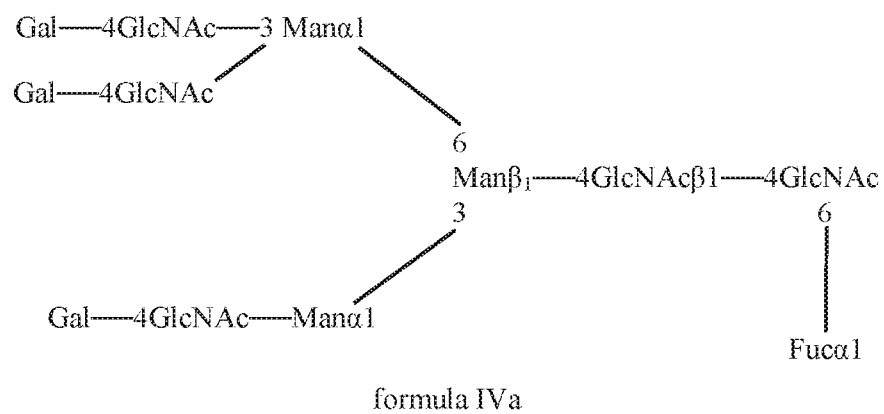
formula IIa

and a glycan of formula IIIa:

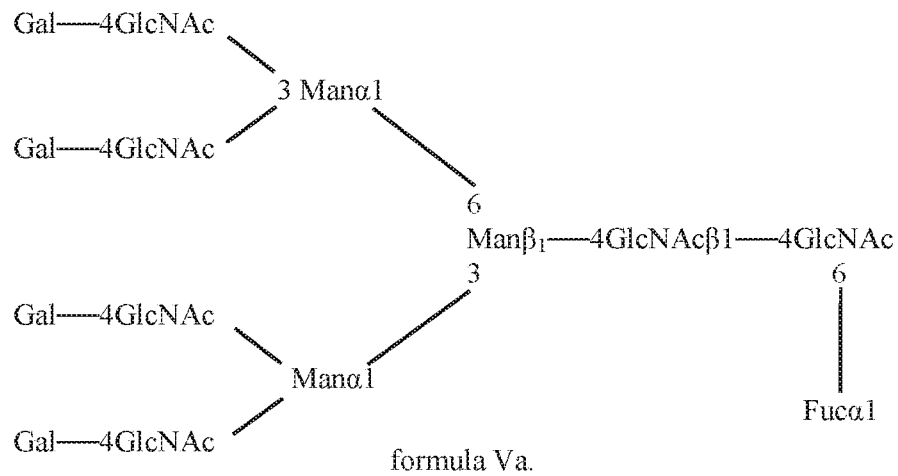


In a further embodiment, the Factor VII or Factor VIIa glycoprotein described immediately above has a glycosylation pattern that further comprises:

(1) a glycan of formula IVa

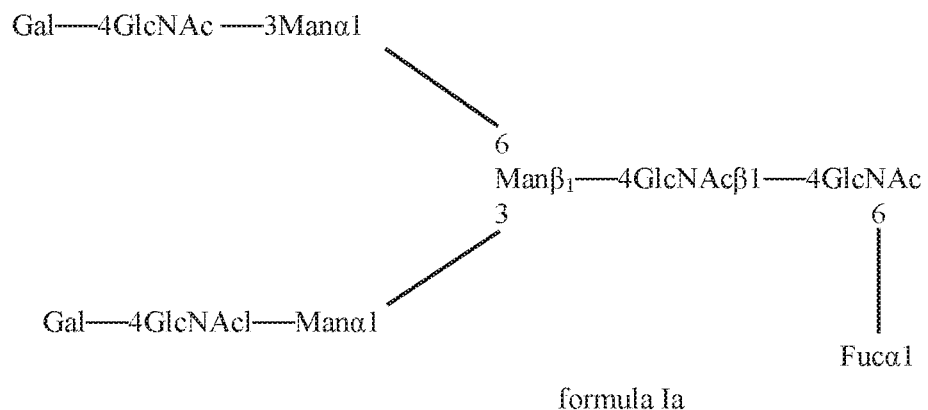


and/or (2) a glycan of formula Va



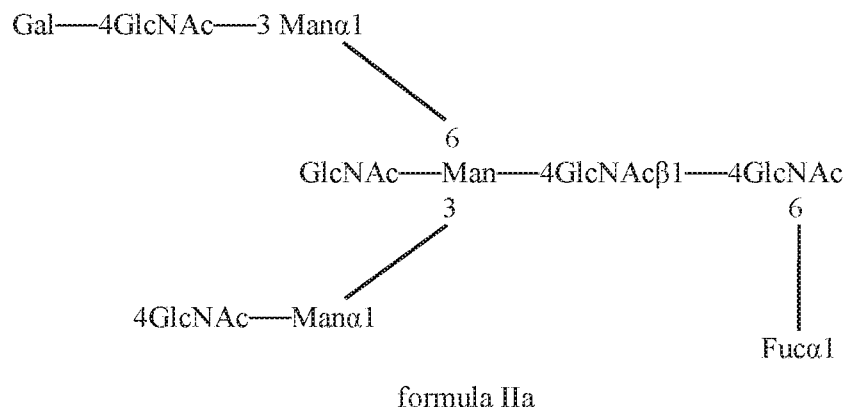
The Factor VII or Factor VIIa glycoprotein described above may have trace amounts or less of the glycan of formula IIa and/or the glycan of formula IIIa. In another embodiment of the invention, the Factor VIIa glycoprotein comprises a heavy chain; wherein the heavy chain has an N-linked glycosylation pattern comprising glycans; and further wherein the glycosylation pattern comprises:

- (1) substantially no glycans with terminal N-acetylneuraminic acid and
- (2) a substantial glycan that is a glycan of formula Ia:

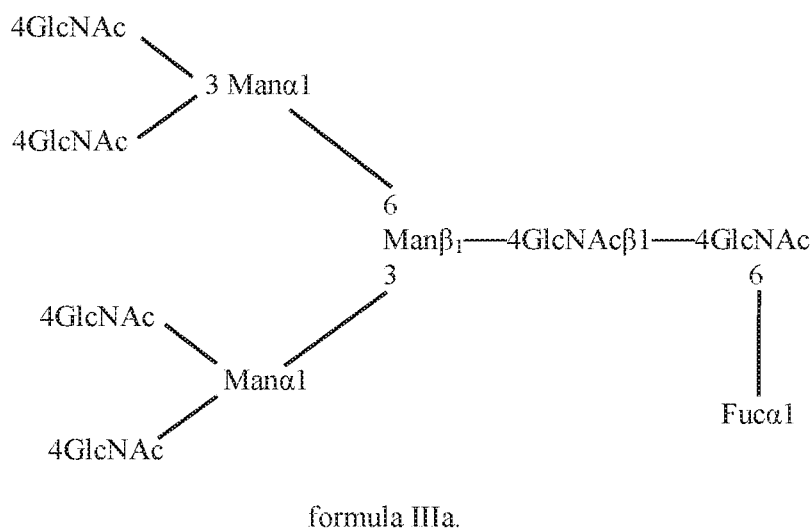


and

(3) trace amounts or less of one or more glycans selected from the group consisting of a glycan of formula IIa:



and a glycan of formula IIIa:



[0051] In certain embodiments, the amino acid sequence of the Factor VII or Factor VIIa glycoprotein comprises, contains, consists essentially of or consists of the amino acid sequence of wild type human Factor VII or Factor VIIa (SEQ ID NO: 2). In one embodiment, the glycoprotein has an amino acid sequence with 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid differences from the amino acid sequence of SEQ ID NO: 2, and preferably has conservative amino acid substitutions when substitutions are present. In another embodiment, the amino acid sequence of the Factor VII or Factor VIIa glycoprotein comprises, contains, consists essentially of or consists of the amino acid sequence of SEQ ID NO: 2 and has an amino acid

sequence with 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid differences from the amino acid sequence of SEQ ID NO: 2, provided that the amino acid sequence does not comprise or consist of the amino acid sequence of SEQ ID NO: 2 having the mutations P10Q, K32E, A34E, R36E, T106N, and V253. In one embodiment, the glycoprotein has an amino acid sequence with 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid differences from the amino acid sequence of SEQ ID NO: 2 and the amino acid sequence does not comprise or consist of the amino acid sequence of SEQ ID NO: 2 having only the mutations P10Q, K32E, T106N and V253N, or only the mutations P10Q, K32E, A34E, R36E, T106N, and V253N. It is to be understood that “having only the mutations” means that amino acid sequences with additional mutations as well as the listed mutations are outside the scope of the disclaimer.

[0052] In another embodiment, the glycoprotein has an amino acid sequence that has 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 89, 88, 87, 86, 85, 84, 83, 82, 81, 80, 75, or 70% homology with the amino acid sequence of SEQ ID NO: 2 and has Factor VII or Factor VIIa activity. In another embodiment, the glycoprotein has an amino acid sequence that has 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 89, 88, 87, 86, 85, 84, 83, 82, 81, 80, 75, or 70% homology with the amino acid sequence of SEQ ID NO: 2 and has factor VII or Factor VIIa activity, provided that the amino acid sequence does not comprise or consist of SEQ ID NO: 2 having only mutations P10Q, K32E, A34E, R36E, T106N, and V253. In a further embodiment, the glycoprotein has an amino acid sequence that has 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 89, 88, 87, 86, 85, 84, 83, 82, 81, 80, 75, or 70% homology with the amino acid sequence of SEQ ID NO: 2 and the amino acid sequence does not comprise or consist of the amino acid sequence of SEQ ID NO: 2 having mutations at only P10Q, K32E, T106N and V253N, or at only P10Q, K32E, A34E, R36E, T106N, and V253N.

[0053] In another embodiment the Factor VII or Factor VIIa glycoprotein is encoded by a nucleic acid sequence that encodes the amino acid sequence of SEQ ID NO: 2, or encodes a polypeptide having an amino acid sequence having 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 89, 88, 87, 86, 85, 84, 83, 82, 81, 80, 75, or 70% homology with the amino acid sequence of SEQ ID NO: 2 and having Factor VII or Factor VIIa activity. In another embodiment, the Factor VII or Factor VIIa glycoprotein is encoded by a nucleic acid sequence that encodes the amino acid sequence of SEQ ID NO: 2, or encodes a polypeptide having an amino acid sequence having 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 89, 88, 87, 86, 85, 84, 83, 82, 81, 80, 75, or 70% homology with the amino acid sequence of SEQ ID NO: 2

and having Factor VII or Factor VIIa activity, and the amino acid sequence does not comprise or consist of the amino acid sequence of SEQ ID NO: 2 having the mutations P10Q, K32E, T106N and V253N, or having the mutations P10Q, K32E, T106N and V253N and P10Q, K32E, A34E, R36E, T106N, and V253N.

[0054] In one embodiment the Factor VII or Factor VIIa glycoprotein is encoded by a nucleic acid that comprises, contains, consists essentially of, or consists of the nucleotide sequence encoding wild type human Factor VII (SEQ ID NO:1). In one embodiment, the nucleotide sequence encoding the glycoprotein has 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 nucleic acid differences from the nucleotide sequence of SEQ ID NO:1 and encodes a polypeptide that has Factor VII or Factor VIIa activity, in one embodiment provided that the amino acid sequence of the polypeptide does not consist of SEQ ID NO: 2 having only mutations P10Q, K32E, A34E, R36E, T106N, and V253; and in another embodiment provided that the amino acid sequence of the polypeptide does not have only the mutations P10Q, K32E, T106N and V253N. In another embodiment, the nucleotide sequence encoding the glycoprotein has a nucleotide sequence that has 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 89, 88, 87, 86, 85, 84, 83, 82, 81, 80, 75, or 70% homology with the nucleotide sequence of SEQ ID NO:1 and encodes a protein having Factor VII or Factor VIIa activity, in one embodiment provided that the amino acid sequence of the protein does not consist of SEQ ID NO: 2 having only mutations P10Q, K32E, A34E, R36E, T106N, and V253N; and in another embodiment provided that the amino acid sequence does not only having mutations P10Q, K32E, T106N and V253N. In another embodiment, the Factor VII glycoprotein is encoded by a nucleic acid that encodes wild type human Factor VII and whose sequence is determined by codon optimization for the host cell. In one embodiment, the nucleic acid encoding the Factor VII glycoprotein of the present disclosure comprises, contains, or consists of the nucleic acid sequence of SEQ ID NO: 3 or a nucleic acid sequence that comprises, contains, or consists of a nucleotide sequence that has 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 89, 88, 87, 86, 85, 84, 83, 82, 81, 80, 75, or 70% homology with the nucleotide sequence of SEQ ID NO: 3 and encodes a protein having Factor VII or Factor VIIa activity, in one embodiment provided that the amino acid sequence of the protein does not consist of SEQ ID NO: 2 having only mutations P10Q, K32E, A34E, R36E, T106N, and V253N; and in another embodiment provided that the amino acid sequence of the protein does not consist of SEQ ID NO: 2 having only mutations P10Q, K32E, T106N and V253N.

[0055] The glycoproteins of the present disclosure may be recombinantly produced in a Chinese Hamster Ovary (CHO) cell line that is a CHO K-1 or CHO-S cell line under conditions such as those in the examples or under other culture conditions that allow for suitable cell productivity for recombinant protein expression and recovery. Other suitable CHO cell lines may be identified by one of ordinary skill in the art by routine testing in view of the knowledge of various CHO cell lines. See, e.g., Wurm, Florian, "CHO Quasispecies – Implications for Manufacturing Processes," *Processes* (2013), 1, 296-311; doi: 10.3390/pr1030296. The CHO host cell is recombinantly transfected with a nucleic acid encoding Factor VII and a nucleic acid encoding sialidase. In certain embodiments, the sialidase nucleic acid is from the species *Artherobacter urefaciens* or derived therefrom, such as by codon optimization, for example, having the nucleic acid sequence of SEQ ID NO: 4, in either case expressing a sialidase comprising the amino acid sequence of SEQ ID NO: 5 as shown in Figure 5 with or without the secretion signal sequence. In another embodiment, the host cell is transfected with a nucleic acid encoding sialidase having at least 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, or 80% identity to the amino acid sequence of SEQ ID NO: 5 with or without the secretion signal sequence. The transfected host cell may be cultured under the following conditions: CD-Fusion medium (Sigma-Aldrich) with 6 mM GlutaMAX® media (Gibco), which contains L-glutamine, and 30 ng/mL vitamin K. It is believed the glycoproteins of the present disclosure may be produced from CHO host cells cultured in ActiCHO cell culture media (GE Healthcare) supplemented with glutamine and vitamin K as appropriate for the CHO cell line. Other media in which CHO cells can grow may potentially also be suitable (e.g., CD-CHO medium from ThermoFisher Scientific).

[0056] Pharmaceutical formulations of the Factor VII or Factor VIIa glycoproteins and compositions thereof comprising the Factor VII or Factor VIIa polypeptide and a pharmaceutically acceptable excipient or carrier are also useful. In certain examples, the pharmaceutical formulations are for parenteral administration, such as by intravenous, subcutaneous or intramuscular administration, and dosing may be as a single bolus dose, intermittent dosing, or as a continuous intravenous infusion. Topical formulations are also useful. One embodiment comprises a pharmaceutical formulation comprising a Factor VII or Factor VIIa glycoprotein as described herein in a lyophilized preparation that is reconstituted at the time of use. Alternatively, the pharmaceutical formulation can be a stable liquid ready-to-use formulation not requiring reconstitution. The pharmaceutical formulation can be a

lyophilized powder in single-use vials of 1, 2, 5, or 8 mg of Factor VII polypeptide. After reconstitution with a specified volume of liquid, such as sterile water containing histidine, the final solution can contain any suitable amount of Factor VII or Factor VIIa glycoprotein that produces a therapeutic effect, such as, without limitation, 0.2 mg/mL, 0.5 mg/mL, 1 mg/mL (1000 micrograms/mL), 2 mg/mL, 3 mg/mL, 4 mg/mL, 5 mg/mL, 1-2 mg/mL, 1-3 mg/mL, 1-5 mg/mL, 1-10 mg/mL, 0.2-1.0 mg/mL, 0.5-1 mg/mL, 0.5-2 mg/mL, 0.5-5 mg/mL, 0.5-4 mg/mL, or 0.5-10 mg/mL of Factor VIIa or Factor VII glycoprotein. Proper dosage for administration to a patient can be readily determined by persons skilled in the art based upon, for example, the weight of the patient, the type of bleeding disorder or episode being treated, and the activity of the particular Factor VIIa or Factor VII polypeptide being employed. In certain examples, dosing can be in the range of 70-110 micrograms/kg, 70-90 micrograms/kg, or 80-100 micrograms/kg and can be 90 micrograms/kg. The lyophilized powder may be reconstituted with an aqueous carrier, such as water, buffered water, 0.4% saline, 0.3% glycine, etc. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Company, Easton, Pa. (1990). Topical application, such as can be advisable in the case of trauma, can be carried out by means of a spray, perfusion, catheters, stent, vascular graft or stent, ointment, or other preparation known in the art. In certain examples, topical administration can be by way of a solid or semi-solid matrix, such as a surgical sponge or collagen matrix, which has been treated with, infused with, coated with, or soaked in a composition comprising the Factor VIIa variant. Methods of preparing such matrices are well known in the art (see, e.g., Thrombosis/Hemostasis 12:445, 2006) and the skilled artisan would be able to readily determine an appropriate dose and method of application of the composition onto the given matrix.

[0057] In one embodiment, the present disclosure relates to kits comprising the Factor VII or Factor VIIa glycoprotein. In certain examples, the kit contains a vial containing ready-to-use liquid containing the glycoprotein in a suitable pharmaceutical composition. In other examples, the kit contains a vial containing lyophilized Factor VII or Factor VIIa glycoprotein, or a lyophilized formulation comprising the glycoprotein, and also a diluent for reconstitution. In other examples, the kit contains a topical formulation of the Factor VII or Factor VIIa glycoprotein, for example, an ointment, spray, or liquid, and a matrix such as a

sponge or other medical matrix to which the topical formulation may be applied before administration to the patient.

[0058] The Factor VII or Factor VIIa glycoproteins and compositions described herein are useful for the treatment of blood clotting disorders, and those disorders that benefit from blood coagulation, particularly for coagulation with a drug having a shorter half-life than wild type Factor VII, and are useful in making medicaments to treat these disorders. Accordingly, the Factor VII or Factor VIIa glycoproteins and compositions herein are useful for hemorrhage, penetrating traumatic injury; blunt traumatic injury; bleeding in elective surgery; bleeding in cardiac surgery; bleeding in spinal surgery; orthopedic surgery; neurosurgery; oncology surgery; postpartum surgery; postpartum hemorrhage; menorrhagia; bleeding in stem cell transplantation; bleeding in liver transplantation; gastrointestinal bleeding; active variceal bleeding in cirrhosis; non variceal bleeding in cirrhosis; diffuse alveolar hemorrhage; aortic aneurysm; intracerebral hemorrhage; traumatic brain injury; brain contusion; reversal of warfarin; reversal of heparin; reversal of anticoagulants; reversal of anti-thrombotics; Factor VII deficiency; burns; prophylaxis in hemophilia patients with inhibitors; partial hepatectomy for non-cirrhotic and cirrhotic patients; acquired hemophilia; idiopathic thrombocytopenic purpura; Glanzmann's Thrombasthenia; Glanzmann's Thrombasthenia refractory to platelet transfusion and Bernard-Soulier Syndrome. In certain embodiments, the Factor VII glycoforms of the present disclosure are useful for treating hemorrhage, gastrointestinal bleeding, uncontrolled bleeding, bleeding in a mammal undergoing transplantation or resection or surgery, variceal bleeding, thrombocytopenia, hemophilia, intracranial hemorrhage, aortic aneurysm, and over administration of an anticoagulant.

[0059] Postpartum hemorrhage is a leading cause of maternal mortality. Thus, there is a need for effective treatment that can be met by the Factor VII glycoforms of the present disclosure. Postpartum hemorrhage has been defined as a blood loss of more than 500 mL following vaginal delivery or more than 1000 mL following cesarean delivery. A woman with pre-existing anemia or certain other disorders may have less ability to cope with such a blood loss and so may suffer from postpartum hemorrhage with lower amounts of blood loss than those identified above. In view of this, some clinicians suggest that postpartum hemorrhage should be defined to include any amount of blood loss that threatens the hemodynamic stability of the woman. Rapid recognition and diagnosis of postpartum

hemorrhage followed by rapid initiation of appropriate management is essential to successful management of the disorder.

Examples

[0060] A Chinese Hamster Ovary (CHO-K1) cell line acquired from SAFC (Sigma-Aldrich) was used as the parent cell line for transfection. A cryopreserved cell bank of this CHO-K1 cell line was prepared in CD-Fusion medium and sterility, mycoplasma and non-endogenous virus (adventitious virus and minute mouse virus) were tested before its being used for cell line construction.

[0061] Cell line construction was performed in CD-Fusion medium with 6 mM GlutaMAX® media (Gibco), and 30 ng/mL vitamin K. The CHO-K1 cells were transfected with linearized pMB246 plasmid DNA using an Amaxa Nucleofector II device with an Amaxa Cell Line Nucleofector Kit V (Lonza Biologics, Slough, UK) using the U-023 program. The pMB246 plasmid contained a nucleic acid sequence encoding human wild type Factor VII of SEQ ID NO: 2. The nucleic acid was codon optimized. Twenty-four hours after transfection the transfectants were directly plated out in 96-well plates with CD-Fusion selection medium containing 10 ug/ml puromycin (Invitrogen). Following drug selection and screening cells from the best-producing, drug-resistant, single foci wells were selected for expansion. After further characterization of the top clones based on specific chromogenic activity the final candidate cell line was selected and a Research Cell Bank (RCB) was produced. These cells were super-transfected with linearized pMB279 plasmid DNA (see Figure 6) using a similar method as described above. Twenty-four hours after transfection the cells were directly plated out in 96-well plates and with CD-Fusion selection medium containing 30 or 50 ug/ml blasticidin selection antibiotic (Invitrogen). After drug selection and screening, the drug-resistant, single foci wells were selected for expansion. After further characterization of the top eight clones based on protein quality and productivity, the final five candidate cell lines were selected and Research Cell Banks (RCB) of those five cell lines were generated.

[0062] Both sialidase and Factor VII are secreted from the engineered cell line. As discussed in detail below, the Factor VII zymogen in the harvest was purified and activated to active Factor VIIa using a modification of known methods. See, e.g., U.S. Pat. App. Pub.

20090047723, "Method for Purification of Factor VII," (Rikke Bolding Jensen and Frank Bech Nygaard).

[0063] Factor VII expressed in mammalian cells has a number of post-translational modifications, including modification of up to 10 glutamate residues within its N-terminal Gla domain by vitamin K-dependent carboxylation to form γ -carboxyglutamate (Gla) residues. Since Factor VII can be expressed with a variable number of Gla residues, a key goal of the purification is to isolate desialylated Factor VII with a high content of Gla modifications (mostly 9 to 10 for wild type).

[0064] In the first step of purification, the harvest media was changed into a buffer solution, concentrated and frozen for later processing. The second step, capture of the product by anion exchange chromatography (AIEC), allows for further concentration of the product and preparation for the third step, hydroxyapatite chromatography (HAC). This later step provides a substantial purification of the product, and can also remove forms of FVII present with a low content of Gla modifications. A fourth step is a second AIEC, which prepares the protein product for the in-solution activation step such as by altering protein concentration and changing the buffer.

[0065] Because Factor VII is expressed in the single chain zymogen form, an activation step is needed to activate the protein by cleavage between the R and the I (residue 153 of the mature protein). An "in solution activation step" was used that involves an incubation of the protein at room temperature under conditions that promote the auto proteolytic cleavage of Factor VII to Factor VIIa. The activation step was followed with a polishing step (third AIEC) and dialysis of the final product into formulation buffer. The final protein is stored at -70 deg C.

[0066] N-linked glycans were removed by enzyme treatment, and the glycans were analyzed using MALDI mass spectrometry. The results are shown in Figure 7. The legend for the Roman numerals used in Figure 7 is found in Figure 3. As shown in Figure 7, the N-linked glycans from desialylated clones are mostly fucosylated bi-antennary (see, e.g., formula I peak) and tri- and tetra-antennary structures (see, e.g., formula IV and V peaks). The mass and the intensity of the peak can be used to determine the glycan composition, and the relative abundance of detectable glycan structures. The data in Figures 7 and 8 is for CHO-K1 cell line subclones 9, 11, and (for Figure 8) 9 DS, which is subclone 9 purified to a

greater extent than the sample marked subclone 9, compared to desialylated NOVOSEVEN. Subclone 9 and subclone 11 were purified and auto-activated. Subclone 9 DS was further purified using tangential flow filtration. Thus all the samples were activated (i.e., desialylated wild type FVIIa).

[0067] Figure 8 shows analysis of glycans from NOVOSEVEN and from clones of the engineered CHO K-1 cell by the method of HPLC after 2-AA labeling. N-linked glycans are enzymatically released, labeled with 2-AA using standard methods, and then analyzed by HPLC. “ds” as in the title of Figure 8 refers to a glycoprotein substantially desialylated. Glycans were quantified as shown in the following table:

FVIIa clone	Percent of total glycans			
	I	IV	V	other
Novo7	45%	nd*	nd	35
Clone 9	34.1	35.7	24.2	6
Clone 11	45.8	28.0	13.9	12.2
Clone 9 DS	27.1	26.9	18.0	28

nd= not detectable

[0068] The N-glycosylation pattern of the heavy chain of recombinant Factor VIIa was analyzed using LC-mass spectrometry for structural characterization, resolved by HPLC after reduction of the activated Factor VIIa molecule. These data therefore, show only those N-linked glycans on the HC, which are a subset of the total pool of N-linked glycans present on the intact HC and HL heterodimer. Figure 9 reports the glycosylation profile for the desialylated heavy chain (“HC”) of the reference product, NOVOSEVEN recombinant Factor VIIa and that of a Factor VIIa glycoprotein of the present disclosure. Desialylated NOVOSEVEN recombinant Factor VIIa may be produced by gentle mixing of a slurry of neuraminidase-agarose beads (Sigma N5254) and a buffer solution of rFVIIa at room temperature for about 16-24 hours. One of ordinary skill can envision additional methods to generate such an enzymatic desialylation. The N-linked glycosylation on the heavy chain of the reference desialylated NOVOSEVEN Factor VIIa product is mostly bi-antennary structure with bisecting and different sialylation (terminal sialic acid content: 0, 1, or 2). Very small amounts of tri-antennary structure were observed.

[0069] In contrast, the heavy chain of the Factor VIIa glycoprotein of the present disclosure has a different glycosylation pattern from the reference desialylated NOVOSEVEN product. Figure 9 (top panel) shows the N-glycan distribution for the HC on a desialylated Factor VIIa according to the present invention produced in a research cell line using a CHO-S cell transfected with nucleotides encoding recombinant wild type human Factor VII and sialidase active domain of *A. urefaciens*. In Figure 9, the downward arrows show two glycans present in desialylated NOVOSEVEN but substantially or completely absent from the Factor VII glycoforms of the present invention. As shown in Figure 9, the glycoprotein of the present invention, when analyzed by LC-MS, shows no glycoforms corresponding to formula II and formula III. However, the HC of the reference product shows significant amounts of each of these glycoforms.

[0070] Subclone 9 DS retained activity in vitro as measured by phospholipid-Factor Xa (PL-Xa) activation. Activity of Factor VIIa may also be assessed using standard thrombin generation assay conditions in normal pooled human plasma. 1 pM tissue factor is the activator and the sample is run under 4 micromolar lipid concentration. Clearance may be measured in an in vitro hepatocyte clearance assay. Increased clearance may be measured in vivo in rat and in a HemA PK study.

[0071] The Factor VII and Factor VIIa glycoproteins of the present disclosure have glycans that can serve as ligands for clearance receptors, such as the asialoglycoprotein receptor (ASGPR) in the liver. This receptor binds to glycans having terminal galactose residues. In particular, ASGPR binds glycans with multiple arms terminating with man-GlcNAc-Gal. The presently disclosed glycoproteins have reduced levels of glycans found on other recombinant Factor VII and Factor VIIa molecules such as NOVOSEVEN recombinant Factor VIIa, or glycans ending in GlcNAc. These glycans have the potential for interaction with other cell receptors, such as Man/GlcNAc receptors that facilitate uptake into macrophages and other cells, such as dendritic cells. Without wanting to be limited by theory in any way, it is believed that such glycans can promote a different mechanism of clearance, compared to the presently disclosed glycoproteins, and therefore, have a different clinical utility. For example, the rate of uptake into macrophages can be different than uptake by the ASGPR, resulting in different duration of actions and bio-distributions. Importantly, uptake into dendritic cells via Man/GlcNAc receptors has been associated with higher rates of immunogenicity. The role of different glycan structures may be particularly important for

clearance of molecules further altered to remove sialic acid, such as those of the present invention.

CLAIMS

1. Factor VII or Factor FVIIa glycoprotein having an N-linked glycosylation pattern comprising glycans; wherein the glycosylation pattern comprises:

- (1) substantially no glycans with terminal N-acetylneuraminic acid
- (2) a substantial glycan that is a glycan of formula I: $(\text{GlcNAc})_4(\text{Man})_3(\text{Gal})_2(\text{Fuc})$
- (3) trace amounts or less of one or more glycans selected from the group consisting of a glycan of formula II: $(\text{GlcNAc})_5(\text{Man})_3(\text{Gal})(\text{Fuc})$ and a glycan of formula III: $(\text{GlcNAc})_6(\text{Man})_3(\text{Fuc})$ and
- (4) a glycan of formula V: $(\text{GlcNAc})_6(\text{Gal})_4(\text{Man})_3(\text{Fuc})$.

2. The Factor VII or Factor VIIa glycoprotein of claim 1, wherein the glycosylation pattern further comprises:

a glycan of formula IV: $(\text{GlcNAc})_5(\text{Man})_3(\text{Gal})_3(\text{Fuc})$.

3. The Factor VII or Factor VIIa glycoprotein of claim 1 or 2, wherein the glycosylation pattern has trace amounts or less of the glycan of formula II.

4. The Factor VII or Factor VIIa glycoprotein of claim 1 or 2, wherein the glycosylation pattern has trace amounts or less of the glycan of formula III.

5. The Factor VII or Factor VIIa glycoprotein of one of claims 1-4, wherein the glycoprotein comprises an amino acid sequence having three or less mutations relative to SEQ ID NO: 2.

6. The Factor VII or Factor VIIa glycoprotein of claim 1, wherein the glycoprotein is a Factor VIIa glycoprotein and comprises a heavy chain; wherein the heavy chain has an N-linked glycosylation pattern comprising glycans; and further wherein the glycosylation pattern comprises:

- (1) substantially no glycans with terminal N-acetylneuraminic acid and
 - (2) a substantial glycan that is a glycan of formula I: $(\text{GlcNAc})_4(\text{Man})_3(\text{Gal})_2(\text{Fuc})$;
- and

(3) trace amounts or less of one or more glycans selected from the group consisting of a glycan of formula II: (GlcNAc)₅(Man)₃(Gal)(Fuc) and a glycan of formula III: (GlcNAc)₆(Man)₃(Fuc).

7. The Factor VIIa glycoprotein of claim 6, wherein the glycosylation pattern has trace amounts or less of the glycan of formula II.

8. The Factor VIIa glycoprotein of claim 5 or 6, wherein the glycosylation pattern has trace amounts or less of the glycan of formula III.

9. The Factor VIIa glycoprotein of one of claims 5-8, wherein the glycoprotein comprises an amino acid sequence having three or less mutations relative to SEQ ID NO: 2.

10. A method of making the Factor VII or Factor VIIa glycoprotein of one of claims 2-9 comprising:

expressing a recombinant nucleic acid encoding the amino acid sequence for the Factor VII or Factor VIIa glycoprotein in a CHO K-1 host cell that has been recombinantly engineered to express sialidase; and

isolating the Factor VII or Factor VIIa glycoprotein thereby produced.

11. The method of claim 10, wherein the sialidase comprises an amino acid sequence of SEQ ID NO: 5.

12. An effective amount of the Factor VII glycoprotein of one of claims 1-9, for use in the treatment of a disease or a disorder wherein blood clot formation is desirable, wherein the Factor VII glycoprotein is to be administered to a mammal in need thereof and wherein the disease or disorder is selected from the group consisting of a hemorrhage, gastrointestinal bleeding, uncontrolled bleeding, bleeding in a mammal undergoing transplantation or resection or surgery, variceal bleeding, thrombocytopenia, hemophilia, intracranial hemorrhage, aortic aneurysm, over-administration of an anticoagulant, penetrative traumatic injury, blunt traumatic injury, bleeding in elective surgery, bleeding in cardiac surgery, bleeding in spinal surgery, orthopedic surgery, neurosurgery, oncology surgery, post-partum surgery, menorrhagia, bleeding in stem cell transplantation, bleeding in liver transplantation, gastrointestinal bleeding, active variceal bleeding in cirrhosis, non variceal bleeding in cirrhosis, diffuse alveolar hemorrhage, aortic aneurysm, intracerebral hemorrhage, traumatic

brain injury, brain contusion, reversal of warfarin, reversal of heparin, reversal of anticoagulants, reversal of anti-thrombotics, Factor VII deficiency, burns, prophylaxis in hemophilia patients with inhibitors, partial hepatectomy for non-cirrhotic and cirrhotic patients, acquired hemophilia, idiopathic thrombocytopenic purpura, Glanzmann's Thrombasthenia, Glanzmann's Thrombasthenia refractory to platelet transfusion, and Bernard-Soulier Syndrome.

13. Use of an effective amount of the Factor VII glycoprotein of one of claims 1-9, in the manufacture of a medicament for the treatment of a disease or a disorder wherein blood clot formation is desirable, wherein the Factor VII glycoprotein is to be administered to a mammal in need thereof and wherein the disease or disorder is selected from the group consisting of a hemorrhage, gastrointestinal bleeding, uncontrolled bleeding, bleeding in a mammal undergoing transplantation or resection or surgery, variceal bleeding, thrombocytopenia, hemophilia, intracranial hemorrhage, aortic aneurysm, over-administration of an anticoagulant, penetrative traumatic injury, blunt traumatic injury, bleeding in elective surgery, bleeding in cardiac surgery, bleeding in spinal surgery, orthopedic surgery, neurosurgery, oncology surgery, post-partum surgery, menorrhagia, bleeding in stem cell transplantation, bleeding in liver transplantation, gastrointestinal bleeding, active variceal bleeding in cirrhosis, non variceal bleeding in cirrhosis, diffuse alveolar hemorrhage, aortic aneurysm, intracerebral hemorrhage, traumatic brain injury, brain contusion, reversal of warfarin, reversal of heparin, reversal of anticoagulants, reversal of anti-thrombotics, Factor VII deficiency, burns, prophylaxis in hemophilia patients with inhibitors, partial hepatectomy for non-cirrhotic and cirrhotic patients, acquired hemophilia, idiopathic thrombocytopenic purpura, Glanzmann's Thrombasthenia, Glanzmann's Thrombasthenia refractory to platelet transfusion, and Bernard-Soulier Syndrome.

14. The use of claim 13, wherein the disease or disorder is hemorrhage that is postpartum hemorrhage.

15. A pharmaceutical composition comprising the Factor VII or Factor VIIa glycoprotein of one of claims 1-9 and a pharmaceutically acceptable excipient.