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(54) **CONJUGATED FACTOR VIII MOLECULES**

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(57) **ABSTRACT**

The present invention relates to B-domain truncated Factor VIII molecules with a modified circulatory half-life, said molecule being covalently conjugated with a hydrophilic polymer. The invention furthermore relates to methods for obtaining such molecules as well as use of such molecules.

FIGURE 1

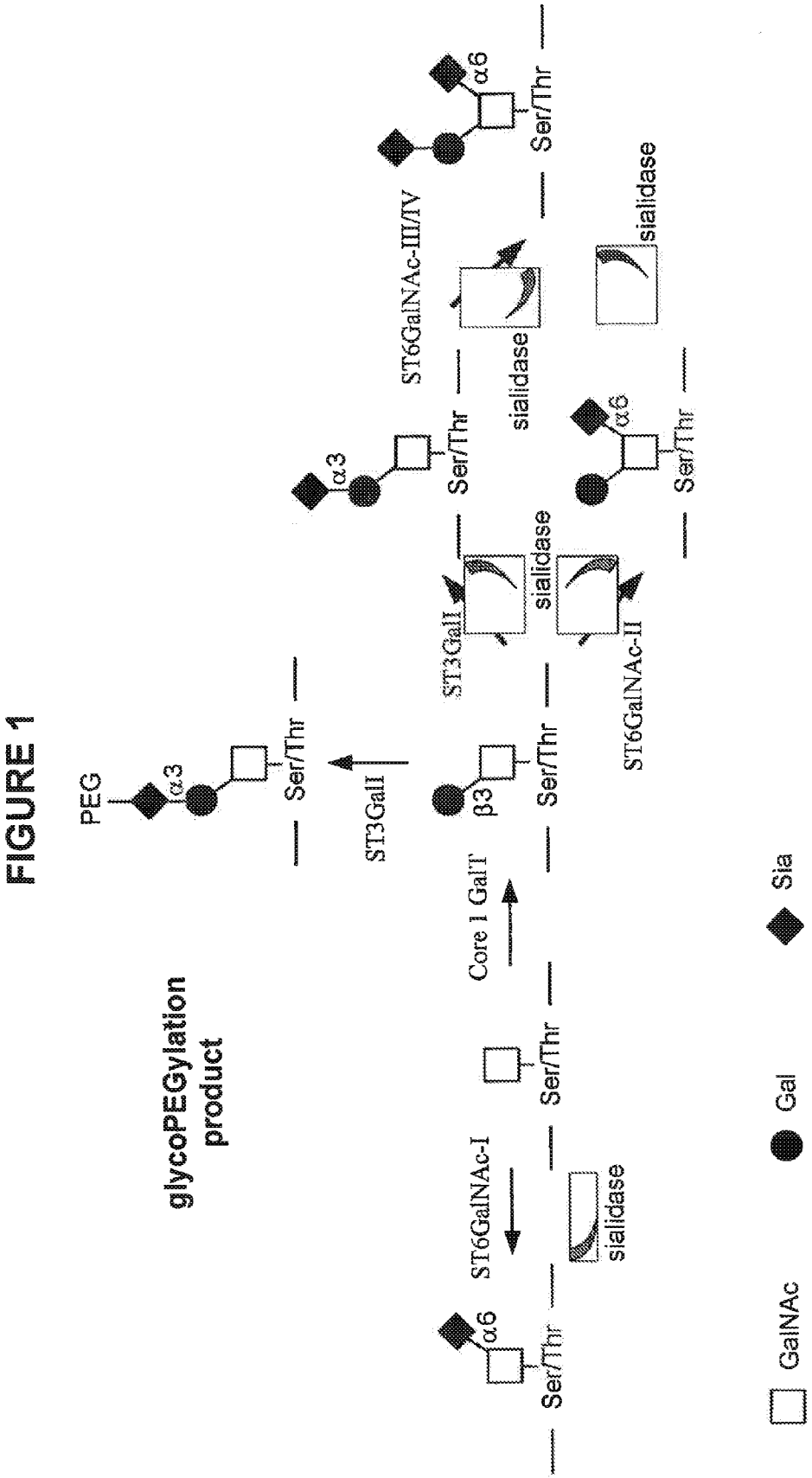
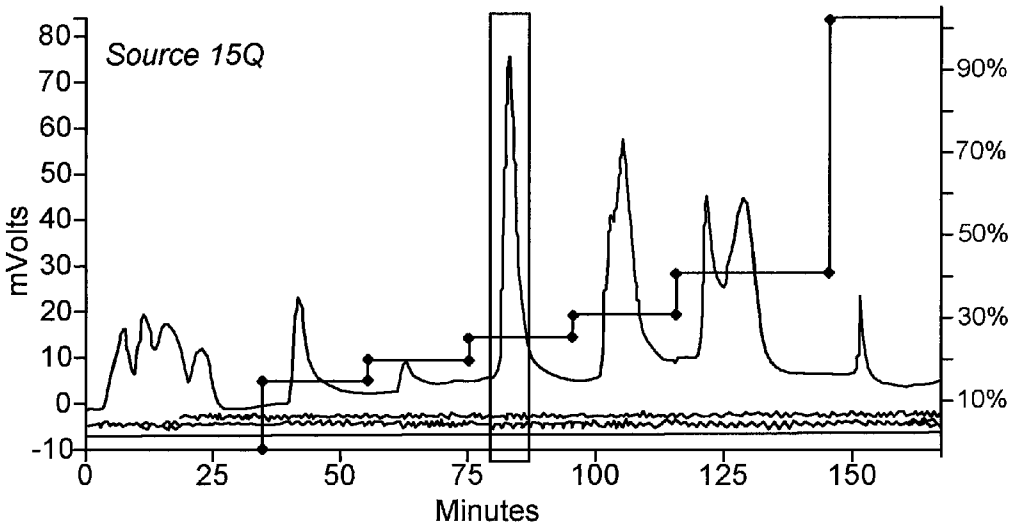
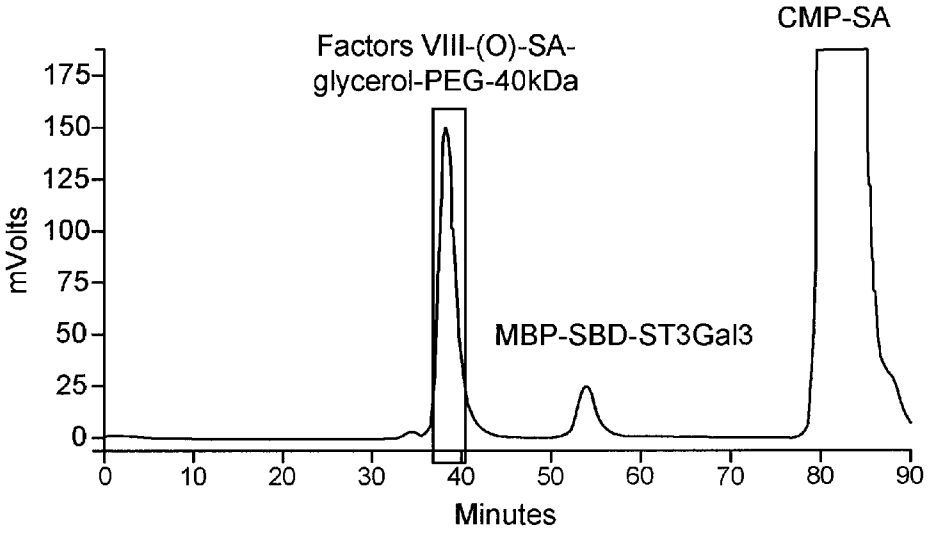

glycoPEGylation product
PEG
α3
Ser/Thr
ST3GalI
β3
Core 1 GalT
ST6GalNAc-I
α6
sialidase
ST3GalI
sialidase
ST6GalNAc-II
α6
sialidase
ST6GalNAc-III/IV
α6
sialidase
GalNAc
Gal
Sia

FIGURE 2



Product Fraction

FIGURE 3



Lane 1; Factor VIII (concentrated)
Lane 2; GlycoPEGylation, 0hrs
Lane 3; GlycoPEGylation, 30hrs
Lane 4; GlycoPEGylatioin, 43hrs
Lane 5; GlycoPEGylation, 49hrs
Lane 6; Source 15Q Product
Lane 7; Capping Reaction (11 hrs)
Lane 8; Superdex 200 Purified Product

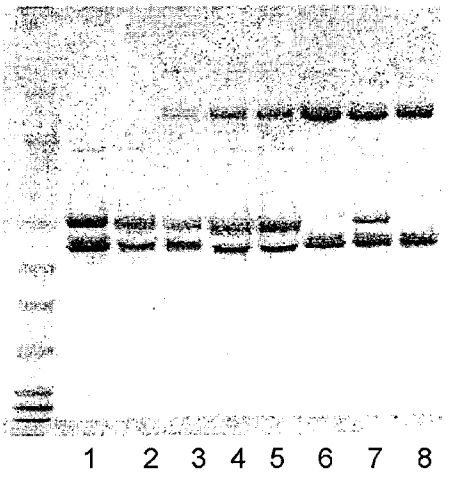


FIGURE 4A

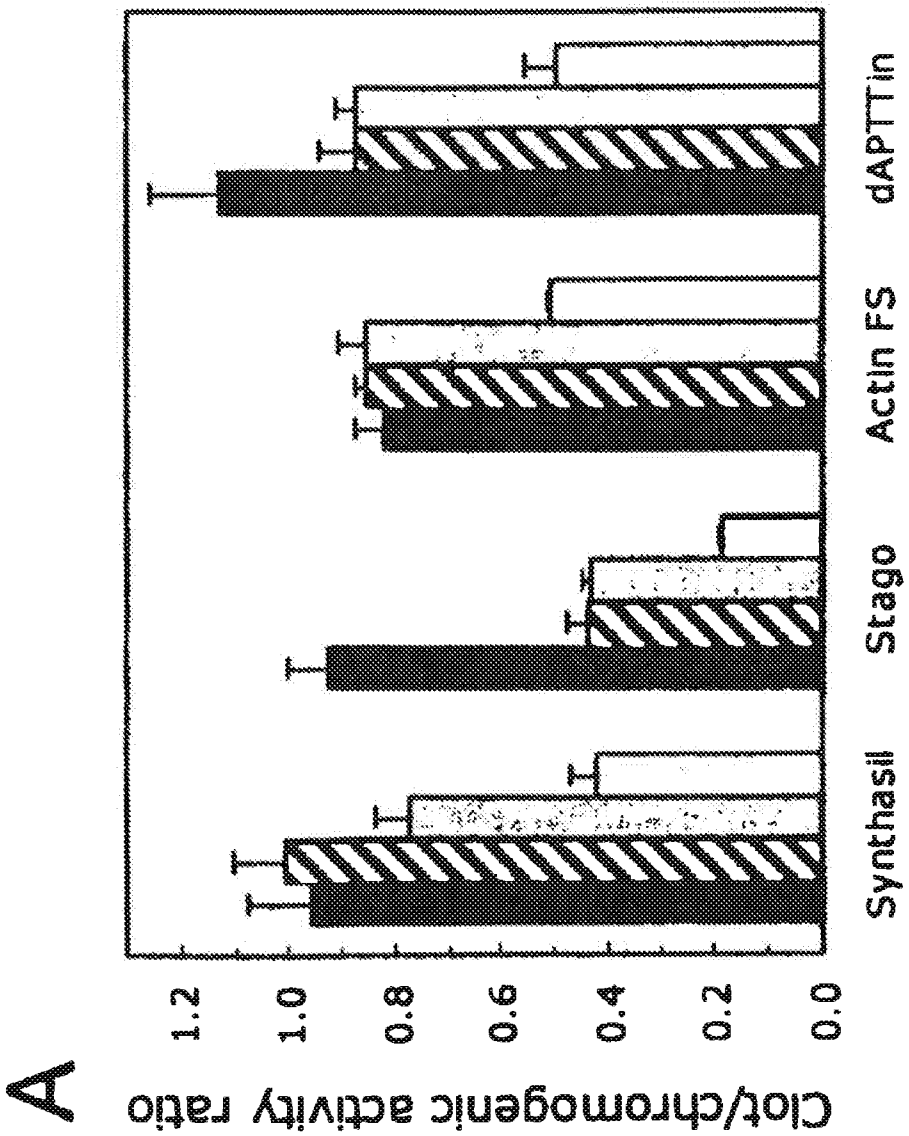

A
Clot/chromogenic activity ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Synthasil
Stago
Actin FS
dAPTTin

FIGURE 4B

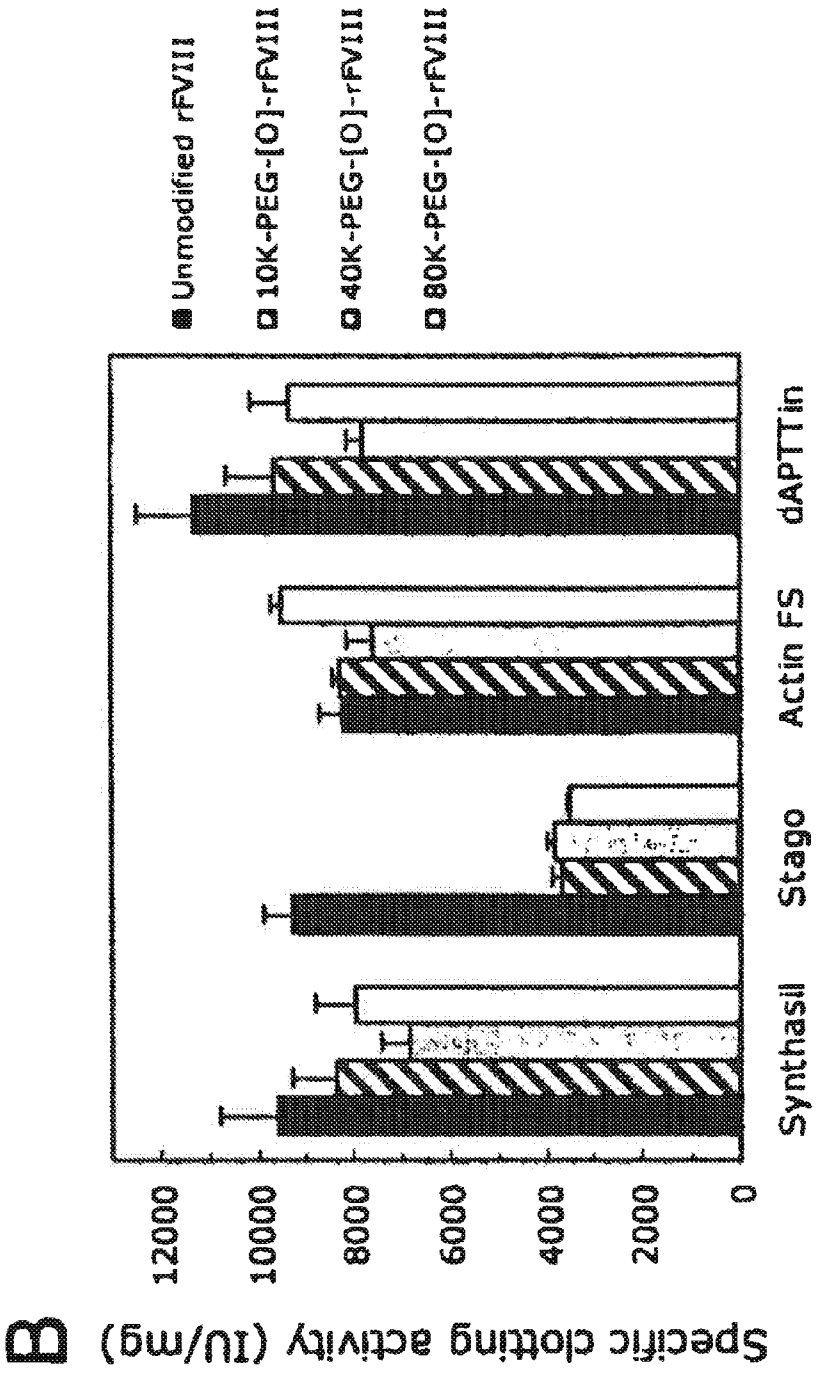

B
Specific clotting activity (IU/mg)
12000
10000
8000
6000
4000
2000
0
Synthasil
Stago
Actin FS
dAPTTin
Unmodified rFVIII
10K-PEG-[O]-rFVIII
40K-PEG-[O]-rFVIII
80K-PEG-[O]-rFVIII

FIGURE 5

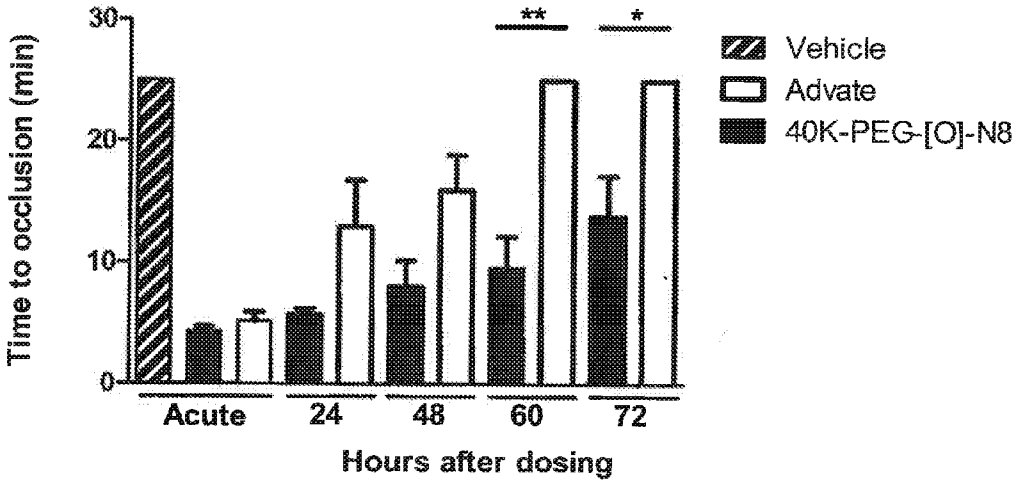

Time to occlusion (min)
30
20
10
0
**
*
Vehicle
Advate
40K-PEG-[O]-N8
Acute
24
48
60
72
Hours after dosing

FIGURE 6

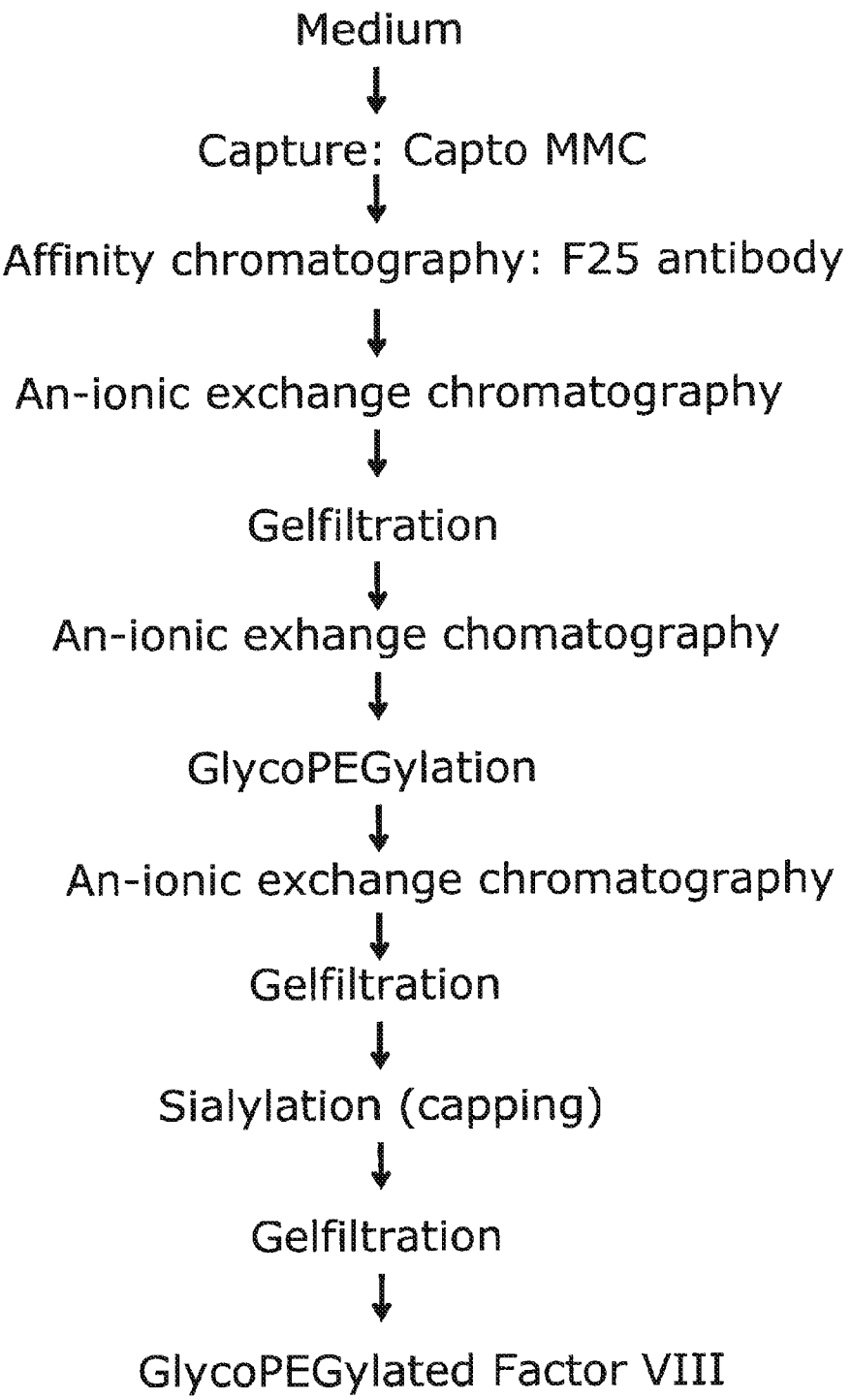
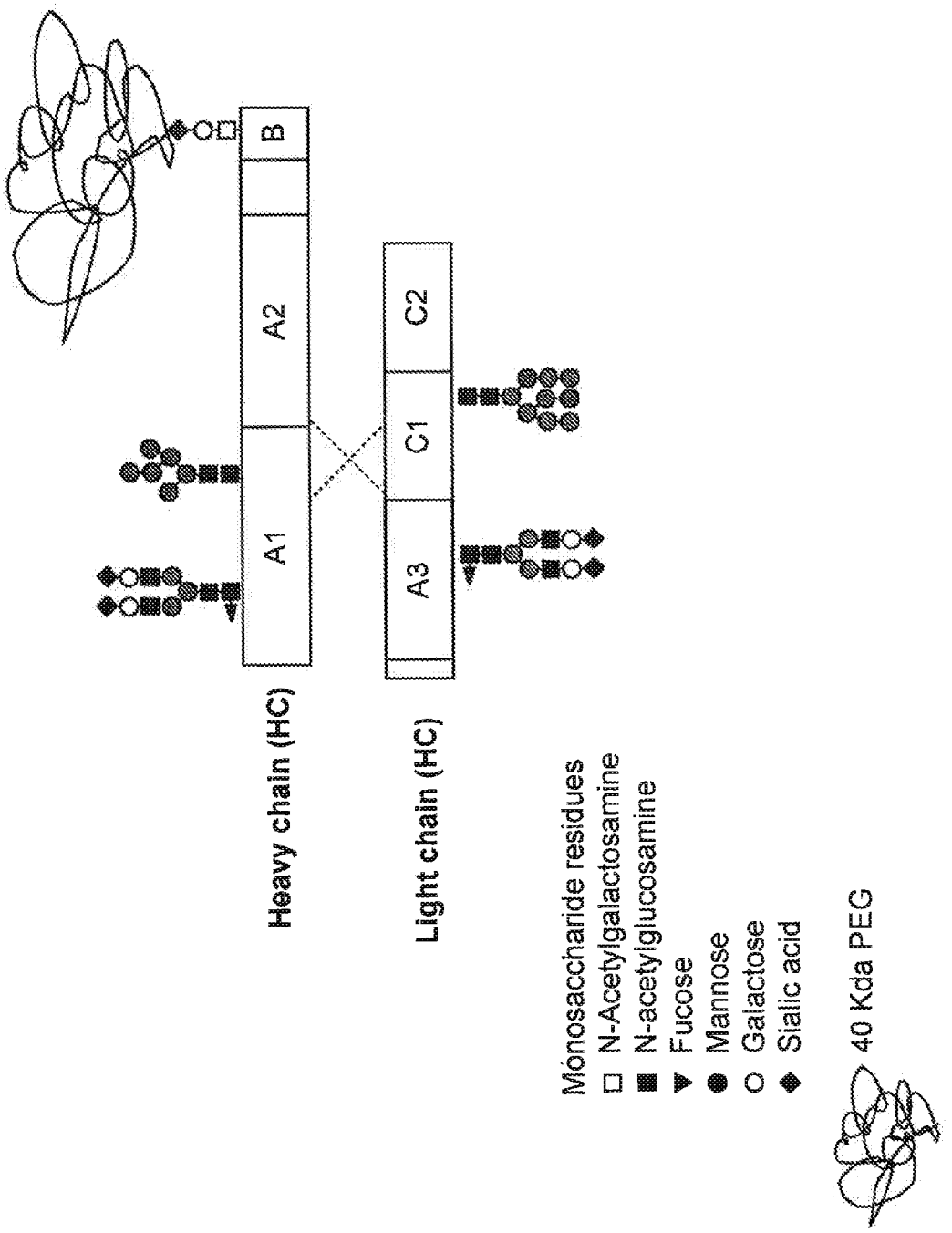

Medium
Capture: Capto MMC
Affinity chromatography: F25 antibody
An-ionic exchange chromatography
Gelfiltration
An-ionic exhange chomatography
GlycoPEGylation
An-ionic exchange chromatography
Gelfiltration
Sialylation (capping)
Gelfiltration
GlycoPEGylated Factor VIII

FIGURE 7


B
A2
A1
C2
C1
A3
Heavy chain (HC)
Light chain (HC)
Monosaccharide residues
N-Acetylgalactosamine
N-acetylglucosamine
Fucose
Mannose
Galactose
Sialic acid
40 Kda PEG

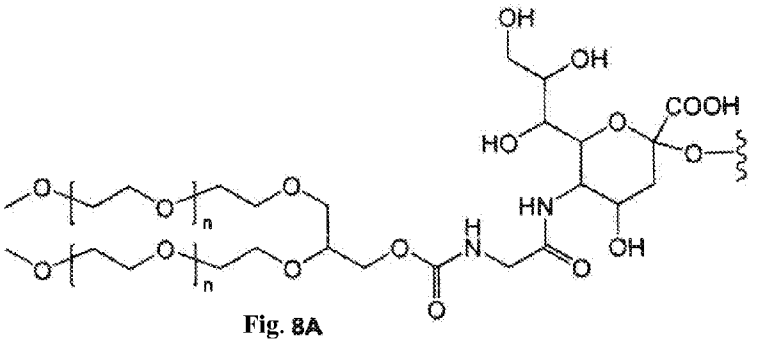


Fig. 8A

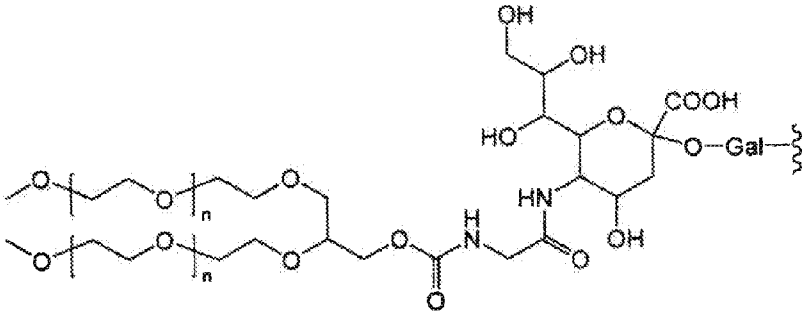


Fig. 8B

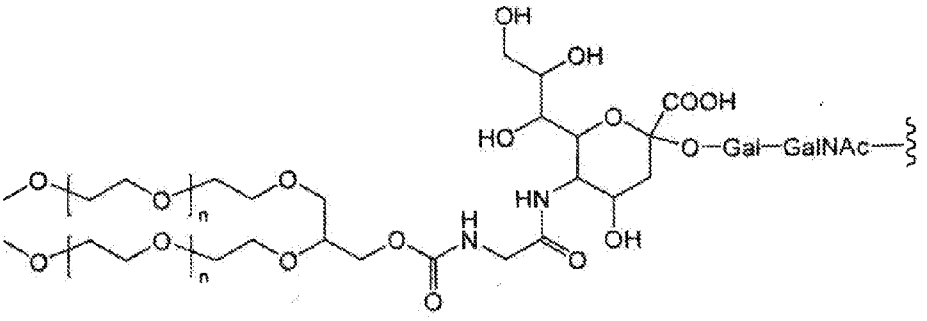


Fig. 8C

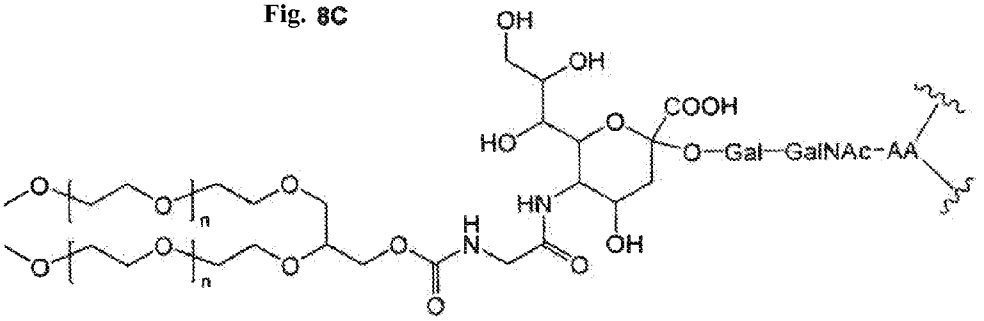


Fig. 8D

n > 400 (eg ~ 455); n > 900 (eg ~ 910)

CONJUGATED FACTOR VIII MOLECULES

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of U.S. patent application Ser. No. 14/272,726, filed May 8, 2014 (Notice of Allowance Received), which is a continuation of U.S. patent application Ser. No. 12/597,473, filed Mar. 1, 2010 (Abandoned), which is a 35 U.S.C. §371 national stage application of International Patent Application PCT/US2009/035339 (published as WO 2009/108806), filed Feb. 26, 2009; this application further claims priority under 35 U.S.C §119 of U.S. Provisional Application 61/032,006, filed Feb. 27, 2008; the contents of all above-named applications are incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present invention relates to conjugated coagulation Factor VIII molecules. In particular, the present invention relates to conjugated Factor VIII molecules having a modified circulatory half-life.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Aug. 19, 2015, is named 7982US02_SeqList_ST25.txt and is 33,925 bytes in size.

BACKGROUND OF THE INVENTION

[0004] Haemophilia A is an inherited bleeding disorder caused by deficiency or dysfunction of coagulation Factor VIII (FVIII) activity. The clinical manifestation is not on primary haemostasis—formation of the blood clot occurs normally—but the clot is unstable due to a lack of secondary thrombin formation. The disease is treated by intravenous injection of coagulation Factor FVIII which is either isolated from blood or produced recombinantly.

[0005] Current treatment recommendations are moving from traditional on-demand treatment towards prophylaxis. The circulatory half-life of endogenous FVIII is 12-14 hours and prophylactic treatment is thus to be performed several times a week in order to obtain a virtually symptom-free life for the patients. IV administration is for many, especially children and young persons, associated with significant inconvenience and/or pain. There is thus a need in the art for novel Factor VIII products with Factor VIII activity that are preferably homogenous in structure, preferably safe and preferably having a significantly prolonged circulatory half-life in order to reduce the number of Factor VIII administration per week. There is furthermore a need in the art for relatively simple methods for obtaining and producing such molecules.

[0006] PEGylation of Factor VIII in order to prolong circulatory half-life is known in the art. It has however been an obstacle to obtain safe products having a homogenous structure as well as a significantly improved circulatory half-life. The available methods of producing conjugated Factor VIII molecules are often laborious, and/or tend to result in low yields and/or products that are not homogenous in structure. The use of artificially engineered O-linked glycosylation sites for obtaining therapeutic proteins having a prolonged circulatory half-life of therapeutic proteins has been suggested in WO2008011633, however, no conjugated Factor VIII molecules are disclosed therein.

SUMMARY OF THE INVENTION

[0007] In a first aspect, the present invention relates to a B domain truncated Factor VIII molecule with a modified circulatory half-life, said molecule being covalently conjugated with a hydrophilic polymer via an O-linked oligosaccharide in the truncated B domain, wherein Factor VIII activation results in removal of the covalently conjugated side group.

[0008] In other aspects, the present invention furthermore relates to methods for obtaining such molecules, use of such molecules and pharmaceutical compositions comprising such molecules.

[0009] What is thus provided is a conjugated Factor VIII molecule with modified circulatory half-life, wherein the conjugated side group (e.g. hydraphilic polymer) is removed upon activation. The molecules according to the invention are preferably homogenous in structure—at least with regard to position of the hydrophilic polymer in the truncated B-domain—and preferably have an advantageous safety profile. Likewise, relatively simple methods for obtaining such molecules are furthermore provided herein. Preferably, activated Factor VIII molecules according to the invention are similar to endogenous activated Factor VIII.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0010] Factor VIII molecules: FVIII/Factor VIII is a large, complex glycoprotein that primarily is produced by hepatocytes. FVIII consists of 2351 amino acids, including signal peptide, and contains several distinct domains, as defined by homology. There are three A-domains, a unique B-domain, and two C-domains. The domain order can be listed as NH2-A1-A2-B-A3-C1-C2-COOH. FVIII circulates in plasma as two chains, separated at the B-A3 border. The chains are connected by bivalent metal ion-bindings. The A1-A2-B chain is termed the heavy chain (HC) while the A3-C1-C2 is termed the light chain (LC).

[0011] Endogenous Factor VIII molecules circulate in vivo as a pool of molecules with B domains of various sizes. What probably occurs in vivo is a gradual enzymatic removal of the B domain resulting in a pool of molecules with B-domains of various sizes. It is generally believed that cleavage at position 740, by which the last part of the B-domain is removed, occurs in connection with thrombin activation. However, it cannot be ruled out that a Factor VIII variant in which e.g. the cleavage site at position 740 has been impaired may be active.

[0012] "Factor VIII" or "FVIII" as used herein refers to a human plasma glycoprotein that is a member of the intrinsic coagulation pathway and is essential to blood coagulation. "Native FVIII" is the full length human FVIII molecule as shown in SEQ ID NO. 1 (amino acid 1-2332). The B-domain spans amino acids 741-1648 in SEQ ID NO 1.

SEQ ID NO 1:
ATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPKSFPFNTSVVYKKTL

FVEFTDHLFNIAKPRPPWMGLLGPTIQAEVYDTVVITLKNMASHPVSLHA

VGVSYWKASEGAEYDDQTSQREKEDDKVFPGGSHTYVWQVLKENGPMASD

-continued

PLCLTYSYLSHVDLVKDLNSGLIGALLVCREGSLAKEKTQTLHKFILLFA

VFDEGKSWHSETKNSLMQDRDAASARAWPKMHTVNGYVNRSLPGLIGCHR

KSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITFLTAQTLL

MDLGQFLLFCHISSHQHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDL

TDSEMDVVRFDDDNSPSFIQIRSVAKKHPKTWVHYIAAEEEDWDYAPLVL

APDDRSYKSQYLNNGPQRIGRKYKKVRFMAYTDETFKTREAIQHESGILG

PLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVRPLYSRRLPKGVKHLKD

FPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFVNMERDLASGLIGP

LLICYKESVDQRGNQIMSDKRNVILFSVFDENRSWYLTENIQRFLPNPAG

VQLEDPEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLS

VFFSGYTFKHKMVYEDTLTLFPFSGETVFMSMENPGLWILGCHNSDFRNR

GMTALLKVSSCDKNTGDYYEDSYEDISAYLLSKNNAIEPRSFSQNSRHPS

TRQKQFNATTIPENDIEKTDPWFAHRTPMPKIQNVSSSDLLMLLRQSPTP

HGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFT

PESGLQLRLNEKLGTTAATELKKLDFKVSSTSNNLISTIPSDNLAAGTDN

TSSLGPPSMPVHYDSQLDTTLFGKKSSPLTESGGPLSLSEENNDSKLLES

GLMNSQESSWGKNVSSTESGRLFKGKRAHGPALLTKDNALFKVSISLLKT

NKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTPLIHDRM

LMDKNATALRLNHMSNKTTSSKNMEMVQQKKEGPIPPDAQNPDMSFFKML

FLPESARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKV

VVGKGEFTKDVGLKEMVFPSSRNLFLTNLDNLHENNTHNQEKKIQEEIEK

KETLIQENVVLPQIHTVTGTKNFMKNLFLLSTRQNVEGSYDGAYAPVLQD

FRSLNDSTNRTKKHTAHFSKKGEEENLEGLGNQTKQIVEKYACTTRISPN

TSQQNFVTQRSKRALKQFRLPLEETELEKRIIVDDTSTQWSKNMKHLTPS

TLTQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSFPSIR

PIYLTRVLFQDNSSHLPAASYRKKDSGVQESSHFLQGAKKNNLSLAILTL

EMTGDQREVGSLGTSATNSVTYKKVENTVLPKPDLPKTSGKVELLPKVHI

YQKDLFPTETSNGSPGHLDLVEGSLLQGTEGAIKWNEANRPGKVPFLRVA

TESSAKTPSKLLDPLAWDNHYGTQIPKEEWKSQEKSPEKTAFKKKDTILS

LNACESNHAIAAINEGQNKPEIEVTWAKQGRTERLCSQNPPVLKRHQREI

TRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQSPRSFQKKTRHYFI

AAVERLWDYGMSSSPHVLRNRAQSGSVPQFKKVVFQEFTDGSFTQPLYRG

ELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFYSSLISYEEDQRQGA

EPRKNFVKPNETKTYFWKVQHHMAPTKDEFDCKAWAYFSDVDLEKDVHSG

LIGPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCR

APCNIQMEDPTFKENYRFHAINGYIMDTLPGLVMAQDQRIRWYLLSMGSN

ENIHSIHFSGHVFTVRKKEEYKMALYNLYPGVFETVEMLPSKAGIWRVEC

LIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKL

ARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFSSLYISQ

-continued

FIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIR

LHPTHYSIRSTLRMELMGCDLNSCSMPLGMESKAISDAQITASSYFTNMF

ATWSPSKARLHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTGVTTQGVKS

LLTSMYVKEFLISSSQDGHQWTLFFQNGKVKVFQGNQDSFTPVVNSLDPP

LLTRYLRIHPQSWVHQIALRMEVLGCEAQDLY

[0013] The Factor VIII molecules according to the present invention are B domain truncated Factor FVIII molecules wherein the remaining domains correspond to the sequence as set forth in amino acid no 1-740 and 1649-2332 in SEQ ID NO. 1. It follows that molecules according to the invention are recombinant molecules produced in transformed host cells, preferably of mammalian origin. However, the remaining domains (i.e. the three A-domains and the two C-domains) may differ slightly e.g. about 1%, 2%, 3%, 4% or 5% from the amino acid sequence as set forth in SEQ ID NO 1 (amino acids 1-740 and 1649-2332). In particular, it is plausible that amino acid modifications (substitutions, deletions, etc.) are introduced in the remaining domains e.g. in order to modify the binding capacity of Factor VIII with various other components such as e.g. vW factor, LPR, various receptors, other coagulation factors, cell surfaces, etc. Furthermore, it is plausible that the Factor VIII molecules according to the invention comprise other post-translational modifications in e.g. the truncated B-domain and/or in one or more of the other domains of the molecules. These other post-translational modifications may be in the form of various molecules conjugated to the Factor VIII molecule according to the invention such as e.g. polymeric compounds, peptidic compounds, fatty acid derived compounds, etc.

[0014] Factor VIII molecules according to the present invention, regardless of whether they are modified outside the B domain or not, have other posttranslational modifications or not, all have Factor VIII activity, meaning the ability to function in the coagulation cascade in a manner functionally similar or equivalent to FVIII, induce the formation of FXa via interaction with FIXa on an activated platelet, and support the formation of a blood clot. The activity can be assessed in vitro by techniques well known in the art such as e.g. clot analysis, endogenous thrombin potential analysis, etc. Factor VIII molecules according to the present invention have FVIII activity being at least about 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, and 100% or even more than 100% of that of native human FVIII.

[0015] B domain: The B-domain in Factor VIII spans amino acids 741-1648 in SEQ ID NO 1. The B-domain is cleaved at several different sites, generating large heterogeneity in circulating plasma FVIII molecules. The exact function of the heavily glycosylated B-domain is unknown. What is known is that the domain is dispensable for FVIII activity in the coagulation cascade. This apparent lack of function is supported by the fact that B domain deleted/truncated FVIII appears to have in vivo properties identical to those seen for full length native FVIII. That being said there are indications that the B-domain may reduce the association with the cell membrane, at least under serum free conditions.

[0016] B domain truncated/deleted Factor VIII molecule: Endogenous full length FVIII is synthesized as a single-chain precursor molecule. Prior to secretion, the precursor is

cleaved into the heavy chain and the light chain. Recombinant B domain-deleted FVIII can be produced from two different strategies. Either the heavy chain without the B-domain and the light chain are synthesized individually as two different polypeptide chains (two-chain strategy) or the B-domain deleted FVIII is synthesized as a single precursor polypeptide chain (single-chain strategy) that is cleaved into the heavy and light chains in the same way as the full-length FVIII precursor.

[0017] In a B domain-deleted FVIII precursor polypeptide, the heavy and light chain moieties are normally separated by a linker. To minimize the risk of introducing immunogenic epitopes in the B domain-deleted FVIII, the sequence of the linker is preferable derived from the FVIII B-domain. The linker must comprise a recognition site for the protease that separates the B domain-deleted FVIII precursor polypeptide into the heavy and light chain. In the B domain of full length FVIII, amino acid 1644-1648 constitutes this recognition site. The thrombin site leading to removal of the linker on activation of B domain-deleted FVIII is located in the heavy chain. Thus, the size and amino acid sequence of the linker is unlikely to influence its removal from the remaining FVIII molecule by thrombin activation. Deletion of the B domain is an advantage for production of FVIII. Nevertheless, parts of the B domain can be included in the linker without reducing the productivity. The negative effect of the B domain on productivity has not been attributed to any specific size or sequence of the B domain.

[0018] The truncated B-domain may contain several O-glycosylation sites. However, according to a preferred embodiment, the molecule comprises only one, alternatively two, three or four O-linked oligosaccharides in the truncated B-domain.

[0019] According to a preferred embodiment, the truncated B domain comprises only one potential O-glycosylation site and the hydrophilic polymer is covalently conjugated to this O-glycosylation site.

[0020] The O-linked oligosaccharides in the B-domain truncated molecules according to the invention may be attached to O-glycosylation sites that were either artificially created by recombinant means and/or by exposure of "hidden" O-glycosylation sites by truncation of the B-domain. In both cases, such molecules may be made by designing a B-domain trunctated Factor VIII amino acid sequence and subsequently subjecting the amino acid sequence to an in silico analysis predicting the probability of O-glycosylation sites in the truncated B-domain. Molecules with a relatively high probability of having such glycosylation sites can be synthesized in a suitable host cell followed by analysis of the glycosylation pattern and subsequent selection of molecules having O-linked glycosylation in the truncated B-domain. Suitable host cells for producing recombinant Factor VIII protein are preferably of mammalian origin in order to ensure that the molecule is glycosylated. In practicing the present invention, the cells are mammalian cells, more preferably an established mammalian cell line, including, without limitation, CHO (e.g., ATCC CCL 61), COS-1 (e.g., ATCC CRL 1650), baby hamster kidney (BHK), and HEK293 (e.g., ATCC CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) cell lines. A preferred BHK cell line is the tk-ts13 BHK cell line (Waechter and Baserga, Proc.Natl.Acad.Sci.USA 79:1106-1110, 1982), hereinafter referred to as BHK 570 cells. The BHK 570 cell line is available from the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Md. 20852, under ATCC accession number CRL 10314. A tk-ts13 BHK cell line is also available from the ATCC under accession number CRL 1632. A preferred CHO cell line is the CHO K1 cell line available from ATCC under accession number CC161 as well as cell lines CHO-DXB11 and CHO-DG44.

[0021] Other suitable cell lines include, without limitation, Rat Hep I (Rat hepatoma; ATCC CRL 1600), Rat Hep II (Rat hepatoma; ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC HB 8065), NCTC 1469 (ATCC CCL 9.1); DUKX cells (CHO cell line) (Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216-4220, 1980) (DUKX cells also being referred to as DXB11 cells), and DG44 (CHO cell line) (Cell, 33: 405, 1983, and Somatic Cell and Molecular Genetics 12: 555, 1986). Also useful are 3T3 cells, Namalwa cells, myelomas and fusions of myelomas with other cells. In some embodiments, the cells may be mutant or recombinant cells, such as, e.g., cells that express a qualitatively or quantitatively different spectrum of enzymes that catalyze post-translational modification of proteins (e.g., glycosylation enzymes such as glycosyl transferases and/or glycosidases, or processing enzymes such as propeptides) than the cell type from which they were derived. DUKX cells (CHO cell line) are especially preferred.

[0022] Currently preferred cells are HEK293, COS, Chinese Hamster Ovary (CHO) cells, Baby Hamster Kidney (BHK) and myeloma cells, in particular Chinese Hamster Ovary (CHO) cells.

[0023] The inventors of the present invention have thus shown that it is possible to activate "hidden" O-glycosylation sites in the Factor VIII B-domain by truncating the B-domain. While not wishing to be bound by any theory, this phenomenon could be attributable to the tertiary structure of the molecule in the truncated B-domain being altered. "Hidden" O-glycosylation sites are thus "made accessible" to glycosylation in the truncated B-domain. One advantage of this approach is the provision of recombinant molecules with an advantageous safety profile with respect to e.g. allergenicity. Another advantage could be that it may represent a simpler approach of obtaining B-domain truncated variants with an O-linked oligosaccharide in the B-domain due to the inherent abundance of glycosylation sites in the B-domain as it has previously proven difficult to engineer artificial O-glycosylation sites in recombinant proteins.

[0024] The length of the B domain in the wt FVIII molecule is about 907 amino acids. The length of the truncated B domain in molecules according to the present invention may vary from about 10 amino acids to about 700 acids, such as e.g. about 12-500 amino acids, 12-400 amino acids, 12-300 amino acids, 12-200 amino acids, 15-100 amino acids, 15-75 amino acids, 15-50 amino acids, 15-45 amino acids, 20-45 amino acids, 20-40 amino acids, or 20-30 amino acids. The truncated B-domain may comprise fragments of the heavy chain and/or the light chain and/or an artificially introduced sequence that is not found in the wt FVIII molecule. The terms "B-domain truncated" and "B-domain deleted" may be used interchangeably herein.

[0025] Modified circulatory half life: Molecules according to the present invention have a modified circulatory half-life compared to the wild type Factor VIII molecule, preferably an increased circulatory half-life. Circulatory half-life is preferably increased at least 10%, preferably at least 15%, preferably at least 20%, preferably at least 25%, preferably at least 30%, preferably at least 35%, preferably at least 40%, preferably at least 45%, preferably at least 50%, preferably at

least 55%, preferably at least 60%, preferably at least 65%, preferably at least 70%, preferably at least 75%, preferably at least 80%, preferably at least 85%, preferably at least 90%, preferably at least 95%, preferably at least 100%, more preferably at least 125%, more preferably at least 150%, more preferably at least 175%, more preferably at least 200%, and most preferably at least 250% or 300%. Even more preferably, such molecules have a circulatory half-life that is increased at least 400%, 500%, 600%, or even 700% relative to the circulatory half-life of the wild type FVIII.

[0026] Hydrophilic polymer: The modifying group/hydrophilic polymer according to the present invention is preferably non-naturally occurring. In one example, the "non-naturally occurring modifying group" is a polymeric modifying group, in which at least one polymeric moiety is non-naturally occurring. In another example, the non-naturally occurring modifying group is a modified carbohydrate. The locus of functionalization with the modifying group is selected such that it does not prevent the "modified sugar" from being added enzymatically to a polypeptide. "Modified sugar" also refers to any glycosyl mimetic moiety that is functionalized with a modifying group and which is a substrate for a natural or modified enzyme, such as a glycosyltransferase.

[0027] The polymeric modifying group added to a polypeptide can alter a property of such polypeptide, for example, its bioavailability, biological activity or its half-life in the body. Exemplary polymers according to the invention include water-soluble polymers that can be linear or branched and can include one or more independently selected polymeric moieties, such as poly(alkylene glycol) and derivatives thereof. The polymeric modifying group according to the invention may include a water-soluble polymer, e.g. poly(ethylene glycol) and derivatives thereof (PEG, m-PEG), poly(propylene glycol) and derivatives thereof (PPG, m-PPG) and the like.

[0028] The term "water-soluble" refers to moieties that have some detectable degree of solubility in water. Methods to detect and/or quantify water solubility are well known in the art. Exemplary water-soluble polymers according to the invention include peptides, saccharides, poly(ethers), poly (amines), poly(carboxylic acids) and the like. Peptides can have mixed sequences and be composed of a single amino acid, e.g., poly(lysine). An exemplary polysaccharide is poly (sialic acid). An exemplary poly(ether) is poly(ethylene glycol), e.g., m-PEG. Poly(ethylene imine) is an exemplary polyamine, and poly(acrylic) acid is a representative poly (carboxylic acid).

[0029] The polymer backbone of the water-soluble polymer according to the invention can be poly(ethylene glycol) (i.e. PEG). The term PEG in connection with the present invention includes poly(ethylene glycol) in any of its forms, including alkoxy PEG, difunctional PEG, multiarmed PEG, forked PEG, branched PEG, pendent PEG (i.e. PEG or related polymers having one or more functional groups pendent to the polymer backbone), or PEG with degradable linkages therein.

[0030] The polymer backbone can be linear or branched. Branched polymer backbones are generally known in the art. Typically, a branched polymer has a central branch core moiety and a plurality of linear polymer chains linked to the central branch core. PEG is commonly used in branched forms that can be prepared by addition of ethylene oxide to various polyols, such as glycerol, pentaerythritol and sorbitol. The central branch moiety can also be derived from several amino acids, such as lysine or cysteine. In one example, the branched poly(ethylene glycol) can be represented in general form as R(-PEG-OH)m in which R represents the core moiety, such as glycerol or pentaerythritol, and m represents the number of arms. Multi-armed PEG molecules, such as those described in U.S. Pat. No. 5,932,462, which is incorporated by reference herein in its entirety, can also be used as the polymer backbone.

[0031] FIGS. **8**A-D show a representative branched PEG polymer of use in embodiments of the invention, referred to herein as "SA-glycerol-PEG." FIG. **8**A shows an exemplary SA-glycerol-PEG component of CMP-SA-glycerol-PEG or of a SA-glycerol-PEG linked to a glycan or an amino acid of a polypeptide. FIG. **8**B shows the SA-glycerol-PEG moiety linked to a glycan or polypeptide through a Gal residue. FIG. **8**C shows the SA-glycerol-PEG moiety linked to a glycan or polypeptide through a Gal-GalNAc residue. FIG. **8**D shows the SA-glycerol-PEG moiety linked to an amino acid of a polypeptide through a Gal-GalNAc moiety. In various embodiments, AA is threonine or serine. In an exemplary embodiment, AA is converted to an O-linked glycosylation site by deletion of the B-domain of the FVIII polypeptide. The discussion regarding the molecular weight of the polymer herein below under this definition is generally applicable to the branched PEG shown in FIG. **8**D. In FIGS. **8**A-D, the index "n" represents any integer providing a linear (and thus a branched) m-PEG of the desired molecular weight. In various embodiments, "n" is selected such that the linear m-PEG moiety is about 20 KDa to about 40 KDa, for example, about 20 KDa, about 30 KDa or about 40 KDa. Integers corresponding to these m-PEG molecular weights correspond to about 400 (e.g. about 455) to about 900 (e.g. about 910). Accordingly, "n" is selected to provide a branched PEG that is about 40 KDa to about 80 KDa, e.g., about 40 KDa, about 50 KDa, about 60 KDa, about 70 KDa, or about 80 KDa.

[0032] Many other polymers are also suitable for the invention. Polymer backbones that are non-peptidic and water-soluble, are particularly useful in the invention. Examples of suitable polymers include, but are not limited to, other poly (alkylene glycols), such as poly(propylene glycol) ("PPG"), copolymers of ethylene glycol and propylene glycol and the like, poly(oxyethylated polyol), poly(olefinic alcohol), poly (vinylpyrrolidone), poly(hydroxypropylmethacrylamide), poly([alpha]-hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazoline, poly(N-acryloylmorpholine), such as described in U.S. Pat. No. 5,629,384, which is incorporated by reference herein in its entirety, as well as copolymers, terpolymers, and mixtures thereof.

[0033] Although the molecular weight of each chain of the polymer backbone can vary, it is typically in the range of from about 100 Da to about 160,000 Da, such as e.g., from about 5,000 Da to about 100,000 Da. More specifically, the size of each conjugated hydrophilic polymer according to the present invention may vary from about 500 Da to about 80,000 Da, such as e.g. about 1000 Da to about 80,000 Da; about 2000 Da to about 70,000 Da; about 5000 to about 70,000 Da; about 5000 to about 60,000 Da; about 10,000 to about 70,000 Da; about 20,000 to about 60,000 Da; about 30,000 to about 60,000 Da; about 30,000 to about 50,000 Da; or about 30,000 to about 40,000 Da. It should be understood that these sizes represent estimates rather than exact measures. According to a preferred embodiment, the molecules according to the invention are conjugated with a heterogeneous population of hydrophilic polymers, such as e.g. PEG

of a size of e.g. 10,000, 40,000, or 80,000 Da+/− about 5000, about 4000, about 3000, about 2000, or about 1000 Da.

[0034] O-linked oligosaccharide: Both N-glycans and O-glycans are attached to proteins by the cells producing the protein. The cellular N-glycosylation machinery recognizes and glycosylates N-glycosylation signals (N—X-S/T motifs) in the amino acid chain, as the nascent protein is translocated from the ribosome to the endoplasmic reticulum (Kiely et al. 1976; Glabe et al. 1980).

[0035] Likewise, O-glycans are attached to specific O-glycosylation sites in the amino acid chain, but the motifs triggering O-glycosylation are much more heterogeneous than the N-glycosylation signals, and our ability to predict O-glycosylation sites in amino acid sequences is still inadequate (Julenius et al. 2004). The construction of artificial O-glycosylation sites it is thus associated with some uncertainty. The general assumption is that the native FVIII molecule does not contain any O-glycosylation sites, and the skilled man would therefore expect that at least one artificial O-glycosylation site would have to be constructed and inserted into the B domain in connection with practicing the present invention.

[0036] The O-linked oligosaccharide in a truncated Factor VIII B domain may thus be covalently linked to a naturally occurring O-linked glycosylation sequence or an O-linked glycosylation sequence which has been artificially constructed by recombinant techniques.

[0037] According to a preferred embodiment of the present invention, the O-linked oligosaccharide is linked to a naturally occurring O-linked glycosylation sequence which is not exposed to glycosylation in the wild type Factor VIII molecule but is becoming accessible to O-glycosylation as a consequence of truncation of the B domain. An example thereof is shown in the examples and in SEQ ID NO 2 (the truncated B-domain corresponds to amino acids 742-763). It is plausible that the "hidden" O-glycosylation site in SEQ ID NO 2 will also become glycosylated even if the B-domain is truncated at a somewhat different place, i.e. if the truncated B domain is somewhat shorter (e.g. 1, 2, 3, 4, or 5 amino acids shorter than SEQ ID NO 2) or longer (such as e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids) compared to SEQ ID NO 2. This approach by activating a "hidden" O-glycosylation site by truncation of a B-domain rather than creation of an artificial O-glycosylation site has the advantage of creating a molecule with an advantageous safety profile (i.e. reduced allergenicity, etc.). Other O-glycosylation sites in the Factor VIII B-domain may likewise become activated by truncating the molecules in different ways.

[0038] Glyco-PEGylation of O-linked oligosaccharide: The biosynthesis of O-glycans can be modified and terminated with the addition of sialic acid residues relatively early in biosynthesis. Certain sialyltransferase enzymes are capable of acting on GalNAcα-Ser/Thr, or early O-glycan core subtypes after Core 1 GalT action. The term T antigenis associated with the presence of the Galβ1-3GalNAcα-Ser/Thr disaccharide. Production of these structures involves a competition among glycosyltransferases for the same substrate and thus the expression levels and subcellular distributions of glycosyltransferases within the Golgi apparatus determines the structural outcome in O-glycan biosynthesis and diversification. As illustrated in FIG. **1**, only the Galβ1-3GalNAcα-Ser/Thr disaccharide is amenable for glycoPEGylation.

[0039] However, the available amount of this structure may be greatly enhanced through treatment of the protein with sialidase or Corel GalT or a combination thereof. As a result of the glycoPEGylation process the Sialic acid PEG is added to the native structure through an α3 bond to the Galβ1-3GalNAcα-Ser/Thr disaccharide of the target protein (FIG. **1**).

[0040] Other hydrophilic polymers can also be attached to O-linked oligosaccharides. The basic requirement for enzymatically conjugating other hydrophilic polymers to FVIII via the O-glycan is the ability to couple them to the glycyl-Sialic acid derivative via the free amino group as disclosed in WO03031464. This may be achieved through a large variety of coupling chemistries known to those skilled in the art. Examples of activated biocompatible polymer includes polyalkylene oxides such as without limitation polyethylene glycol (PEG), 2-(methacryloyloxy)ethyl phosphorylcholine (mPC) polymers (as described in WO03062290), dextrans, colominic acids or other carbohydrate based polymers, polymers of amino acids or of specific peptides sequences, biotin derivatives, polyvinyl alcohol (PVA), polycarboxylates, polyvinylpyrrolidone, polyethylene-co-maleic acid anhydride, polystyrene-co-malic acid anhydride, polyoxazoline, poly-acryloylmorpholine, heparin, albumin, celluloses, hydrolysates of chitosan, starches such as hydroxyethyl-starches and hydroxy propyl-starches, glycogen, agaroses and derivatives thereof, guar gum, pullulan, inulin, xanthan gum, carrageenan, pectin, alginic acid hydrolysates, other bio-polymers and any equivalents thereof.

[0041] Pharmaceutical composition: A pharmaceutical composition is herein preferably meant to encompass compositions comprising Factor VIIII molecules according to the present invention suitable for parenteral administration, such as e.g. ready-to-use sterile aqueous compositions or dry sterile compositions that can be reconstituted in e.g. water or an aqueous buffer. The compositions according to the invention may comprise various pharmaceutically acceptable excipients, stabilizers, etc.

[0042] Additional ingredients in such compositions may include wetting agents, emulsifiers, antioxidants, bulking agents, tonicity modifiers, chelating agents, metal ions, oleaginous vehicles, proteins (e.g., human serum albumin, gelatine or proteins) and a zwitterion (e.g., an amino acid such as betaine, taurine, arginine, glycine, lysine and histidine). Such additional ingredients, of course, should not adversely affect the overall stability of the pharmaceutical formulation of the present invention. Parenteral administration may be performed by subcutaneous, intramuscular, intraperitoneal or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a solution or suspension for the administration of the FVIII compound in the form of a nasal or pulmonal spray. As a still further option, the pharmaceutical compositions containing the FVIII compound of the invention may also be adapted to transdermal administration, e.g. by needle-free injection or from a patch, optionally an iontophoretic patch, or transmucosal, e.g. buccal, administration.

[0043] In a first aspect the present invention thus relates to a B-domain truncated Factor VIII molecule with a modified circulatory half-life, said molecule being covalently conjugated with a hydrophilic polymer via an O-linked oligosaccharide in the truncated B domain, wherein Factor VIII acti-

vation (activation of the molecule) results in removal of the covalently conjugated hydrophilic polymer.

[0044] According to one embodiment, the hydrophilic polymer is PEG. The size of the PEG polymer may vary from about 10,000 to about 160,000 Da; such as 10,000 to 80,000 Da, such as e.g. about 10,000; 15,000, 20,000; 25,000; 30,000; 35,000; 40,000; 45,000; 50,000; 55,000; 60,000; 65,000, 70,000; 75,000; or 80,000 Da. Preferably, the O-linked oligosaccharide is attached to an O-glycosylation site that is made by truncation of the B-domain and not by inserting an artificial O-glycosylation site that is not found in the wt FVIII molecule.

[0045] According to a particularly preferred embodiment, the molecule according to the present invention comprises the amino acid sequence as set forth in SEQ ID NO 2. Such molecules have a unique feature in that the activated FVIII molecule is identical to the native active FVIII molecule. This feature appears to have advantageous properties in safety assessments.

[0046] The present invention also relates to pharmaceutical compositions comprising molecules according to the present invention.

[0047] The present invention furthermore relates to a method of obtaining a molecule according to the present invention, wherein said method comprises conjugating a B-domain truncated Factor VIII molecule with a hydrophilic polymer, such as e.g. a PEG group, via an O-linked oligosaccharide in the truncated B domain. It follows that the present invention also relates to molecules obtained by or obtainable by such methods.

[0048] In another aspect, the present invention relates to a method of treatment of a haemophilic disease comprising administering to a patient in need thereof a therapeutically effective amount of a molecule according to the invention.

[0049] The term "treatment", as used herein, refers to the medical therapy of any human or other animal subject in need thereof. Said subject is expected to have undergone physical examination by a medical practitioner, who has given a tentative or definitive diagnosis which would indicate that the use of said specific treatment is beneficial to the health of said human or other animal subject. The timing and purpose of said treatment may vary from one individual to another, according to the status quo of the subject's health. Thus, said treatment may be prophylactic, palliative, symptomatic and/or curative.

[0050] In yet another aspect, the present invention relates to use of a molecule according to the invention as a medicament as well as use of a molecule according to the invention for manufacture of a medicament for treatment of haemophilia.

[0051] In a final aspect, the present invention relates to a method of engineering a B-domain truncated Factor VIII molecule according to the present invention, said method comprising (i) truncating the B-domain and optionally subjecting the amino acid sequence of this truncated Factor VIII molecule to an analysis identifying potential O-linked glycosylation sites, (ii) producing the molecule in a suitable host cell and (iii) selecting molecules having O-linked glycans in the truncated B-domain.

BRIEF DESCRIPTION OF THE DRAWINGS

[0052] In the figures, the size of the conjugated groups is sometimes referred to as "K", which is herein meant to be equivalent to KDa (kilo Dalton).

[0053] FIG. **1**: Schematic drawing of glycol PEGylation process of O-linked oligosaccharides. The figure does not represent an exhaustive list of possible ways to arrive at the products obtained in the examples.

[0054] FIG. **2**: Ion-exchange chromatography of the reaction mixture on Source 15Q (A). SDS-PAGE with molecular markers (left) of collected fraction (B).

[0055] FIG. **3**: Purification of the capped product on superdex 200 size-exclusion chromatography.

[0056] FIGS. **4**A-B: Clotting activity of O-glycoPEGylated rFVIII using various aPTT reagents. Figure A shows the ration between the clotting activity and the chromogenic activity. Figure B shows the specific clotting activity.

[0057] FIG. **5**: In vivo effects (time to occlusion) in FVIII KO mice of 40K-PEG-[O]—N8.

[0058] FIG. **6**: Flow diagram showing the process steps involved in production of glycoPEGylated Factor FVIII according to the invention.

[0059] FIG. **7**: Schematic representation of a Factor VIII molecule according to the present invention produced in the Examples.

[0060] FIGS. **8**A-D: FIGS. **8**A-D show a representative branched PEG polymer of use in embodiments of the invention, referred to herein as "SA-glycerol-PEG." FIG. **8**A shows an exemplary SA-glycerol-PEG component of CMP-SA-glycerol-PEG or of a SA-glycerol-PEG linked to a glycan or an amino acid of a polypeptide. FIG. **8**B shows the SA-glycerol-PEG moiety linked to a glycan or polypeptide through a Gal residue. FIG. **8**C shows the SA-glycerol-PEG moiety linked to a glycan or polypeptide through a Gal-GalNAc residue. FIG. **8**D shows the SA-glycerol-PEG moiety linked to an amino acid of a polypeptide through a Gal-GalNAc moiety.

EXAMPLES

Example 1

Production of Recombinant B Domain Truncated O-Glycosylated Factor VIII

[0061] An example of the amino acid sequence of a B-domain deleted Factor VIII molecule is given in SEQ ID NO 2. This polypeptide may also be referred to as "N8". This molecule comprises a 21 amino acid residue linker sequence (SFSQNSRHPSQNPPVLKRHQR (SEQ ID NO 3)—the underlined S is the Serine residue with the O-glygan that is pegylated in Example 2).

[0062] Factor VIII molecules according to the present invention may in the Examples be referred to in various ways—but all references to Factor VIII molecules refer to Factor VIII molecules according to the invention, or alternatively Factor VIII molecules in the process of being converted to Factor VIII molecules according to the invention.

SEQ ID NO 2:
ATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPKSFPFNTSVVYKKTL

FVEFTDHLFNIAKPRPPWMGLLGPTIQAEVYDTVVITLKNMASHPVSLHA

VGVSYWKASEGAEYDDQTSQREKEDDKVFPGGSHTYVWQVLKENGPMASD

PLCLTYSYLSHVDLVKDLNSGLIGALLVCREGSLAKEKTQTLHKFILLFA

VFDEGKSWHSETKNSLMQDRDAASARAWPKMHTVNGYVNRSLPGLIGCHR

-continued

KSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITFLTAQTLL

MDLGQFLLFCHISSHQHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDL

TDSEMDVVRFDDDNSPSFIQIRSVAKKHPKTWVHYIAAEEEDWDYAPLVL

APDDRSYKSQYLNNGPQRIGRKYKKVRFMAYTDETFKTREAIQHESGILG

PLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVRPLYSRRLPKGVKHLKD

FPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFVNMERDLASGLIGP

LLICYKESVDQRGNQIMSDKRNVILFSVFDENRSWYLTENIQRFLPNPAG

VQLEDPEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLS

VFFSGYTFKHKMVYEDTLTLFPFSGETVFMSMENPGLWILGCHNSDFRNR

GMTALLKVSSCDKNTGDYYEDSYEDISAYLLSKNNAIEPRSFSQNSRHPS

QNPPVLKRHQREITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQS

PRSFQKKTRHYFIAAVERLWDYGMSSSPHVLRNRAQSGSVPQFKKVVFQE

FTDGSFTQPLYRGELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFYS

SLISYEEDQRQGAEPRKNFVKPNETKTYFWKVQHHMAPTKDEFDCKAWAY

FSDVDLEKDVHSGLIGPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETK

SWYFTENMERNCRAPCNIQMEDPTFKENYRFHAINGYIMDTLPGLVMAQD

QRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMALYNLYPGVFETVE

MLPSKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDFQI

TASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQ

GARQKFSSLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKH

NIFNPPIIARYIRLHPTHYSIRSTLRMELMGCDLNSCSMPLGMESKAISD

AQITASSYFTNMFATWSPSKARLHLQGRSNAWRPQVNNPKEWLQVDFQKT

MKVTGVTTQGVKSLLTSMYVKEFLISSSQDGHQWTLFFQNGKVKVFQGNQ

DSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRMEVLGCEAQDLY

Cell Line and Culture Process:

[0063] Using Factor VIII cDNA a mammalian expression plasmid encoding B-domain deleted Factor VIII having an amino acid sequence as set forth in SEQ ID NO 2 was constructed. The plasmid is encoding Factor VIII heavy chain comprising amino acid 1-740 of full length human Factor VIII and Factor VIII light chain comprising amino acid 1649-2332 of full length human Factor VIII. The heavy and light chain sequences are connected by a 21 amino acid linker with the sequence of amino acid 741-750 and 1638-1648 of full length human Factor VIII. Chinese hamster ovary (CHO) cells were transfected with the BDD Factor VIII coding plasmid and selected with the dihydrofolate reductase system eventually leading to a clonal suspension producer cell cultivated in animal component-free medium.

[0064] The first step in the process is the inoculation of a cell vial, from a working cell bank vial, into a chemically defined and animal component free growth medium. Initially after thawing, the cells are incubated in a T-flask. One or two days after thawing, the cells are transferred to a shaker flask, and the culture volume is expanded by successive dilutions in order to keep the cell density between 0.2-3.0×10^6 cells/ml. The next step is the transfer of the shaker flask culture into seed bioreactors. The culture volume is here further expanded before the final transfer to the production bioreactor. The same chemically defined and animal component free medium is used for all the inoculum expansion steps. After transfer to the production bioreactor, the medium is supplemented with components that increase the product concentration. In the production bioreactor the cells are cultured in a repeated batch process with a cycle time of three days. At harvest, 80-90% of the culture volume is transferred to a harvest tank. The remaining culture fluid is then diluted with fresh medium, in order to obtain the initial cell density, and then a new growth period is initiated.

[0065] The harvest batch is clarified by centrifugation and filtration and transferred to a holding tank before initiation of the purification process. A buffer is added to the cell free harvest in the holding tank to stabilise pH.

[0066] By the end of the production run, cells are collected and frozen down, in order to make an end of production cell bank. This cell bank is tested for mycoplasma, sterility and viral contamination.

Purification:

[0067] For the isolation of B-domain-deleted Factor VIII from cell culture media, a four step purification procedure was used including a concentration step on a Capto MMC column, an immunoabsorbent chromatography step, an anionic exchange chromatography and finally a gel filtration step. Typically the following procedure was used: 11 litre of sterile filtered medium was pumped onto at column (1.6×12 cm) of Capto MMC (GE Healthcare, Sweden) equilibrated in buffer A: 20 mM imidazole, 10 mM $CaCl_2$, 50 mM NaCl, 0.02% Tween 80, pH=7.5 at a flow of 15 ml/min. The column was washed with 75 ml of buffer A followed by wash with 75 ml of buffer A containing 1.5 M NaCl. The protein was eluted with 20 mM imidazole, 10 mM $CaCl_2$, 0.02% Tween 80, 2.5 M NaCl, 8 M ethyleneglycol, pH=7.5 at a flow of 1 ml/min. Fractions of 8 ml were collected and assayed for Factor VIII activity (CoA-test). Factor VIII containing fractions were pooled and normally a pool volume of around 50 ml was obtained.

[0068] A monoclonal antibody against Factor VIII has been developed (Kjalke Eur J Biochem 234 773). By epitope mapping (results not shown) this antibody, F25, was found to recognise the far C-terminal sequence of the heavy chain from amino acid residue 725 to 740. The F25 antibody was coupled to NHS-activated Sepharose 4 FF (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) at a density of 2.4 mg per ml of gel essentially as described by the manufacturer. The pool from the previous step was diluted 10 times with 20 mM imidazole, 10 mM $CaCl_2$, 0.02% Tween 80, pH=7.3 and applied to the F25 Sepharose column (1.6×9.5 cm) equilibrated with 20 mM imidazole, 10 mM $CaCl_2$, 150 mM NaCl, 0.02% Tween 80, 1 M glycerol pH=7.3 at a flow of 0.5 ml/min. The column was washed with equilibration buffer until the UV signal was constant and then with 20 mM imidazole, 10 mM $CaCl_2$, 0.65 M NaCl, pH=7.3 until the UV signal was constant again. Factor VIII was eluted with 20 mM imidazole, 10 mM $CaCl_2$, 0.02% Tween 80, 2.5 M NaCl, 50% ethyleneglycol, pH=7.3 at a flow of 1 ml/min. Fractions of 1 ml were collected and assayed for Factor VIII activity (CoA-test). Factor VIII containing fractions were pooled and normally a pool volume of around 25 ml was obtained.

[0069] A buffer A: 20 mM imidazole, 10 mM $CaCl_2$, 0.02% Tween 80, 1 M glycerol, pH=7.3 and a buffer B: 20 mM

imidazole, 10 mM $CaCl_2$, 0.02% Tween 80, 1 M glycerol, 1 M NaCl, pH=7.3 was prepared for the ion-exchange step. A column (1×10 cm) of Macro-Prep 25Q Support (Bio-Rad Laboratories, Hercules, Calif., USA) was equilibrated with 85% buffer A/15% Buffer B at a flow of 2 ml/min. The pool from the previous step was diluted 10 times with buffer A and pumped onto the column with a flow of 2 ml/min. The column was washed with 85% buffer A/15% buffer B at a flow of 2 ml/min and Factor VIII was eluted with a linear gradient from 15% buffer B to 70% buffer B over 120 ml at a flow of 2 ml/min. Fractions of 2 ml were collected and assayed for Factor VIII activity (CoA-test). Factor VIII containing fractions were pooled and normally a pool volume of around 36 ml was obtained.

[0070] The pool from the previous step was applied to a Superdex 200, prep grade (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) column (2.6×60 cm) equilibrated and eluted at 1 ml/min with 20 mM imidazole, 10 mM $CaCl_2$, 0.02% Tween 80, 1 M glycerol, 150 mM NaCl, pH=7.3. Fractions of 3 ml were collected and assayed for Factor VIII activity (CoA-test). Factor VIII containing fractions were pooled and normally a pool volume of around 57 ml was obtained. The pool containing Factor VIII was store at −80° C.

[0071] With the use of the above four-step purification procedure an overall yield of approximately 15% was obtained as judged by CoA activity and ELISA measurements.

[0072] The cell line used for manufacture of N8 is a recombinant Chinese hamster ovary (CHO) cell line stably transfected with expression plasmid #814 F8-500 in pTSV7 consisting of the pTSV7 expression vector with an insert containing cDNA encoding the F8-500 protein. "N8" is herein meant to correspond to a protein having an amino acid sequence as listed in SEQ ID NO 2. Starting at the N-terminus, the F8-500 protein (N8) consists of the FVIII signal peptide (amino acids −19 to −1) followed by the FVIII heavy chain without the B domain (amino acids 1-740), a 21 amino acid linker (SFSQNSRHPSQNPPVLKRHQR), and the FVIII light chain (amino acids 1649-2332 of wild-type human FVIII). The sequence of the 21 amino acid linker is derived from the FVIII B domain and consists of amino acids 741-750 and 1638-1648 of full length FVIII.

[0073] CHO cells were transfected with 814 F8-500 in pTSV7 and selected with the dihydrofolate reductase system eventually leading to a clonal suspension producer cell cultivated in animal component-free medium. A production run is initiated by thawing a working cell bank vial and expanding the cells until transfer to a production bioreactor. The same chemically defined and animal component free medium is used for all the inoculum expansion steps. After transfer to the production bioreactor, the medium is supplemented with components that increase the product concentration. In the production bioreactor the cells are cultured in a repeated batch process with a cycle time of three days. At harvest, 80-90% of the culture volume is transferred to a harvest tank. The remaining culture fluid is then diluted with fresh medium, in order to obtain the initial cell density, and then a new growth period is initiated. The harvest batch is clarified by centrifugation and filtration and transferred to a holding tank before initiation of the purification process. A buffer is added to the cell free harvest in the holding tank to stabilize pH.

Example 2

PEGylation of Recombinant B Domain Truncated and O-glycosylated Factor VIII

[0074] The recombinant Factor VIII molecules obtained in Example 1 are conjugated with polyethylenglycol (PEG) using the following procedure:

[0075] For the glycoPEGylation of the recombinant Factor VIII molecules obtained in Example 1 to be efficient a FVIII concentration >5 mg/ml is preferred. Since FVIII is not normally soluble at the concentration a screening of selected buffer compositions was conducted (see some of these results in table 1).

[0076] Based on these considerations, a buffer containing 50 mM MES, 50 mM CaCl2, 150 mM NaCl, 20% glycerol, pH 6.0 was found to be a suitable reaction buffer.

TABLE 1

Evaluation of impact of reaction conditions on FVIII solubility and aggregation.

Reaction buffer composition	Precipitate	% Aggregate
10 mM Histidine, 260 mM Glycine, 1% Sucrose, 10 mM CaCl2	YES	n. d.
50 mM HEPES, 10 mM CaCl2, 150 mM NaCl, pH 7;	YES	n. d.
50 mM MES, 10 mM CaCl2, 150 mM NaCl, pH 6.0	YES	n. d.
50 mM MES, 50 mM CaCl2, 150 mM NaCl, pH 6.0	NO	8
50 mM MES, 50 mM CaCl2, 150 mM NaCl, 10% glycerol, pH 6.0	NO	5
50 mM MES, 50 mM CaCl2, 150 mM NaCl, 20% glycerol, pH 6.0	NO	1.0-1.7

[0077] Recombinant FVIII which had been purified as described above was concentrated in reaction buffer either by ion exchange on a Poros 50 HQ column using step elution, on a Sartorius Vivaspin (PES) filter, 10 kDa cut-off or on an Amicon 10 kDa MWCO PES filter to a concentration of 6-10 mg/mL. The glycoPEGylation of FVIII was initiated by mixing Factor VIII (BDD) (~4.7 mg/mL final) with Sialidase (*A. urifaciens*) (159 mU/mL), CMP-SA-glycerol-PEG-40 kDa (5 mol.eq.) and MBP-ST3Gal1 (540 mU) in reaction buffer (50 mM MES, 50 mM CaCl2, 150 mM NaCl, 20% glycerol, 0.5 mM antipain, pH 6.0). The reaction mixture was incubated at 32° C. until a conversion yield of ~20-30% of total.

[0078] Following the incubation the sample was diluted with Buffer A (25 mM Tris, 5 mM $CaCl_2$, 20 mM NaCl, 20% glycerol, pH 7.5) and applied onto a Source 15Q column (1 cm id×6 cm, 4.7 mL, 1 mL/min, 280 nm). The bound material was washed with Buffer A and eluted using a step gradient with Buffer B (25 mM Tris, 5 mM $CaCl_2$, 1 M NaCl, 20% glycerol, pH 7.5). GlycoPEGylated Factor VIII-(O)-SA-glycerol-PEG-40 kDa was eluted from the column at ~25% Buffer B. FIG. **2** shows ion-exchange chromatography of the reaction mixture on Source 15Q.

[0079] In order to block free galactose moieties which had been exposed on the N-glycans during the sialidase treatment the pooled fraction of Factor VIII-SA-glycerol-PEG-40 kDa (1.0 mg/mL final) was mixed with CMP-SA (2,000 mol eq) and MBP-SBD-ST3Gal3 (400 mU/mL) in reaction buffer 50 mM MES, 20 mM CaCl2, 150 mM NaCl, 10 mM MnCl2, 20% glycerol, pH 6.0 and incubated at 32° C. for 11 hours.

[0080] The resulting capped, glycoPEGylated Factor VIII-SA-glycerol-PEG-40 kDa was separated from CMP-SA and ST3GalIII by gel-filtration on a Superdex 200 column (10 cm id×300 mm; 280 nm) equilibrated with 50 mM MES, 50 mM CaCl2, 150 mM NaCl, 10% glycerol, pH 6.0; flow rate of 0.25 mL/min. The product Factor VIII-SA-glycerol-PEG-40 kDa elutes at 38 min. FIG. **3** shows purification of the capped product using Superdex 200 size-exclusion chromatography. The peak fraction was collected, aliquoted and subjected to subsequent analysis.

[0081] The purpose of the capping procedure is to reduce in vivo clearance of the conjugated Factor VIII molecule.

Example 3

Activity of O-Glycan PEGylated rFVIII in Chromogenic FVIII Activity Assay

[0082] The activity of the O-glycoPEGylated rFVIII obtained in Example 2 was evaluated in a chromogenic FVIII assay using Coatest SP reagents (Chromogenix) as follows: rFVIII samples and calibrator (the 7th international FVIII standard from NIBSC) were diluted in Coatest assay buffer (50 mM Tris, 150 mM NaCl, 1% BSA, pH 7.3, with preservative). Fifty μl of samples, standards, and buffer negative control were added to 96-well microtiter plates (Nunc) in duplicates. The Factor IXa/Factor X reagent, the phospholipid reagent and $CaCl_2$ from the Coatest SP kit were mixed 5:1:3 (vol:vol:vol) and 75 μl of this added to the wells. After 15 min incubation at room temperature 50 μl of the Factor Xa substrate 5-2765/thrombin inhibitor 1-2581 mix was added and the reactions incubated 10 min at room temperature before 25 μl 1 M citric acid, pH 3, was added. The absorbance at 415 nm was measured on a Spectramax microtiter plate reader (Molecular Devices) with absorbance at 620 nm used as reference wavelength. The value for the negative control was subtracted from all samples and a calibration curve prepared by linear regression of the absorbance values plotted vs. FVIII concentration. The specific activity was calculated by dividing the activity of the samples with the protein concentration determined by size exclusion HPLC by integrating the light chain peak in the HPLC chromatogram, i.e. the PEG-moiety was not included. The data in table 2 demonstrate that the specific chromogenic activity was maintained for the O-glycoPEGylated rFVIII compounds, meaning that Factor VIII activity appear to be retained in the PEGylated variants.

TABLE 2

Specific chromogenic activity of O-glycoPEGylated rFVIII with different PEG group sizes.

rFVIII compound	Specific chromogenic activity (IU/mg)
rFVIII	11819 ± 727 (5)
10 KDa-PEG-[O]-rFVIII	Approx 8331 (1)
40 KDa-PEG-[O]-rFVIII	9760 ± 886 (8)
80 KDa-PEG-[O]-rFVIII	12129 ± 2643 (3)

Data are mean and standard deviations of the numbers of independent determinations noted in parentheses

Example 4

Activity of O-Glycan PEGylated rFVIII in FVIII Clotting Activity Assay

[0083] The activity of the O-glycoPEGylated rFVIII was further evaluated in FVIII clotting assay. rFVIII samples were diluted in HBS/BSA (20 mM hepes, 150 mM NaCl, pH 7.4 with 1% BSA) to approximately 10 U/ml followed by 10-fold dilution in FVIII-deficient plasma containing VWF (Dade Behring). The samples and a calibrated plasma standard (HemosIL Calibration Plasma from Instrumentation Laboratory) were subsequently diluted in HBS/BSA to four (samples) or six (calibrator) different concentrations. The clotting time was measured on an ACL9000 instrument (Instrumentation laboratory) using the single factor program, where samples/standards were mixed with equal volumes of FVIII-deficient plasma with VWF (Dade Behring), calcium and aPTT reagents, and the clotting time measured. As reagents the following were used: Synthasil (HemosIL, Instrumentation Laboratory), Actin FS (Activated PTT Reagent, Dade Behring) Stago (STA® PTT-A, Stago), and dAPPTin (DAPPTIN®TC, Technoclone). The activities of the samples were calculated based on a semi-log plot of clotting time versus concentration of the calibrator.

[0084] The clotting activity (FIG. **4**) of the O-glycoPE-Gyated rFVIII compounds (control, 10, 40, and 80 kDA PEG, respectively) was decreased to various extend depending on the PEG size and the aPTT reagents used. Using Synthasil or dAPPTin as aPTT reagents resulted in a gradual decrease in clotting activity with PEG-size. With Stago's aPTT reagent, a 50% lower specific clotting activity was observed for all three O-glycoPEGylated N8 compounds evaluated. When Actin FS was used as aPTT reagent a specific clotting activity around 10,000 IU/mg was maintained. The data indicates that the aPTT assay is influenced by the presence of a PEG moiety, however, using a selected aPTT reagents e.g. Actin FS the specific clotting activity of rFVIII is not impaired upon O-glycoPEGylation.

Example 5

Effect of O-Linked PEGylation of rFVIII on Co-Factor Activity and Rate of FVIII Activation

[0085] Incorporation of activated FVIII into the FIXa-FVIIIa complex enhances the catalytic efficiency of FIXa-catalyzed FX activation five orders of magnitude (van Dieij en et al. (1981) *J Biol Chem* 256:3433) and characterization of FIXa-FVIIIa complex assembly and FX activation kinetics is a sensitive measure of the functional integrity of FVIIIa molecules. The co-factor activity of thrombin-activated rFVIII or PEG-rFVIII was characterized by determining the kinetic parameters of FIXa-catalyzed FX activation in the presence of phospholipids and thrombin-activated rFVIII or PEG-rFVIII. Using the FVIIIa activity assay (FIXa-cofactor activity assay), reciprocal titrations of FIXa and FVIIIa against a fixed concentration (0.1 nM) of rFVIIIa or FIXa, respectively, were performed to obtain apparent affinity of FIXa for rFVIIIa ($K_{1/2FIXa}$) and functional FVIIIa concentration. The Michaelis constant (k_m) and turn-over number (k_{cat}) of FX activation were obtained from titrations of FX against a fixed concentration of FIXa-FVIIIa complex.

[0086] The FIXa-cofactor activity assays was carried out as follows: Thrombin-activated rFVIII and PEG-rFVIII variants were prepared freshly for each test by incubating rFVIII (usually 0.7 nM, 1 U/mL) with 5 nM human α-thrombin for exactly 30 seconds at 37° C. Subsequently, the rate of FX activation was quantified by subsampling the activation reaction above into a prepared mixture of FIXa, phospholipid vesicles (Phospholipid TGT from Rossix [Mölndal, Sweden]), hirudin, Pefabloc Xa and $CaCl_2$; FX activation was

initiated by addition of FX and allowed to proceed for either 30 seconds or 60 seconds at 37° C. Activation was stopped by dilution of the FX activation reaction into ice cold buffer containing EDTA. Using a FXa specific chromogenic substrate, the concentration of FXa was quantified by reading absorbance at 405 nM in an ELISA reader. A reference curve prepared using purified FXa was used to convert absorbance to FXa concentration. The turn-over number of FIXa-rFVIIIa complexes assembled from activated rFVIII or PEG-rFVIII variants was used to convert the rate of FX activation to rFVIIIa concentration.

[0087] The rate of thrombin-catalyzed rFVIII activation was measured by quantifying the initial (0 to 3 min) formation of rFVIIIa in a mixture containing 0.7 nM rFVIII or PEG-rFVIII and 0.13 nM human α-thrombin. Formation of FVIIIa was linear in time. The rate of FVIIIa activation was expressed as moles rFVIIIa formed per minute per mole of rFVIII initially present ($v/[rFVIII]_0$).

[0088] O-linked glycoPEGylation of rFVIII did not affect the rate of thrombin-catalyzed rFVIII activation or the k_m or k_{cat} of FIXa-catalyzed activation of FX in the presence of activated rFVIII (see Table 3). Furthermore, O-linked glycoPEGylation did not affect the apparent K_d of rFVIIIa-FIXa interaction ($K_{1/2FIXa}$).

[0089] FIGS. **4**A-B show clotting activity of O-glycoPEGylated rFVIII using various aPTT reagents.

Data are shown as the ratio between the clotting activity and the chromogenic activity (FIG. **4**A) or as the specific clotting activity (FIG. **4**B). Mean and standard deviations of values from three independent experiments are shown.

TABLE 3

Rate of rFVIII activation and kinetic constants of FX activation by FIXa

FVIII molecule	Rate of FVIII activation	$K_{1/2FIXa}$	FX Activation K_m	FX Activation k_{cat}
	10^{-3} × min^{-1}	nM	nM	s^{-1}
rFVIII	10.4 ± 1.9	0.88 ± 0.46	7.9 ± 1.7	4.5 ± 1.9
40 K-PEG-[O]-rFVIII	9.9 ± 3.8	0.42 ± 0.02	6.4 ± 0.8	4.7 ± 0.2
80 K-PEG-[O]-rFVIII	9.8 ± 3.4	1.11 ± 0.12	8.2 ± 0.6	3.7 ± 0.4

Data are mean and standard deviations of 3-6 measurements.

Example 6

Pharmacokinetics of glycoPEGylated B-Domain Deleted (BDD)-FVIII in FVIII KO Mice and vWF KO Mice

[0090] The pharmacokinetics of BDD-FVIII glycoPEGylated with various PEG sizes was studied following i.v. administration of 280 IU/kg to FVIII KO mice.

[0091] The following compounds were studied: BDD-FVIII, BDD-FVIII-10K PEG (O-glycan, 0129-0000-1005), BDD-FVIII-40K PEG (O-glycan, 0129-0000-1003), BDD-FVIII-2×40K PEG (O and N-glycan 0129-0000-1008-1A), BDD-FVIII-80K PEG (N-glycan, 0129-0000-1012, 0-glycan 0129-0000-1009).

Design of Animal Studies:

[0092] Factor VIII knock out (FVIII KO) mice were bred at Taconic M&B, based on exon 16 KO in C57B1/6 background. A mixture of male and female (app.1:1) with an approximate weight of 25 g and age range of 19-26 weeks were employed. The mice were not fully back-crossed. No FVIII is detected in this mouse strain.

[0093] The mice were given single i.v. injections of 280 IU/kg in the tail vein with the compounds listed above. If a mouse was dosed peri-veneously, the mouse was exchanged with another mouse. After dosing, orbital plexus blood samples were collected from pre-dose until 64 hours after dosing using non-coated capillary glass tubes. Three samples were taken from each mouse, and 2, 3 or 4 samples were collected at each time point. Blood was stabilised in sodium citrate (9:1) and diluted in FVIII COA SP buffer (1:4) before centrifugation for 5 minutes at 4000 g. Plasma obtained from diluted blood was frozen at dry ice at kept at −80° C. before quantitative analysis by means of FVIII chromogenic activity and/or FVIII antigen analysis.

Quantitative Plasma Analysis:

[0094] The FVIII chromogenic activity was determined by the use of reagents from the Coatest SP kit (Chromogenix). Diluted plasma samples, calibrators (ILS calibration plasma) in Coatest SP-buffer, and buffer negative control (50 μl) were added to 96-well microtiter plates (Nunc) in duplicates. The Factor IXa/Factor X reagent, the phospholipid reagent and CaCl2 from the Coatest SP kit were mixed 5:1:3 (vol:vol:vol) and 75 l of this added to the wells. After 15 min incubation at room temperature 50 l of the Factor Xa substrate 5-2765/thrombin inhibitor 1-2581 mix was added and the reactions incubated 10 min at room temperature before 25 μl 2% citric acid was added. The absorbance at 405 nm was measured on a Spectramax microtiter plate reader (Molecular Devices). FVIII activity in the plasma samples was calculated from the calibration curve made by dilutions of the calibrated international plasma standard (ILS).

[0095] The FVIII antigen assay was a commercial available ELISA kit from Diagnostica Stago (Asserachrom VIII:CAg) using two monoclonal antibodies directed against the light chain of human FVIII. Calibrators (dilutions of the compounds) or plasma samples were diluted at least 50-fold in coatest SP dilution buffer supplied by the kit were applied to the precoated wells and the ELISA performed according to the manufactures instructions. The values used for reporting the pharmacokinetic study are based on the standard curve made from the compounds themselves.

Pharmacokinetic Parameters Estimations:

[0096] Pharmacokinetic analysis was carried out by non-compartmental methods (NCA) of data using ILS as calibrator (data based on chromogenic activity), using the compounds themselves as calibrator (data based on ELISA). From the data the following parameters were estimated: Cmax (maximum concentration, after i.v. administration this is at the first sampling time point), Tmax (time of maximum concentration, after i.v. administration this is the first time point), AUCO-∞ (area under the curve from time 0 to infinity), T½, (terminal half-live), CL (clearance) and Vss (volume of distribution at steady state). All calculations were performed using WinNonlin Pro version 4.1.

[0097] After i.v. injection of 280 IU/Kg BDD-FVIII, BDD-FVIII-10 KDa PEG, BDD-FVIII-40 KDa PEG, BDD-FVIII-2×40 KDa PEG and BDD-FVIII-80 KDa PEG to FVIII KO mice, the half-life increased along with increasing PEG size in the range of 7.8 h (BDD-FVIII) to 15-16 h (Table 4), which corresponds to a 2-fold increase. Similarly, the clearance was reduced and the MRT increased with increasing PEG sizes (Table 4).

TABLE 4

Pharmacokinetic parameters estimates of FVIII glycoPEGylated with different sizes of PEG after i.v. administration to FVIII KO mice based on chromogenic activity (BDD: B-domain deleted).

Compound	Dose (IU/kg)	T½ (h)	CL (ml/h/kg)	MRT (h)	Prolongation (fold)
BDD-FVIII	280	6.7-9.3	8.1-10	9.9-11	1
BDD-FVIII 10 KDa PEG (O-glycan)	280	10	8.5	16	1.3
BDD-FVIII-2x40 KDa PEG	280	13	5.8	19	1.9-2.1
BDD-FVIII 40 KDa PEG (O-glycan)	280	15-16	3.6-3.8	20-22	1.7
BDD-FVIII 80 KDa PEG (O-glycan)	280	15	6.4	21	1.9

CONCLUSION

[0098] GlycoPEGylation of BDD-FVIII increased the T½ 1.3-2.1 fold as compared to BDD-FVIII after i.v. administration of 280 IU/kg to FVIII KO mice. An increasing T½ was observed as the size of the PEG group was increased in the range of 10 KDa to 80 KDa PEG.

Example 7

Prolonged Haemostatic Effect of 40K-PEG-[O]—N8 Compared to Advate in a FeCl3 Induced Injury Model in Haemophilia a Mice

[0099] The duration of action of 40K-PEG-[O]—N8 vs. recombinant FVIII (Advate) was investigated in a FeCl3 induced injury model in haemophilia A (F8-KO) mice.

[0100] Mice were anesthetized and placed on a heating pad (37° C.) to maintain body temperature. The carotid artery was exposed and a flow-probe (0.5PSB Nanoprobe) that measures blood flow by ultrasound was placed around the artery. The injury (an iron-mediated chemical oxidation) was induced by applying a filter paper (2×5 mm) briefly soaked in a 10% FeCl3 solution around the exposed carotid artery. The filter paper was removed after 3 min. The artery was then washed three times with 0.9% NaCl and finally Surgilube (an acoustic coupler) was applied in order to displace air in the flow-probe and secure an optimised measurement of the blood flow. Blood flow (ml/min) was recorded for 25 min after removing the FeCl3 saturated filter paper and the time to occlusion was determined by measuring the time (in min) from removal of FeCl3 saturated filter paper until the blood flow was 0 ml/min. If occlusion did not occur after 25 min the occlusion time was reported as 25 min even though no occlusion occurred during the observation period. F8-KO mice (n=6-10) were treated with Advate (280 U/kg), 40K-PEG-[O]—N8 (280 U/kg), or vehicle. The FeCl3 induced injury was made 5 min (acute effect) or 24, 48, 60, and 72 hours after dosing. The blood flow (ml/min) was recorded for 25 min after removal of FeCl3, and subsequently the time to occlusion was determined.

[0101] No occlusion occurred in vehicle treated F8-KO mice, whereas occlusion occurred in all mice treated with 40 KDa-PEG-[O]—N8 and Advate 5 min after dosing (acute effect) with a mean occlusion time of 4.3±0.4 min and 5.2±0.7 min, respectively. In 40 KDa-PEG-[O]—N8 treated F8-KO mice the average occlusion time increased to 13.8±3.4 min at 72 hours after dosing. In contrast the Advate treated F8-KO mice had an occlusion time of 13.0±3.4 min and 15.9±2 9 min after 24 and 48 hours, respectively. Importantly no occlusions were observed 60 and 72 hours after administration of Advate. In all mice treated with 40 KDa-PEG-[O]—N8 occlusion was observed 24 hours after dosing whereas only 67% of the mice treated with Advate occluded. After 72 hours occlusion was still seen in 63% of the mice treated with 40 KDa-PEG-[O]—N8, whereas no occlusion was observed 60 and 72 hours after administration of Advate.

Prolonged Effect of 40 KDa-PEG-[O]—N8 in F8-KO Mice.

[0102] The FeCl3 induced injury was made 5 min (acute effect), 24, 48, 60, and 72 hours after dosing 280 IU/kg 40 KDa-PEG-[O]—N8, 280 IU/kg Advate, or vehicle. The blood flow (ml/min) was recorded for 25 min after removal of FeCl3, and subsequently the time to occlusion was determined. At 60 and 72 hours after dosing no occlusion occurred in mice dosed with Advate. Mean and SEM of 6-10 mice per group are shown. Time to occlusion between the different groups was compared using Kruskal-Wallis test including Dunn's post test. * : $p<0.05$; **: $p<0.01$.

[0103] In conclusion, the haemostatic effect of 40 KDa-PEG-[O]—N8 is significantly prolonged compared to Advate in a FeCl3 induced injury model in F8-KO mice.

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His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
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Glu Thr  Glu Leu Glu Lys Arg  Ile Ile Val Asp Asp  Thr Ser Thr
    1325                1330                1335

Gln Trp  Ser Lys Asn Met Lys  His Leu Thr Pro Ser  Thr Leu Thr
    1340                1345                1350

Gln Ile  Asp Tyr Asn Glu Lys  Glu Lys Gly Ala Ile  Thr Gln Ser
    1355                1360                1365

Pro Leu  Ser Asp Cys Leu Thr  Arg Ser His Ser Ile  Pro Gln Ala
    1370                1375                1380

Asn Arg  Ser Pro Leu Pro Ile  Ala Lys Val Ser Ser  Phe Pro Ser
    1385                1390                1395

Ile Arg  Pro Ile Tyr Leu Thr  Arg Val Leu Phe Gln  Asp Asn Ser
    1400                1405                1410

Ser His  Leu Pro Ala Ala Ser  Tyr Arg Lys Lys Asp  Ser Gly Val
    1415                1420                1425

Gln Glu  Ser Ser His Phe Leu  Gln Gly Ala Lys Lys  Asn Asn Leu
    1430                1435                1440

Ser Leu  Ala Ile Leu Thr Leu  Glu Met Thr Gly Asp  Gln Arg Glu
    1445                1450                1455

Val Gly  Ser Leu Gly Thr Ser  Ala Thr Asn Ser Val  Thr Tyr Lys
    1460                1465                1470

Lys Val  Glu Asn Thr Val Leu  Pro Lys Pro Asp Leu  Pro Lys Thr
    1475                1480                1485

Ser Gly  Lys Val Glu Leu Leu  Pro Lys Val His Ile  Tyr Gln Lys
    1490                1495                1500

Asp Leu  Phe Pro Thr Glu Thr  Ser Asn Gly Ser Pro  Gly His Leu
    1505                1510                1515

Asp Leu  Val Glu Gly Ser Leu  Leu Gln Gly Thr Glu  Gly Ala Ile
    1520                1525                1530

Lys Trp  Asn Glu Ala Asn Arg  Pro Gly Lys Val Pro  Phe Leu Arg
    1535                1540                1545

Val Ala  Thr Glu Ser Ser Ala  Lys Thr Pro Ser Lys  Leu Leu Asp
    1550                1555                1560

Pro Leu  Ala Trp Asp Asn His  Tyr Gly Thr Gln Ile  Pro Lys Glu
    1565                1570                1575

Glu Trp  Lys Ser Gln Glu Lys  Ser Pro Glu Lys Thr  Ala Phe Lys
    1580                1585                1590

Lys Lys  Asp Thr Ile Leu Ser  Leu Asn Ala Cys Glu  Ser Asn His
    1595                1600                1605
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Ala Ile  Ala Ala Ile Asn Glu  Gly Gln Asn Lys Pro  Glu Ile Glu
    1610                1615                1620

Val Thr  Trp Ala Lys Gln Gly  Arg Thr Glu Arg Leu  Cys Ser Gln
    1625                1630                1635

Asn Pro  Pro Val Leu Lys Arg  His Gln Arg Glu Ile  Thr Arg Thr
    1640                1645                1650

Thr Leu  Gln Ser Asp Gln Glu  Glu Ile Asp Tyr Asp  Asp Thr Ile
    1655                1660                1665

Ser Val  Glu Met Lys Lys Glu  Asp Phe Asp Ile Tyr  Asp Glu Asp
    1670                1675                1680

Glu Asn  Gln Ser Pro Arg Ser  Phe Gln Lys Lys Thr  Arg His Tyr
    1685                1690                1695

Phe Ile  Ala Ala Val Glu Arg  Leu Trp Asp Tyr Gly  Met Ser Ser
    1700                1705                1710

Ser Pro  His Val Leu Arg Asn  Arg Ala Gln Ser Gly  Ser Val Pro
    1715                1720                1725

Gln Phe  Lys Lys Val Val Phe  Gln Glu Phe Thr Asp  Gly Ser Phe
    1730                1735                1740

Thr Gln  Pro Leu Tyr Arg Gly  Glu Leu Asn Glu His  Leu Gly Leu
    1745                1750                1755

Leu Gly  Pro Tyr Ile Arg Ala  Glu Val Glu Asp Asn  Ile Met Val
    1760                1765                1770

Thr Phe  Arg Asn Gln Ala Ser  Arg Pro Tyr Ser Phe  Tyr Ser Ser
    1775                1780                1785

Leu Ile  Ser Tyr Glu Glu Asp  Gln Arg Gln Gly Ala  Glu Pro Arg
    1790                1795                1800

Lys Asn  Phe Val Lys Pro Asn  Glu Thr Lys Thr Tyr  Phe Trp Lys
    1805                1810                1815

Val Gln  His His Met Ala Pro  Thr Lys Asp Glu Phe  Asp Cys Lys
    1820                1825                1830

Ala Trp  Ala Tyr Phe Ser Asp  Val Asp Leu Glu Lys  Asp Val His
    1835                1840                1845

Ser Gly  Leu Ile Gly Pro Leu  Leu Val Cys His Thr  Asn Thr Leu
    1850                1855                1860

Asn Pro  Ala His Gly Arg Gln  Val Thr Val Gln Glu  Phe Ala Leu
    1865                1870                1875

Phe Phe  Thr Ile Phe Asp Glu  Thr Lys Ser Trp Tyr  Phe Thr Glu
    1880                1885                1890

Asn Met  Glu Arg Asn Cys Arg  Ala Pro Cys Asn Ile  Gln Met Glu
    1895                1900                1905

Asp Pro  Thr Phe Lys Glu Asn  Tyr Arg Phe His Ala  Ile Asn Gly
    1910                1915                1920

Tyr Ile  Met Asp Thr Leu Pro  Gly Leu Val Met Ala  Gln Asp Gln
    1925                1930                1935

Arg Ile  Arg Trp Tyr Leu Leu  Ser Met Gly Ser Asn  Glu Asn Ile
    1940                1945                1950

His Ser  Ile His Phe Ser Gly  His Val Phe Thr Val  Arg Lys Lys
    1955                1960                1965

Glu Glu  Tyr Lys Met Ala Leu  Tyr Asn Leu Tyr Pro  Gly Val Phe
    1970                1975                1980

Glu Thr  Val Glu Met Leu Pro  Ser Lys Ala Gly Ile  Trp Arg Val
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    1985                1990                1995

Glu Cys  Leu Ile Gly Glu His  Leu His Ala Gly Met  Ser Thr Leu
    2000                2005                2010

Phe Leu  Val Tyr Ser Asn Lys  Cys Gln Thr Pro Leu  Gly Met Ala
    2015                2020                2025

Ser Gly  His Ile Arg Asp Phe  Gln Ile Thr Ala Ser  Gly Gln Tyr
    2030                2035                2040

Gly Gln  Trp Ala Pro Lys Leu  Ala Arg Leu His Tyr  Ser Gly Ser
    2045                2050                2055

Ile Asn  Ala Trp Ser Thr Lys  Glu Pro Phe Ser Trp  Ile Lys Val
    2060                2065                2070

Asp Leu  Leu Ala Pro Met Ile  Ile His Gly Ile Lys  Thr Gln Gly
    2075                2080                2085

Ala Arg  Gln Lys Phe Ser Ser  Leu Tyr Ile Ser Gln  Phe Ile Ile
    2090                2095                2100

Met Tyr  Ser Leu Asp Gly Lys  Lys Trp Gln Thr Tyr  Arg Gly Asn
    2105                2110                2115

Ser Thr  Gly Thr Leu Met Val  Phe Phe Gly Asn Val  Asp Ser Ser
    2120                2125                2130

Gly Ile  Lys His Asn Ile Phe  Asn Pro Pro Ile Ile  Ala Arg Tyr
    2135                2140                2145

Ile Arg  Leu His Pro Thr His  Tyr Ser Ile Arg Ser  Thr Leu Arg
    2150                2155                2160

Met Glu  Leu Met Gly Cys Asp  Leu Asn Ser Cys Ser  Met Pro Leu
    2165                2170                2175

Gly Met  Glu Ser Lys Ala Ile  Ser Asp Ala Gln Ile  Thr Ala Ser
    2180                2185                2190

Ser Tyr  Phe Thr Asn Met Phe  Ala Thr Trp Ser Pro  Ser Lys Ala
    2195                2200                2205

Arg Leu  His Leu Gln Gly Arg  Ser Asn Ala Trp Arg  Pro Gln Val
    2210                2215                2220

Asn Asn  Pro Lys Glu Trp Leu  Gln Val Asp Phe Gln  Lys Thr Met
    2225                2230                2235

Lys Val  Thr Gly Val Thr Thr  Gln Gly Val Lys Ser  Leu Leu Thr
    2240                2245                2250

Ser Met  Tyr Val Lys Glu Phe  Leu Ile Ser Ser Ser  Gln Asp Gly
    2255                2260                2265

His Gln  Trp Thr Leu Phe Phe  Gln Asn Gly Lys Val  Lys Val Phe
    2270                2275                2280

Gln Gly  Asn Gln Asp Ser Phe  Thr Pro Val Val Asn  Ser Leu Asp
    2285                2290                2295

Pro Pro  Leu Leu Thr Arg Tyr  Leu Arg Ile His Pro  Gln Ser Trp
    2300                2305                2310

Val His  Gln Ile Ala Leu Arg  Met Glu Val Leu Gly  Cys Glu Ala
    2315                2320                2325

Gln Asp  Leu Tyr
    2330


<210> SEQ ID NO 2
<211> LENGTH: 1445
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<220> FEATURE:
<223> OTHER INFORMATION: B-domain truncated human Factor VIII

<400> SEQUENCE: 2

Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1 5 10 15

Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
20 25 30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
35 40 45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
50 55 60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65 70 75 80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
85 90 95

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
100 105 110

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
115 120 125

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
130 135 140

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145 150 155 160

His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
165 170 175

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
180 185 190

His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
195 200 205

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
210 215 220

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
225 230 235 240

Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
245 250 255

Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
260 265 270

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
275 280 285

Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
290 295 300

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
305 310 315 320

Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
325 330 335

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
340 345 350

Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
355 360 365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
370 375 380

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Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385                 390                 395                 400

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
                405                 410                 415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
            420                 425                 430

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
        435                 440                 445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
    450                 455                 460

Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465                 470                 475                 480

Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
                485                 490                 495

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
            500                 505                 510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
        515                 520                 525

Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
    530                 535                 540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545                 550                 555                 560

Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
                565                 570                 575

Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
            580                 585                 590

Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
        595                 600                 605

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
    610                 615                 620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625                 630                 635                 640

Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
                645                 650                 655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
            660                 665                 670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
        675                 680                 685

Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
    690                 695                 700

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705                 710                 715                 720

Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
                725                 730                 735

Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro Ser Gln Asn
            740                 745                 750

Pro Pro Val Leu Lys Arg His Gln Arg Glu Ile Thr Arg Thr Thr Leu
        755                 760                 765

Gln Ser Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile Ser Val Glu
    770                 775                 780
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Met Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser
785                 790                 795                 800

Pro Arg Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val
                805                 810                 815

Glu Arg Leu Trp Asp Tyr Gly Met Ser Ser Ser Pro His Val Leu Arg
            820                 825                 830

Asn Arg Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe
        835                 840                 845

Gln Glu Phe Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu
    850                 855                 860

Leu Asn Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val
865                 870                 875                 880

Glu Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr
                885                 890                 895

Ser Phe Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly
            900                 905                 910

Ala Glu Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr
        915                 920                 925

Phe Trp Lys Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp
    930                 935                 940

Cys Lys Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val
945                 950                 955                 960

His Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu
                965                 970                 975

Asn Pro Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu Phe
            980                 985                 990

Phe Thr Ile Phe Asp Glu Thr Lys  Ser Trp Tyr Phe Thr  Glu Asn Met
        995                 1000                1005

Glu Arg  Asn Cys Arg Ala Pro  Cys Asn Ile Gln Met  Glu Asp Pro
    1010                1015                1020

Thr Phe  Lys Glu Asn Tyr Arg  Phe His Ala Ile Asn  Gly Tyr Ile
    1025                1030                1035

Met Asp  Thr Leu Pro Gly Leu  Val Met Ala Gln Asp  Gln Arg Ile
    1040                1045                1050

Arg Trp  Tyr Leu Leu Ser Met  Gly Ser Asn Glu Asn  Ile His Ser
    1055                1060                1065

Ile His  Phe Ser Gly His Val  Phe Thr Val Arg Lys  Lys Glu Glu
    1070                1075                1080

Tyr Lys  Met Ala Leu Tyr Asn  Leu Tyr Pro Gly Val  Phe Glu Thr
    1085                1090                1095

Val Glu  Met Leu Pro Ser Lys  Ala Gly Ile Trp Arg  Val Glu Cys
    1100                1105                1110

Leu Ile  Gly Glu His Leu His  Ala Gly Met Ser Thr  Leu Phe Leu
    1115                1120                1125

Val Tyr  Ser Asn Lys Cys Gln  Thr Pro Leu Gly Met  Ala Ser Gly
    1130                1135                1140

His Ile  Arg Asp Phe Gln Ile  Thr Ala Ser Gly Gln  Tyr Gly Gln
    1145                1150                1155

Trp Ala  Pro Lys Leu Ala Arg  Leu His Tyr Ser Gly  Ser Ile Asn
    1160                1165                1170

Ala Trp  Ser Thr Lys Glu Pro  Phe Ser Trp Ile Lys  Val Asp Leu
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    1175                1180                1185

Leu Ala  Pro Met Ile Ile His  Gly Ile Lys Thr Gln  Gly Ala Arg
    1190                1195                1200

Gln Lys  Phe Ser Ser Leu Tyr  Ile Ser Gln Phe Ile  Ile Met Tyr
    1205                1210                1215

Ser Leu  Asp Gly Lys Lys Trp  Gln Thr Tyr Arg Gly  Asn Ser Thr
    1220                1225                1230

Gly Thr  Leu Met Val Phe Phe  Gly Asn Val Asp Ser  Ser Gly Ile
    1235                1240                1245

Lys His  Asn Ile Phe Asn Pro  Pro Ile Ile Ala Arg  Tyr Ile Arg
    1250                1255                1260

Leu His  Pro Thr His Tyr Ser  Ile Arg Ser Thr Leu  Arg Met Glu
    1265                1270                1275

Leu Met  Gly Cys Asp Leu Asn  Ser Cys Ser Met Pro  Leu Gly Met
    1280                1285                1290

Glu Ser  Lys Ala Ile Ser Asp  Ala Gln Ile Thr Ala  Ser Ser Tyr
    1295                1300                1305

Phe Thr  Asn Met Phe Ala Thr  Trp Ser Pro Ser Lys  Ala Arg Leu
    1310                1315                1320

His Leu  Gln Gly Arg Ser Asn  Ala Trp Arg Pro Gln  Val Asn Asn
    1325                1330                1335

Pro Lys  Glu Trp Leu Gln Val  Asp Phe Gln Lys Thr  Met Lys Val
    1340                1345                1350

Thr Gly  Val Thr Thr Gln Gly  Val Lys Ser Leu Leu  Thr Ser Met
    1355                1360                1365

Tyr Val  Lys Glu Phe Leu Ile  Ser Ser Ser Gln Asp  Gly His Gln
    1370                1375                1380

Trp Thr  Leu Phe Phe Gln Asn  Gly Lys Val Lys Val  Phe Gln Gly
    1385                1390                1395

Asn Gln  Asp Ser Phe Thr Pro  Val Val Asn Ser Leu  Asp Pro Pro
    1400                1405                1410

Leu Leu  Thr Arg Tyr Leu Arg  Ile His Pro Gln Ser  Trp Val His
    1415                1420                1425

Gln Ile  Ala Leu Arg Met Glu  Val Leu Gly Cys Glu  Ala Gln Asp
    1430                1435                1440

Leu Tyr
    1445


<210> SEQ ID NO 3
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Truncated B-domain of human factor VIII

<400> SEQUENCE: 3

Ser Phe Ser Gln Asn Ser Arg His Pro Ser Gln Asn Pro Pro Val Leu
1               5                   10                  15

Lys Arg His Gln Arg
            20
```

What is claimed:

1. A Factor VIII molecule with a modified circulatory half-life comprising a FVIII molecule with a truncated B-domain consisting of amino acids 741-760 of SEQ ID N0:2, wherein the FVIII molecule is covalently conjugated to a hydrophilic polymer via an O-linked oligosaccharide on a serine residue corresponding to position 750 of SEP ID N0:2.

2. The FVIII molecule of claim **1** made by a process comprising: a) transfecting a mammalian host cell with a vector encoding the FVIII molecule of claim **1**; b) culturing the host cell of step (a) under conditions suitable to express the FVIII molecule in the host cell; c) harvesting the FVIII molecule from the host cell culture of step (b); and d) covalently conjugating the FVIII molecule with the hydrophilic polymer via an O-linked oligosaccharide on the serine residue corresponding to position 750 of SEQ ID N0:2.

3. The FVIII molecule of claim **1**, wherein the hydrophilic polymer is a polysaccharide.

4. The FVIII molecule of claim **1**, wherein the hydrophilic polymer is heparin.

5. The FVIII molecule of claim **2**, wherein the mammalian host cell is a CHO cell.

6. A pharmaceutical composition comprising the FVIII molecule of claim **1**.

7. A pharmaceutical composition comprising the FVIII molecule of claim **2**.

8. A method of treating a haemophilic disease comprising administering to a patient in need thereof a therapeutically effective amount of the pharmaceutical composition of claim **6**.

9. A method of treating a haemophilic disease comprising administering to a patient in need thereof a therapeutically effective amount of the pharmaceutical composition of claim **7**.

10. A method according to claim **8**, wherein the pharmaceutical composition is administered subcutaneously.

11. A method according to claim **9**, wherein the pharmaceutical composition is administered intravenously.

12. The FVIII molecule of claim **2**, wherein the hydrophilic polymer is a polysaccharide.

13. The FVIII molecule of claim **2**, wherein the hydrophilic polymer is heparin.

* * * * *