SITE-DIRECTED MODIFICATION OF FVIII

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims benefit of priority to US Patent App. Ser. No. 60/627,277 filed on November 12, 2004, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] This invention relates to Factor VIII (FVIII) muteins that allow coupling, at a defined site, to one or more biocompatible polymers such as polyethylene glycol. In addition, related formulations, dosages and methods of administration thereof for therapeutic purposes are provided. These modified FVIII variants, and associated compositions and methods are useful in providing a treatment option with reduced injection frequency and reduced immunogenic response for individuals afflicted with hemophilia A.

BACKGROUND OF THE INVENTION

[0003] Hemophilia A is the most common hereditary coagulation disorder, with an estimated incidence of 1 per 5000 males. It is caused by deficiency or structural defects in FVIII, a critical component of the intrinsic pathway of blood coagulation. The current treatment for hemophilia A involves intravenous injection of human FVIII. Human FVIII has been produced recombinantly as a single-chain molecule of approximately 300 kD. It consists of the structural domains A1-A2-B-A3-C1-C2 (Thompson, 2003, Semin. Hematol. 29, pp. 11-22). The precursor product is processed into two polypeptide chains of 200 kD (heavy) and 80 kD (light) in the Golgi Apparatus, with the two chains held together by metal ions (Kaufman et al., 1988, J. Biol. Chem. 263, p. 6352; Andersson et al., 1986, Proc. Natl. Acad. Sci. 83, p. 2979).

[0004] The B-domain of FVIII seems to be dispensable as B-domain deleted FVIII (BDD, 90 kD A1-A2 heavy chain plus 80 kD light chain) has also been shown to be effective as a replacement therapy for hemophilia A. The B-domain deleted FVIII sequence contains a deletion of all but 14 amino acids of the B-domain.

[0005] Hemophilia A patients are currently treated by intravenous administration of FVIII on demand or as a prophylactic therapy administered several times a week. For prophylactic treatment 15-25 IU/kg bodyweight is given of factor VIII three times a week. It is constantly required in the patient. Because of its short half-life in man, FVIII must be administered frequently. Despite its large

size of greater than 300 kD for the full-length protein, FVIII has a half-life in humans of only about 11 hours. (Ewenstein et al, 2004, Semin. Hematol. 41, pp.1-16). The need for frequent intravenous injection creates tremendous barriers to patient compliance. It would be more convenient for the patients if a FVIII product could be developed that had a longer half-life and therefore required less frequent administration. Furthermore, the cost of treatment could be reduced if the half-life were increased because fewer dosages may then be required.

[0006] An additional disadvantage to the current therapy is that about 25-30% of patients develop antibodies that inhibit FVIII activity (Saenko et al, 2002, Haemophilia 8, pp. 1-11). The major epitopes of inhibitory antibodies are located within the A2 domain at residues 484-508, the A3 domain at residues 1811-1818, and the C2 domain. Antibody development prevents the use of FVIII as a replacement therapy, forcing this group of patients to seek an even more expensive treatment with high-dose recombinant Factor VIIa and immune tolerance therapy.

[0007] The following studies identified FVIII epitopes of inhibitory antibodies. In a study of 25 inhibitory plasma samples, 11 were found to bind to the thrombin generated 73 kD light chain fragment A3C1C2, 4 to the A2 domain, and 10 to both (Fulcher, C. et al., 1985, Proc. Natl. Acad. Sci. 2(22), pp. 7728-32). In another study, six of eight A2 domain inhibitors from patients were neutralized by a recombinant A2 polypeptide (Scandella, D. et al., 1993, Blood 82(6), pp.1767-75). Epitopes for six of nine inhibitors from patients were mapped to A2 residues 379-538 (Scandella, D. et al., 1988, Proc. Natl. Acad. Sci. 85(16), pp. 6152-6). An epitope for 18 heavy-chain inhibitors was localized to the same N-terminal 18.3 kD region of the A2 domain (Scandella, D. et al., 1989, Blood 74(5), pp.1618-26).

[0008] An active, recombinant hybrid human/porcine FVIII molecule, generated by replacing human A2 domain residues 387-604 with the homologous porcine sequence, was resistant to a patient A2 inhibitor (Lubin, I. et al., 1994, J. Biol. Chem. 269(12), pp. 8639-41) and resistant to a murine monoclonal antibody mAB 413 IgG that competes with patient A2 inhibitors for binding to A2 (Scandella, D. et al., 1992, Thromb Haemost. 67(6), pp.665-71). This A2 domain epitope was further localized to the A2 domain residues 484-508 when experiments showed that mAB 413 IgG and four patient inhibitors did not inhibit a hybrid human/porcine FVIII in which the A2 domain residues 484-508 were replaced with that of porcine (Healey, J. et al., 1995, J. Biol. Chem. 270(24), pp.14505-9). This hybrid FVIII was also more resistant to at least half of 23 patient plasmas screened (Barrow, R. et al., 2000, Blood 95(2), pp. 564-8). Alanine scanning mutagenesis identified residue 487 to be critical for binding to all five patient inhibitors tested, while residues 484, 487, 489, and 492 were all important to interaction with mAB 413 IgG (Lubin, I., J. Biol. Chem. 272(48), pp. 30191-5). Inhibitory antibody titers in mice receiving the R484A/R489A/P492A mutant, but not the R484A/R489A mutant, were significantly lower than in mice receiving control human BDD FVIII (Parker, E. et al., 2004, Blood

104(3), pp. 704-10). In sum, the 484-508 region of the A2 domain seems to be a binding site for inhibitors of FVIII activity.

[0009] In addition to the development of an immune response to FVIII, another problem with conventional therapy is that it requires frequent dosaging because of the short half-life of FVIII in vivo. The mechanisms for clearance of FVIII from the circulation have been studied.

[0010] FVIII clearance from circulation has been partly attributed to specific binding to the low-density lipoprotein receptor-related protein (LRP), a hepatic clearance receptor with broad ligand specificity (Oldenburg et al., 2004, Haemophilia 10 Suppl 4, pp. 133-139). Recently, the low-density lipoprotein (LDL) receptor was also shown to play a role in FVIII clearance, such as by cooperating with LRP in regulating plasma levels of FVIII (Bovenschen et al., 2005, Blood 106, pp. 906-910). Both interactions are facilitated by binding to cell-surface heparin sulphate proteoglycans (HSPGs). Plasma half-life in mice can be prolonged by 3.3-fold when LRP is blocked or 5.5-fold when both LRP and cell-surface HSPGs are blocked (Sarafanov et al., 2001, J. Biol. Chem. 276, pp. 11970-11979). HSPGs are hypothesized to concentrate FVIII on the cell surface and to present it to LRP. LRP binding sites on FVIII have been localized to A2 residues 484-509 (Saenko et al., 1999, J. Biol. Chem. 274, pp. 37685-37692), A3 residues 1811-1818 (Bovenschen et al., 2003, J. Biol. Chem. 278, pp. 9370-9377) and an epitope in the C2 domain (Lenting et al., 1999, J. Biol. Chem. 274, pp. 23734-23739).

[0011] FVIII is also cleared from circulation by the action of proteases. To understand this effect, one must understand the mechanism by which FVIII is involved in blood coagulation. FVIII circulates as a heterodimer of heavy and light chains, bound to vWF. VWF binding involves FVIII residues 1649-1689 (Foster et al., 1988, J. Biol. Chem. 263, pp. 5230-5234), and parts of C1 (Jacquemin et al., 2000, Blood 96, pp. 958-965) and C2 domains (Spiegel, P. et al., 2004, J. Biol. Chem. 279(51), pp. 53691-8). FVIII is activated by thrombin, which cleaves peptide bonds after residues 372, 740, and 1689 to generate a heterotrimer of A1, A2, and A3-C1-C2 domains (Pittman et al., 2001, Proc. Natl. Acad. Sci. 276, pp. 12434-12439). Upon activation, FVIII dissociates from vWF and is concentrated to the cell surface of platelets by binding to phospholipid. Phospholipid binding involves FVIII residues 2199, 2200, 2251, and 2252 (Gilbert et al., 2002, J. Biol. Chem. 277, pp. 6374-6381). There it binds to FIX through interactions with FVIII residues 558-565 (Fay et al., 1994, J. Biol. Chem. 269, pp. 20522-20527) and 1811-1818 (Lenting et al., 1996, J. Biol. Chem. 271, pp. 1935-1940) and FX through interactions with FVIII residues 349-372 (Nogami et al., 2004, J. Biol. Chem. 279, pp. 15763-15771) and acts as a cofactor for FIX activation of FX, an essential component of the intrinsic coagulation pathway. Activated FVIII (FVIIIa) is partly inactivated by the protease activated protein C (APC) through cleavage after FVIII residues 336 and 562 (Regan et al., 1996, J. Biol. Chem.

271, pp. 3982-3987). The predominant determinant of inactivation, however, is the dissociation of the A2 domain from A1 and A3-C1-C2 (Fay et al., 1991, J. Biol. Chem. 266, pp. 8957-8962).

[0012] One method that has been demonstrated to increase the in vivo half-life of a protein is PEGylation. PEGylation is the covalent attachment of long-chained polyethylene glycol (PEG) molecules to a protein or other molecule. The PEG can be in a linear form or in branched form to produce different molecules with different features. Besides increasing the half-life of peptides or proteins, PEGylation has been used to reduce antibody development, protect the protein from protease digestion and keep the material out of the kidney filtrate (Harris et al., 2001, Clinical Pharmacokinetics 40, pp. 539-51). In addition, PEGylation may also increase the overall stability and solubility of the protein. Finally, the sustained plasma concentration of PEGylated proteins can reduce the extent of adverse side effects by reducing the trough to peak levels of a drug, thus eliminating the need to introduce super-physiological levels of protein at early time-points.

[0013] Random modification of FVIII by targeting primary amines (N-terminus and lysines) with large polymers such as PEG and dextran has been attempted with varying degree of success (WO94/15625, US Patent 4970300, US Patent 6048720). The most dramatic improvement, published in a 1994 patent application (WO94/15625), shows a 4-fold half-life improvement but at a cost of 2-fold activity loss after reacting full-length FVIII with 50-fold molar excess of PEG. WO2004/075923 discloses conjugates of FVIII and polyethylene glycol that are created through random modification. Randomly PEGylated proteins, such as interferon-alpha (Kozlowski et al, 2001, BioDrugs 15, pp. 419-429) have been approved as therapeutics in the past.

[0014] This random approach, however, is much more problematic for the heterodimeric FVIII. FVIII has hundreds of potential PEGylation sites, including the 158 lysines, the two N-termini, and multiple histidines, serines, threonines, and tyrosines, all of which could potentially be PEGylated with reagents primarily targeting primary amines. For example, the major positional isomer for PEGylated interferon Alpha-2b was shown to be a histidine (Wang et al., 2000, Biochemistry 39, pp. 10634-10640). Furthermore, heterogeneous processing of full length FVIII can lead to a mixture of starting material that leads to further complexity in the PEGylated products. An additional drawback to not controlling the site of PEGylation on FVIII is a potential activity reduction if the PEG were to be attached at or near critical active sites, especially if more than one PEG or a single large PEG is conjugated to FVIII. Because random PEGylation will invariably produce large amounts of multiply PEGylated products, purification to obtain only mono-PEGylated products will drastically lower overall yield. Finally, the enormous heterogeneity in product profile will make consistent synthesis and characterization of each lot nearly impossible. Since good manufacturing requires a consistent, well-characterized product, product heterogeneity is a barrier to commercialization. For all these reasons, a more specific method for PEGylating FVIII is desired.

[0015] Various site-directed protein PEGylation strategies have been summarized in a recent review (Kochendoerfer, G., Curr. Opin. Chem. Biol. 2005, available online as of Oct. 15, 2005, direct object identifier doi:10.1016/j.cbpa.2005.10.007). One approach involves incorporation of an unnatural amino acid into proteins by chemical synthesis or recombinant expression followed by the addition of a PEG derivative that will react specifically with the unnatural amino acid. For example, the unnatural amino acid may be one that contains a keto group not found in native proteins. However, chemical synthesis of proteins is not feasible for a protein as large as FVIII. Current limit of peptide synthesis is about 50 residues. Several peptides can be ligated to form a larger piece of polypeptide, but to produce even the B-domain deleted FVIII would require greater than 20 ligations, which would result in less than 1% recovery even under ideal reaction condition. Recombinant expression of proteins with unnatural amino acids has so far mainly been limited to non-mammalian expression systems. This approach is expected to be problematic for a large and complex protein such as FVIII that needs to be expressed in mammalian systems.

[0016] Another approach to site-specific PEGylation of proteins is by targeting N-terminal backbone amine with PEG-aldehydes. The low pH required under this process to achieve specificity over other amine groups, however, is not compatible with the narrow near-neutral pH range needed for the stability of FVIII (Wang et al., 2003, International J. Pharmaceutics 259, pp. 1-15). Moreover, N-terminal PEGylation of FVIII may not lead to improved plasma half-life if this region is not involved in plasma clearance. In fact, the N-terminal region of the FVIII light chain has been implicated in binding to the von Willebrand factor (vWF), a carrier protein that is critical for FVIII survival in circulation. By N-terminal modification of factor VIII, the critically important association with vWF may be disrupted or weakened. Thus, N-terminal PEGylation of FVIII may have the opposite effect of reducing plasma half-life of FVIII.

[0017] WO90/12874 discloses site-specific modification of human IL-3, granulocyte colony stimulating factor and erythropoietin polypeptides by inserting or substituting a cysteine for another amino acid, then adding a ligand that has a sulfhydryl reactive group. The ligand couples selectively to cysteine residues. Modification of FVIII or any variant thereof is not disclosed.

[0018] For the reasons stated above, there exists a need for an improved FVIII variant that possesses greater duration of action in vivo and reduced immunogenicity, while retaining functional activity. Furthermore, it is desirable that such a protein be produced as a homogeneous product in a consistent manner.

SUMMARY OF THE INVENTION

[0019] It is an object of the present invention to provide a biocompatible polymer-conjugated functional FVIII polypeptide having improved pharmacokinetic characteristics and therapeutic characteristics.

[0020] It is another object of the present invention to provide a biocompatible polymer-conjugated B domain deleted FVIII protein having improved pharmacokinetic properties.

[0021] It is yet another object of the invention to provide a biocompatible polymer-conjugated functional FVIII polypeptide having reduced binding to the low-density lipoprotein receptor-related protein (LRP), low-density lipoprotein (LDL) receptor, the heparan sulphate proteoglycans (HSPGs) and/or inhibitory antibodies against FVIII.

[0022] It is yet another object of the present invention to provide an improved FVIII variant that possesses greater duration of action in vivo and reduced immunogenicity, which is capable of being produced as a homogeneous product in a consistent manner.

[0023] In one aspect of the invention there is provided a conjugate having factor VIII procoagulant activity comprising a functional factor VIII polypeptide covalently attached at one or more predefined sites on the polypeptide to one or more biocompatible polymers, wherein the predefined site is a not an N-terminal amine. The invention also includes a method for the preparation of this conjugate comprising mutating a nucleotide sequence that encodes for the functional factor VIII polypeptide to substitute a coding sequence for a cysteine residue at a pre-defined site; expressing the mutated nucleotide sequence to produce a cysteine enhanced mutein; purifying the mutein; reacting the mutein with the biocompatible polymer that has been activated to react with polypeptides substantially only at the introduced cysteine residues such that the conjugate is formed; and purifying the conjugate. The invention is also directed to pharmaceutical compositions comprising the conjugate and a pharmaceutically acceptable adjuvant and methods of treating hemophilia by administering therapeutically effective amounts of these pharmaceutical compositions to a mammal in need thereof.

[0024] The invention also relates to a method for site-directed PEGylation of a factor VIII mutein comprising (a) expressing a site-directed factor VIII mutein wherein the mutein has a cysteine replacement for an amino acid residue on the exposed surface of the factor VIII mutein and that cysteine is capped; (b) contacting the cysteine mutein with a reductant under conditions to mildly reduce the cysteine mutein and to release the cap; (c) removing the cap and the reductant from the cysteine mutein; and (d) at least about 5 minutes after the removal of the reductant, treating the

cysteine mutein with PEG comprising a sulfhydryl coupling moiety under conditions such that PEGylated factor VIII mutein is produced.

BRIEF DESCRIPTION OF THE FIGURES

[0025] FIG. 1. Vector maps and mutagenesis strategy for PEG muteins.

[0026] FIG. 2. A UV absorbance profile at 280nm with respect to time for the PEG2 protein purified over a monoclonal FVIII antibody chromatography column. The chromatography was performed using an AKTA® Explorer 100 chromatography system from Amersham Bioscience.

[0027] FIG. 3 Three-step site-directed PEGylation method. PEG represents a cysteine-reactive PEG such as PEG-maleimide. Closed bars represent disulfide formation while open bars represent reduced cysteines.

[0028] FIG. 4. Site-directed PEGylation of PEG2.

[0029] FIG. 5. Site-directed PEGylation of PEG6.

[0030] FIG. 6a. Site-directed PEGylation of BDD, PEG2, 4, 5, and 6. Upper panels were stained with heavy (H) chain antibody while bottom panels were stained with light (L) chain antibody. "U" is unprocessed material containing both H & L.

[0031] FIG. 6b. PEGylation of PEG15 and PEG7 with PEG2 and PEG6 as controls. Start purified PEG muteins ("S") are reduced with TCEP and PEGylated with a 12 kD ("12") or a 22 kD ("22") PEG after removal of the reductant ("R"). Samples were run on 6% Tris-glycine SDS PAGE and stained with a heavy chain ("HC") antibody on left panel or light chain ("LC") antibody on right panel. "U" is unprocessed material containing both HC & LC. PEGylated bands are highlighted by dots.

[0032] FIG. 6c. PEGylation of PEG2+6 with PEG2 and PEG6 as controls. PEG2, PEG6, or PEG2+6 is reduced with TCEP and PEGylated with a 5 kD ("5") or a 43 kD ("43") PEG after removal of the reductant ("R"). PEG2+6 was also PEGylated with 12, 22, and 33 kD PEGs. Samples were run on 6% Tris-glycine SDS PAGE and stained with coomassie for proteins on the left or heavy chain (H) or light chain (L) antibody. "U" is unprocessed material containing both H & L. PEGylated bands are highlighted by dots.

[0033] FIG. 6d. PEGylation of wildtype full length FVIII (KG-2) with PEG2 as a control. Left gel stained with coomassie stain for proteins and right gel with iodine for PEG. "BDD U" is unprocessed BDD material containing both H & L. PEGylated bands are highlighted by dots.

[0034] FIG. 7. Thrombin cleavage of PEGylated PEG2. The N-terminal half of A2 domain is colored in blue and C-terminal half in green, with the R8B12 antibody epitope highlighted in dark green (right FVIII model). PEG2 (lane 1) and 22 kD PEGylated PEG2 (lane2) were treated with thrombin (lanes 3 and 4, respectively) and then run on a 7% Tris-Acetate gel (Invitrogen) and stained with the R8B12 antibody. Each lane contains about 50 ng of FVIII.

[0035] FIG. 8. Thrombin cleavage of PEGylated wildtype full-length FVIII (KG-2). "S" = starting KG-2 material. "R" = reduced KG-2 and reductant removed. "P" = "R" PEGylated with 43 kD PEG. "Pure" = "P" purified away from excess PEG. "L" = light chain. PEGylated bands are highlighted by dots.

[0036] FIG. 9. Iodine Staining of PEGylated PEG2. 22 or 43 kD PEGylated PEG2 was run on a 6% TrisGlycine gel and stained with the R8B12 FVIII antibody (lanes 1 and 2) or iodine (lanes 3 and 4). The two stains were lined up according to their molecular weight marker lanes. Lanes 1 and 2 each contains about 30 ng of FVIII while lanes 3 and 4 contain about 2 μg.

[0037] FIG. 10. MALDI Mass Spectrometry analysis of PEGylated and UnPEGylated PEG2. MALDI Mass Spectrometry was performed on PEG2 (Fig. 10a) or 22 kD PEGylated PEG2 (Fig. 10b). Upon PEGylation, the heavy (H) chain peak of PEG2 is greatly reduced and a new peak (H+PEG), centered at 111 kD (22 kD PEG + 89 kD heavy chain), appears. No PEGylated light (L) chain peak, expected to be centered at 100 kD (22 kD PEG + 83 kD light chain) is detected.

[0038] FIG. 11. MALDI Mass Spectrometry of PEGylated and unPEGylated PEG2 after thrombin cleavage.

[0039] FIG. 12. MALDI Mass Spectrometry analysis of PEGylated PEG6 before and after thrombin cleavage.

[0040] FIG. 13. The UV absorption profile at 280 nm of PEGylated PEG2 purified on size-exclusion column.

[0041] FIG. 14. The UV absorption profile at 280 nm of PEGylated and UnPEGylated PEG6 purified on cation exchange column.

[0042] FIG. 15. The UV absorption profile at 280 nm of PEGylated and UnPEGylated PEG6 purified on size-exclusion column.

[0043] FIG. 16. Activity of PEGylated protein is compared to activity of the unPEGylated protein as measured by a chromogenic assay and a coagulation assay. Purified full-length FVIII is represented as KG-2. The percent activity reported was determined by dividing the value of sample treated with PEG after reduction and reductant removal by that of the sample treated with buffer control taking into consideration the PEGylation yield.

[0044] FIG. 17. Rabbit PK study of PEGylated PEG2 compared to PEG2.

[0045] FIG 18. Rabbit PK study of PEGylated PEG2 compared to BDD and PEG2. P-values are comparisons between PEGylated PEG2 and BDD.

[0046] FIG 19. Rabbit PK study of PEGylated PEG6 compared to BDD and PEG6.

[0047] FIG 20. Rabbit PK study of PEGylated wildtype full-length ("fl") FVIII compared to unmodified fl FVIII.

[0048] FIG 21. Hemophilic mouse PK study of PEGylated PEG6 compared to PEG6 and BDD.

[0049] FIG 22. Normal mouse PK study of 22 and 43 kD PEGylated PEG2 compared to BDD.

[0050] FIG 23. Normal mouse PK study of 22 kD PEGylated PEG2 compared to BDD, full time course.

[0051] FIG 24. The Hemophilic Mouse (BDD) Factor VIII recovery histogram depicting a pharmacokinetic (PK) assessment of the half-life of two species of BDD Factor VIII in a hemophilic mouse assay.

[0052] FIG 25. Hemophilic mouse kidney laceration study of 22 kD PEGylated PEG2 compared to BDD. Vehicle treated mice have a blood loss of 25 uL/gram body weight.

[0053] FIG 26. Chromogenic Activity of PEGylated PEG2 and BDD in the presence of increasing amounts of FVIII antibodies. Antibody epitope is denoted in parenthesis.

[0054] FIG 27. Chromogenic Activity of PEGylated PEG2 in the presence of increasing amounts of FVIII mAB 413 antibodies.

[0055] FIG 28. Chromogenic activity of BDD, 43 kD PEGylated PEG2, 33 kD PEGylated PEG6, and 33 kD diPEGylated PEG2+6 in the presence of human plasma derived from patients that have developed inhibitors to FVIII. The inhibitor titer and date of blood collection were noted at the top. Top two panels include data collected at patient plasma dilution of 5- to 405-fold. Lower left panel focuses on 1:15-fold dilution for patient HRF-828 plasma. Lower right panel confirms that the 0.064 IU/mL used for each FVIII sample in the top two panels was not a saturating dose.

[0056] FIG 29. PEGylation screening method and validation. Top panel shows a schematic of PEGylation screening of transiently expressed PEG muteins. Bottom panel shows a Western analysis of PEGylated products using a heavy chain ("H")-specific antibody (left) or a light-chain ("L") specific antibody (right). PEGylated bands are highlighted by dots. "U" is unprocessed material containing both H and L.

[0057] FIG 30. PEGylation screening of PEG15-17. Western analysis of PEGylated products using heavy chain ("H")-specific antibodies (R8B12 and 58.12) or light-chain ("L") specific antibodies (C7F7 and GM). All 3 muteins are selective for the heavy chain, with relative PEGylation efficiency of PEG15~PEG16>PEG17. PEGylated bands are highlighted by dots. "U" is unprocessed material containing both H and L.

[0058] FIG 31. Gel showing PEGylation of PEG2+14 as a function of reductant concentration. PEG2+14 was treated with 67 to 670 uM of TCEP for 30 minutes at 4 oC. The reductant was removed by spin-column followed by PEGylation with a 12 kD PEG. Heavy and light chains of FVIII are highlighted by "H" and "L," respectively. The two dots point to the PEGylated heavy and light chains.

[0059] Fig 32. Deconvoluted Mass Spectra of PEG2+14 treated with 67 to 670 uM of TCEP followed by reductant removal.

DETAILED DESCRIPTION OF THE INVENTION

[0060] The present invention is based on the discovery that polypeptides having FVIII activity can be covalently attached at a predefined site to a biocompatible polymer that is not at an N-terminal amine, and that such polypeptides substantially retain their coagulant activity. Furthermore, these polypeptide conjugates have improved circulation time and reduced antigenicity. The conjugates of the invention are advantageous over the prior art conjugates that had random polymer

attachments to FVIII or attachments at an N-terminal. Site-directed attachment allows one to design modifications that avoid the regions required for biological activity and thereby to maintain substantial FVIII activity. It also allows for designing to attach polymers to block binding at sites involved in FVIII clearance. Site-directed attachment also allows for a uniform product rather than the heterogeneous conjugates produced in the art by random polymer coupling. By avoiding attachment at an N-terminal amine of the light chain, the conjugates of the present invention avoid the possible loss of activity from attaching a ligand at an active site of the FVIII polypeptide. The N-terminal region of the light chain is believed to be involved in the association of vWF factor to FVIII, which is a stabilizing association in the circulation.

DEFINITIONS

[0061] Biocompatible polymer. A biocompatible polymer includes polyalkylene oxides such as without limitation polyethylene glycol (PEG), dextrans, colominic acids or other carbohydrate based polymers, polymers of amino acids, biotin derivatives, polyvinyl alcohol (PVA), polycarboxylates, polyvinylpyrrolidone, polyethylene-co-maleic acid anhydride, polystyrene-co-malic acid anhydride, polyoxazoline, polyacryloylmorpholine, 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. Preferred is polyethylene glycol, and still more preferred is methoxypolyethylene glycol (mPEG). Other useful polyalkylene glycol compounds are polypropylene glycols (PPG), polybutylene glycols (PBG), PEG-glycidyl ethers (Epox-PEG), PEG-oxycarbonylimidazole (CDI-PEG), branched polyethylene glycols, linear polyethylene glycols, forked polyethylene glycols and multi-armed or "super branched" polyethylene glycols (star-PEG).

[0062] Polyethylene glycol (PEG). "PEG" and "polyethylene glycol" as used herein are interchangeable and include any water-soluble poly(ethylene oxide). Typically, PEGs for use in accordance with the invention comprise the following structure "--$(OCH_2CH_2)_n$--" where (n) is 2 to 4000. As used herein, PEG also includes "-- CH_2CH_2--$O(CH_2CH_2O)_n$ -- CH_2CH_2--" and "--$(OCH_2CH_2)_nO$--," depending upon whether or not the terminal oxygens have been displaced. Throughout the specification and claims, it should be remembered that the term "PEG" includes structures having various terminal or "end capping" groups, such as without limitation a hydroxyl or a C_{1-20} alkoxy group. The term "PEG" also means a polymer that contains a majority, that is to say, greater than 50%, of –OCH $_2CH_2$--repeating subunits. With respect to specific forms, the PEG can take any number of a variety of molecular weights, as well as structures or geometries such as branched, linear, forked, and multifunctional.

[0063] PEGylation. PEGylation is a process whereby a polyethylene glycol (PEG) is covalently attached to a molecule such as a protein.

[0064] Activated or Active functional group. When a functional group such as a biocompatible polymer is described as activated, the functional group reacts readily with an electrophile or a nucleophile on another molecule.

[0065] B domain deleted FVIII (BDD). As used herein, BDD is characterized by having the amino acid sequence which contains a deletion of all but 14 amino acids of the B-domain of FVIII. The first 4 amino acids of the B-domain (SFSQ, SEQ ID NO:1) are linked to the 10 last residues of the B-domain (NPPVLKRHQR, SEQ ID NO:2). (Lind, P. et al, 1995, Eur. J. Biochem. 232, pp. 19-27). The BDD used herein has the amino acid sequence of SEQ ID NO:3.

[0066] FVIII. Blood clotting Factor VIII (FVIII) is a glycoprotein synthesized and released into the bloodstream by the liver. In the circulating blood, it is bound to von Willebrand factor (vWF, also known as Factor VIII-related antigen) to form a stable complex. Upon activation by thrombin, it dissociates from the complex to interact with other clotting factors in the coagulation cascade, which eventually leads to the formation of a thrombus. Human full-length FVIII has the amino acid sequence of SEQ ID NO:4, although allelic variants are possible.

[0067] Functional factor VIII polypeptide. As used herein, functional factor VIII polypeptide denotes a functional polypeptide or combination of polypeptides that are capable, in vivo or in vitro, of correcting human factor VIII deficiencies, characterized, for example, by hemophilia A. Factor VIII has multiple degradation or processed forms in the natural state. These are proteolytically derived from a precursor, one chain protein, as demonstrated herein. A functional factor VIII polypeptide includes such single chain protein and also provides for these various degradation products that have the biological activity of correcting human factor VIII deficiencies. Allelic variations likely exist. The functional factor VIII polypeptides include all such allelic variations, glycosylated versions, modifications and fragments resulting in derivatives of factor VIII so long as they contain the functional segment of human factor VIII and the essential, characteristic human factor VIII functional activity remains unaffected in kind. Those derivatives of factor VIII possessing the requisite functional activity can readily be identified by straightforward in vitro tests described herein. Furthermore, functional factor VIII polypeptide is capable of catalyzing the conversion of factor X to Xa in the presence of factor IXa, calcium, and phospholipid, as well as correcting the coagulation defect in plasma derived from hemophilia A affected individuals. From the disclosure of the sequence of the human factor VIII amino acid sequences and the functional regions herein, the fragments that can be derived via restriction enzyme cutting of the DNA or proteolytic or other degradation of human factor VIII protein will be apparent to those skilled in the art.

[0068] FIX. As used herein, FIX means Coagulation Factor IX, which is also known as Human Clotting Factor IX, or Plasma Thromboplastin Component.

[0069] FX. As used herein, FX means Coagulation Factor X, which is also known by the names Human Clotting Factor X and by the eponym Stuart-Prower factor.

[0070] Pharmacokinetics. "Pharmacokinetics" ("PK") is a term used to describe the properties of absorption, distribution, metabolism, and elimination of a drug in a body. An improvement to a drug's pharmacokinetics means an improvement in those characteristics that make the drug more effective in vivo as a therapeutic agent, especially its useful duration in the body.

[0071] Mutein. A mutein is a genetically engineered protein arising as a result of a laboratory induced mutation to a protein or polypeptide.

[0072] Protein. As used herein, protein and polypeptide are synonyms.

[0073] FVIII clearance receptor. A FVIII clearance receptor as used herein means a receptor region on a functional FVIII polypeptide that binds or associates with one or more other molecules to result in FVIII clearance from the circulation. Factor VIII clearance receptors include without limitation the regions of the FVIII molecule that bind LRP, LDL receptor and/or HSPG.

DISCUSSION

[0074] It is envisioned that any functional factor VIII polypeptide may be mutated at a predetermined site and then covalently attached at that site to a biocompatible polymer according to the methods of the invention. Useful polypeptides include, without limitation, full-length factor VIII having the amino acid sequence as shown in SEQ ID NO:4 and BDD FVIII having the amino acid sequence as shown in SEQ ID NO:3. Preferred is BDD FVIII.

[0075] The biocompatible polymer used in the conjugates of the invention may be any of the polymers discussed above. The biocompatible polymer is selected to provide the desired improvement in pharmacokinetics. For example, the identity, size and structure of the polymer is selected so as to improve the circulation half-life of the polypeptide having FVIII activity or decrease the antigenicity of the polypeptide without an unacceptable decrease in activity. Preferably, the polymer comprises PEG, and still more preferably has at least 50% of its molecular weight as PEG. In one embodiment, the polymer is a polyethylene glycol terminally capped with an end-capping moiety such as hydroxyl, alkoxy, substituted alkoxy, alkenoxy, substituted alkenoxy, alkynoxy, substituted alkynoxy, aryloxy and substituted aryloxy. Still more preferred are polymers comprising

methoxypolyethylene glycol. Yet more preferred are polymers comprising methoxypolyethylene glycol having a size range from 3 kD to 100 kD, and more preferably from 5 kD to 64 kD or from 5 kD to 43 kD.

[0076] Preferably the polymer has a reactive moiety. For example, in one embodiment, the polymer has a sulfhydryl reactive moiety that can react with a free cysteine on a functional factor VIII polypeptide to form a covalent linkage. Such sulfhydryl reactive moieties include thiol, triflate, tresylate, aziridine, oxirane, S-pyridyl or maleimide moieties. Preferred is a maleimide moiety. In one embodiment, the polymer is linear and has a "cap" at one terminus that is not strongly reactive towards sulfhydryls (such as methoxy) and a sulfhydryl reactive moiety at the other terminus. In a preferred embodiment, the conjugate comprises PEG-maleimide and has a size range from 5 kD to 64 kD.

[0077] Further guidance for selecting useful biocompatible polymers is provided in the examples that follow.

[0078] Site-directed mutation of a nucleotide sequence encoding polypeptide having FVIII activity may occur by any method known in the art. Preferred methods include mutagenesis to introduce a cysteine codon at the site chosen for covalent attachment of the polymer. This may be accomplished using a commercially available site-directed mutagenesis kit such as the Stratagene cQuickChange™ II site-directed mutagenesis kit, the Clontech Transformer site-directed mutagenesis kit no. K1600-1, the Invitrogen GenTaylor site-directed mutagenesis system no. 12397014, the Promega Altered Sites II in vitro mutagenesis system kit no. Q6210, or the Takara Mirus Bio LA PCR mutagenesis kit no. TAK RR016.

[0079] The conjugates of the invention may be prepared by first replacing the codon for one or more amino acids on the surface of the functional FVIII polypeptide with a codon for cysteine, producing the cysteine mutein in a recombinant expression system, reacting the mutein with a cysteine-specific polymer reagent, and purifying the mutein.

[0080] In this system, the addition of a polymer at the cysteine site can be accomplished through a maleimide active functionality on the polymer. Examples of this technology are provided infra. The amount of sulfhydryl reactive polymer used should be at least equimolar to the molar amount of cysteines to be derivatized and preferably is present in excess. Preferably, at least a 5-fold molar excess of sulfhydryl reactive polymer is used, and still more preferably at least a ten-fold excess of such polymer is used. Other conditions useful for covalent attachment are within the skill of those in the art.

[0081] In the examples that follow, the muteins are named in a manner conventional in the art. The convention for naming mutants is based on the amino acid sequence for the mature, full length Factor VIII as provided in SEQ ID NO:4. As a secreted protein, FVIII contains a signal sequence that is proteolytically cleaved during the translation process. Following removal of the 19 amino acid signal sequence, the first amino acid of the secreted FVIII product is an alanine.

[0082] As is conventional and used herein, when referring to mutated amino acids in BDD FVIII, the mutated amino acid is designated by its position in the sequence of full-length FVIII. For example, the PEG6 mutein discussed below is designated K1808C because it changes the lysine (K) at the position analogous to 1808 in the full-length sequence to cysteine (C).

[0083] The predefined site for covalent binding of the polymer is best selected from sites exposed on the surface of the polypeptide that are not involved in FVIII activity or involved in other mechanisms that stabilize FVIII in vivo, such as binding to vWF. Such sites are also best selected from those sites known to be involved in mechanisms by which FVIII is deactivated or cleared from circulation. Selection of these sites is discussed in detail below. Preferred sites include an amino acid residue in or near a binding site for (a) low density lipoprotein receptor related protein, (b) a heparin sulphate proteoglycan, (c) low density lipoprotein receptor and/or (d) factor VIII inhibitory antibodies. By "in or near a binding site" means a residue that is sufficiently close to a binding site such that covalent attachment of a biocompatible polymer to the site would result in steric hindrance of the binding site. Such a site is expected to be within 20 Å of a binding site, for example.

[0084] In one embodiment of the invention, the biocompatible polymer is covalently attached to the functional factor VIII polypeptide at an amino acid residue in or near (a) a factor VIII clearance receptor as defined supra, (b) a binding site for a protease capable of degradation of factor VIII and/or (c) a binding site for factor VIII inhibitory antibodies. The protease may be activated protein C (APC). In another embodiment, the biocompatible polymer is covalently attached at the predefined site on the functional factor VIII polypeptide such that binding of low-density lipoprotein receptor related protein to the polypeptide is less than to the polypeptide when it is not conjugated, and preferably more than twofold less. In one embodiment, the biocompatible polymer is covalently attached at the predefined site on the functional factor VIII polypeptide such that binding of heparin sulphate proteoglycans to the polypeptide is less than to the polypeptide when it is not conjugated, and preferably is more than twofold less. In a further embodiment, the biocompatible polymer is covalently attached at the predefined site on the functional factor VIII polypeptide such that binding of factor VIII inhibitory antibodies to the polypeptide is less than to the polypeptide when it is not conjugated, preferably more than twofold less than the binding to the polypeptide when it is not conjugated. In another embodiment, the biocompatible polymer is covalently attached at the predefined site on the functional factor VIII polypeptide such that binding of low density lipoprotein receptor to the polypeptide is less

than to the polypeptide when it is not conjugated, preferably more than twofold less. In another embodiment, the biocompatible polymer is covalently attached at the predefined site on the functional factor VIII polypeptide such that a plasma protease degrades the polypeptide less than when the polypeptide is not conjugated. In a further embodiment, the degradation of the polypeptide by the plasma protease is more than twofold less than the degradation of the polypeptide when it is not conjugated as measured under the same conditions over the same time period.

[0085] LRP, LDL receptor, or HSPG binding affinity for FVIII can be determined using surface plasmon resonance technology (Biacore). For example, FVIII can be coated directly or indirectly through a FVIII antibody to a Biacore™ chip, and varying concentrations of LRP can be passed over the chip to measure both on-rate and off-rate of the interaction (Bovenschen N. et al., 2003, J. Biol. Chem. 278(11), pp. 9370-7). The ratio of the two rates gives a measure of affinity. A two-fold, preferably five-fold, more preferably ten-fold, and even more preferably 30-fold decrease in affinity upon PEGylation would be desired.

[0086] Degradation of a FVIII by the protease APC can be measured by any of the methods known to those of skill in the art.

[0087] In one embodiment, the biocompatible polymer is covalently attached to the polypeptide at one or more of the factor VIII amino acid positions 81, 129, 377, 378, 468, 487, 491, 504, 556, 570, 711, 1648, 1795, 1796, 1803, 1804, 1808, 1810, 1864, 1903, 1911, 2091, 2118 and 2284. In another embodiment, the biocompatible polymer is covalently attached to the polypeptide at one or more of factor VIII amino acid positions 377, 378, 468, 491, 504, 556, 1795, 1796, 1803, 1804, 1808, 1810, 1864, 1903, 1911 and 2284 and (1) the binding of the conjugate to low-density lipoprotein receptor related protein is less than the binding of the unconjugated polypeptide to the low-density lipoprotein receptor related protein; (2) the binding of the conjugate to low-density lipoprotein receptor is less than the binding of the unconjugated polypeptide to the low-density lipoprotein receptor; or (3) the binding of the conjugate to both low-density lipoprotein receptor related protein and low-density lipoprotein receptor is less than the binding of the unconjugated polypeptide to the low-density lipoprotein receptor related protein and the low-density lipoprotein receptor.

[0088] In a further embodiment, the biocompatible polymer is covalently attached to the polypeptide at one or more of factor VIII amino acid positions 377, 378, 468, 491, 504, 556 and 711 and the binding of the conjugate to heparin sulphate proteoglycan is less than the binding of the unconjugated polypeptide to heparin sulphate proteoglycan. In a further embodiment, the biocompatible polymer is covalently attached to the polypeptide at one or more of the factor VIII amino acid positions 81, 129, 377, 378, 468, 487, 491, 504, 556, 570, 711, 1648, 1795, 1796, 1803, 1804, 1808, 1810, 1864, 1903, 1911, 2091, 2118 and 2284 and the conjugate has less binding to factor VIII

inhibitory antibodies than the unconjugated polypeptide. In a further embodiment, the biocompatible polymer is covalently attached to the polypeptide at one or more of the factor VIII amino acid positions 81, 129, 377, 378, 468, 487, 491, 504, 556, 570, 711, 1648, 1795, 1796, 1803, 1804, 1808, 1810, 1864, 1903, 1911, 2091, 2118 and 2284, and preferably at one or more of positions 377, 378, 468, 491, 504, 556, and 711 and the conjugate has less degradation from a plasma protease capable of factor VIII degradation than does the unconjugated polypeptide. More preferred, the plasma protease is activated protein C.

[0089] In a further embodiment, the biocompatible polymer is covalently attached to B-domain deleted factor VIII at amino acid position 129, 491, 1804, and/or 1808, more preferably at 491 or 1808. In a further embodiment, the biocompatible polymer is attached to the polypeptide at factor VIII amino acid position 1804 and comprises polyethylene glycol. Preferably, the one or more predefined sites for biocompatible polymer attachment are controlled by site specific cysteine mutation.

[0090] One or more sites, preferably one or two, on the functional factor VIII polypeptide may be the predefined sites for polymer attachment. In particular embodiments, the polypeptide is mono-PEGylated or diPEGylated.

[0091] The invention also relates to a method for the preparation of the conjugate comprising mutating a nucleotide sequence that encodes for the functional factor VIII polypeptide to substitute a coding sequence for a cysteine residue at a pre-defined site; expressing the mutated nucleotide sequence to produce a cysteine enhanced mutein; purifying the mutein; reacting the mutein with the biocompatible polymer that has been activated to react with polypeptides at substantially only reduced cysteine residues such that the conjugate is formed; and purifying the conjugate. In another embodiment, the invention provides a method for site-directed PEGylation of a factor VIII mutein comprising: (a) expressing a site-directed factor VIII mutein wherein the mutein has a cysteine replacement for an amino acid residue on the exposed surface of the factor VIII mutein and that cysteine is capped; (b) contacting the cysteine mutein with a reductant under conditions to mildly reduce the cysteine mutein and to release the cap; (c) removing the cap and the reductant from the cysteine mutein; and (d)at least about 5 minutes, and preferably at least 15 minutes, still more preferably at least 30 minutes after the removal of the reductant, treating the cysteine mutein with PEG comprising a sulfhydryl coupling moiety under conditions such that PEGylated factor VIII mutein is produced. The sulfhydryl coupling moiety of the PEG is selected from the group consisting of thiol, triflate, tresylate, aziridine, oxirane, S-pyridyl and maleimide moieties, preferably maleimide.

[0092] The invention also concerns pharmaceutical compositions for parenteral administration comprising therapeutically effective amounts of the conjugates of the invention and a

pharmaceutically acceptable adjuvant. Pharmaceutically acceptable adjuvants are substances that may be added to the active ingredient to help formulate or stabilize the preparation and cause no significant adverse toxicological effects to the patient. Examples of such adjuvants are well known to those skilled in the art and include water, sugars such as maltose or sucrose, albumin, salts, etc. Other adjuvants are described for example in Remington's Pharmaceutical Sciences by E. W. Martin. Such compositions will contain an effective amount of the conjugate hereof together with a suitable amount of vehicle in order to prepare pharmaceutically acceptable compositions suitable for effective administration to the host. For example, the conjugate may be parenterally administered to subjects suffering from hemophilia A at a dosage that may vary with the severity of the bleeding episode. The average doses administered intraveneously are in the range of 40 units per kilogram for pre-operative indications, 15 to 20 units per kilogram for minor hemorrhaging, and 20 to 40 units per kilogram administered over an 8-hours period for a maintenance dose.

[0093] In one embodiment the inventive method involves replacing one or more surface BDD amino acids with a cysteine, producing the cysteine mutein in a mammalian expression system, reducing a cysteine which has been capped during expression by cysteine from growth media, removing the reductant to allow BDD disulfides to reform, and reacting with a cysteine-specific biocompatible polymer reagent, such as such as PEG-maleimide. Examples of such reagents are PEG-maleimide with PEG sizes such as 5, 22, or 43 kD available from Nektar Therapeutics of San Carlos, CA under Nektar catalog numbers 2D2M0H01 mPEG-MAL MW 5,000 Da, 2D2M0P01 mPEG-MAL MW 20 kD, 2D3X0P01 mPEG2-MAL MW 40 kD, respectively, or 12 or 33 kD available from NOF Corporation, Tokyo, Japan under NOF catalog number Sunbright ME-120MA and Sunbright ME-300MA, respectively. The PEGylated product is purified using ion-exchange chromatography to remove unreacted PEG and using size-exclusion chromatography to remove unreacted BDD. This method can be used to identify and selectively shield any unfavorable interactions with FVIII such as receptor-mediated clearance, inhibitory antibody binding, and degradation by proteolytic enzymes. We noted that the PEG reagent supplied by Nektar or NOF as 5kD tested as 6kD in our laboratory, and similarly the PEG reagent supplied as linear 20 kD tested as 22 kD, that supplied as 40 kD tested as 43 kD and that supplied as 60kD tested as 64kD in our laboratory. To avoid confusion, we use the molecular weight as tested in our laboratory in the discussion herein, except for the 5 kD PEG, which we report as 5kD as the manufacturer identified it.

[0094] In addition to cysteine mutations at positions 491 and 1808 of BDD (disclosed above), positions 487, 496, 504, 468, 1810, 1812, 1813, 1815, 1795, 1796, 1803, and 1804 were mutated to cysteine to potentially allow blockage of LRP binding upon PEGylation. Also, positions 377, 378, and 556 were mutated to cysteine to allow blockage of both LRP and HSPG binding upon PEGylation. Positions 81, 129, 422, 523, 570, 1864, 1911, 2091, and 2284 were selected to be equally spaced on BDD so that site-directed PEGylation with large PEGs (>40 kD) at these positions together with

PEGylation at the native glycosylation sites (41, 239, and 2118) and LRP binding sites should completely cover the surface of BDD and identify novel clearance mechanism for BDD.

[0095] In one embodiment, the cell culture medium contains cysteines that "cap" the cysteine residues on the mutein by forming disulfide bonds. In the preparation of the conjugate, the cysteine mutein produced in the recombinant system is capped with a cysteine from the medium and this cap is removed by mild reduction that releases the cap before adding the cysteine-specific polymer reagent. Other methods known in the art for site-specific mutation of FVIII may also be used, as would be apparent to one of skill in the art.

[0096] EXAMPLES

[0097] STRUCTURE ACTIVITY RELATIONSHIP ANALYSIS OF FVIII. FVIII and BDD FVIII are very large complex molecules with many different sites involved in biological reactions. Previous attempts to covalently modify them to improve pharmacokinetic properties had mixed results. That the molecules could be specifically mutated and then a polymer added in a site-specific manner was surprising. Furthermore, the results of improved pharmacokinetic properties and retained activity were surprising also, given the problems with past polymeric conjugates causing nonspecific addition and reduced activity.

[0098] In one embodiment, the invention concerns site-directed mutagenesis using cysteine-specific ligands such as PEG-maleimide. A non-mutated BDD does not have any available cysteines to react with a PEG-maleimide, so only the mutated cysteine position will be the site of PEGylation. More specifically, BDD FVIII has 19 cysteines, 16 of which form disulfides and the other 3 of which are free cysteines (McMullen et al., 1995, Protein Sci. 4, pp. 740-746). The structural model of BDD suggests that all 3 free cysteines are buried (Stoliova-McPhie et al., 2002, Blood 99, pp. 1215-1223). Because oxidized cysteines cannot be PEGylated by PEG-maleimides, the 16 cysteines that form disulfides in BDD cannot be PEGylated without being first reduced. Based on the structural models of BDD, the 3 free cysteines in BDD may not be PEGylated without first denaturing the protein to expose these cysteines to the PEG reagent. Thus, it does not appear feasible to achieve specific PEGylation of BDD by PEGylation at native cysteine residues without dramatically altering the BDD structure, which will most likely destroy its function.

[0099] The redox state of the 4 cysteines in the B domain of full-length FVIII is unknown. PEGylation of the 4 cysteines in the B domain may be possible if they do not form disulfides and are surface exposed. However, because full-length FVIII and BDD have a similar pharmacokinetic (PK) profile and similar half-lives in vivo (Gruppo et al., 2003, Haemophilia 9, pp. 251-260), B domain

PEGylation is unlikely to result in improved plasma half-life unless the PEG happens to also protect non-B domain regions.

[0100] To determine the predefined site on a polypeptide having FVIII activity for polymer attachment that will retain factor VIII activity and improve pharmacokinetics, the following guidelines are presented based on BDD FVIII. Modifications should be targeted toward clearance, inactivation, and immunogenic mechanisms such as LRP, HSPG, APC, and inhibitory antibody binding sites. Stoilova-McPhie, S. et al., 2002, Blood 99(4), pp. 1215-23 shows the structure of BDD. For example, to prolong half-life, a single PEG can be introduced at a specific site at or near LRP binding sites in A2 residues 484-509 and A3 residues 1811-1818. Introduction of the bulky PEG at these sites should disrupt FVIII's ability to bind LRP and reduce the clearance of FVIII from circulation. It is also believed that to prolong half-life without significantly affecting activity that a PEG can be introduced at residue 1648, which is at the junction of the B domain and the A3 domain in the full-length molecule and in the 14-amino acid liker I the BDD between the A2 and A3 domains.

[0101] Specificity of PEGylation can be achieved by engineering single cysteine residues into the A2 or A3 domains using recombinant DNA mutagenesis techniques followed by site-specific PEGylation of the introduced cysteine with a cysteine-specific PEG reagent such as PEG-maleimide. Another advantage of PEGylating at 484-509 and 1811-1818 is that these two epitopes represent two of the three major classes of inhibitory antigenic sites in patients. To achieve maximal effect of improved circulating half-life and reduction of immunogenic response, both A2 and A3 LRP binding sites can be PEGylated to yield a diPEGylated product. It should be noted that PEGylation within the 1811-1818 region may lead to significant loss of activity since this region is also involved in FIX binding. Site-directed PEGylation within 558-565 should abolish HSPG binding, but may also reduce activity as this region also binds to FIX.

[0102] Additional surface sites can be PEGylated to identify novel clearance mechanism of FVIII. PEGylation of the A2 domain may offer additional advantage in that the A2 domain dissociates from FVIII upon activation and is presumably removed from circulation faster than the rest of FVIII molecule because of its smaller size. PEGylated A2, on the other hand, may be big enough to escape kidney clearance and have a comparable plasma half-life to the rest of FVIII and thus can reconstitute the activated FVIII in vivo.

[0103] IDENTIFICATION OF PEGylation SITES IN A2 AND A3 REGIONS. Five positions (Y487, L491, K496, L504 and Q468 corresponding to PEG1-5 positions) at or near the putative A2 LRP binding region were selected as examples for site-directed PEGylation based on the high surface exposure and outward direction of their Cα to Cβ trajectory. Furthermore, these residues are roughly equidistant from each other in the three-dimensional structure of the molecule, so that together they

can represent this entire region. Eight positions (1808, 1810, 1812, 1813, 1815, 1795, 1796, 1803, 1804 corresponding to PEG6-14) at or near the putative A3 LRP binding region were selected as examples for site-directed PEGylation. PEG6 (K1808) is adjacent to 1811-1818 and the natural N-linked glycosylation site at 1810. PEGylation at position 1810 (PEG7) will replace the sugar with a PEG. Mutation at the PEG8 position T1812 will also abolish the glycosylation site. Although the PEG9 position (K1813) was predicted to be pointing inward, it was selected in case the structure model is not correct. PEG10 (Y1815) is a bulky hydrophobic amino acid within the LRP binding loop, and may be a critical interacting residue since hydrophobic amino acids are typically found at the center of protein-protein interactions. Because the 1811-1818 region has been reported to be involved in both LRP and FIX binding, PEGylation within this loop was thought possibly to result in reduced activity. Thus, PEG11-PEG14 (1795, 1796, 1803, 1804) were designed to be near the 1811-1818 loop but not within the loop so that one can dissociate LRP and FIX binding with different PEG sizes.

[0104] To block both LRP binding sites simultaneously, double PEGylation at, for example, the PEG2 and PEG6 position, can be generated.

[0105] Since the 558-565 region has been shown to bind to both HSPG and FIX, no sites were designed within this region. Instead, PEG15-PEG17 (377, 378, and 556) were designed in between the A2 LRP and HSPG binding regions so that an attached PEG may interfere both interactions and disrupt possible interactions between them. Additional sites that are surface exposed and outwardly pointing could also be selected within or near the LRP and HPSG binding regions. To identify novel clearance mechanisms, FVIII can be systematically PEGylated. In addition to PEG1-17, the three other natural glycosylation sites, namely, N41, N239, and N2118 corresponding to PEG18-20 can be used as tethering points for PEGylation since they should be surface exposed. Surface areas within a 20 angstrom radius from the Cβ atoms of PEG2, PEG6, and the four glycosylation sites were mapped onto the BDD model in addition to functional interaction sites for vWF, FIX, FX, phospholipid, and thrombin.

[0106] PEG21-29 corresponding to Y81, F129, K422, K523, K570, N1864, T1911, Q2091, and Q2284 were then selected based on their ability to cover nearly the entire remaining BDD surface with a 20 angstrom radius from each of their Cβ atoms. These positions were also selected because they are fully exposed, outwardly pointing, and far away from natural cysteines to minimize possible incorrect disulfide formation. The 20 angstrom radius is chosen because a large PEG, such as a 64 kD branched PEG, is expected to have the potential to cover a sphere with about a 20 angstrom radius. PEGylation of PEG21-29 together with PEG2 and PEG6 and glycosylation sites PEG18, 19, and 20 is likely to protect nearly the entire non-functional surface of FVIII.

[0107] PEGylation positions that lead to enhanced properties such as improved PK profile, greater stability, or reduced immunogenicity can be combined to generate multi-PEGylated product with maximally enhanced properties. PEG30 and PEG31 were designed by removing the exposed disulfides in A2 and A3 domain, respectively. PEG30, or C630A, should free up its disulfide partner C711 for PEGylation. Likewise, PEG31, C1899A should allow C1903 to be PEGylated.

[0108] MUTAGENESIS. Substrates for site-directed PEGylation of FVIII may be generated by introducing a cysteine codon at the site chosen for PEGylation. The Stratagene cQuickChange™ II site-directed mutagenesis kit was used to make all of the PEG mutants (Stratagene kit 200523 from Stratagene Corporation, La Jolla, CA). The cQuikChange™ site-directed mutagenesis method is performed using PfuTurbo® DNA polymerase and a temperature cycler. Two complimentary oligonucleotide primers, containing the desired mutation, are elongated using PfuTurbo, which will not displace the primers. dsDNA containing the wildtype FVIII gene is used as a template. Following multiple elongation cycles, the product is digested with DpnI endonuclease, which is specific for methylated DNA. The newly synthesized DNA, containing the mutation, is not methylated, whereas the parental wild-type DNA is methylated. The digested DNA is then used to transform XL-1 Blue super-competent cells.

[0109] The mutagenesis efficiency is almost 80%. The mutagenesis reactions were performed in either pSK207+BDD C2.6 or pSK207+BDD (Figure 1). Successful mutagenesis was confirmed by DNA sequencing and appropriate fragments, containing the mutation, were transferred into the FVIII backbone in the mammalian expression vector pSS207+BDD. After transfer, all of the mutations were again sequence-confirmed. For A3 muteins PEG 6, 7, 8, 9, and 10, mutagenesis was done in the vector pSK207+BDD C2.6. After being confirmed by sequencing, the mutant fragment, KpnI/Pme was subcloned into pSK207+BDD. The BDD mutein was then subcloned into the pSS207+BDD expression vector. For A3 muteins PEG 11, 12, 13, 14, the mutagenesis was done directly in the vector pSK207+BDD and sequence-confirmed mutant BDD were then subcloned into pSS207+BDD. For A2 muteins PEG 1, 2, 3, 4, 5, the mutagenesis was done in the pSK207+ BDD C2.6vector. The sequence confirmed mutant was subcloned into pSK207+BDD and then to pSS207+BDD.

THE PRIMERS (SENSE STAND ONLY) USED FOR MUTAGENESIS
ARE LISTED FOR EACH REACTION:

[0110] PEG1, Y487C: GATGTCCGTCCTTTGTGCTCAAGGAGATTACCA (SEQ ID NO:5)

[0111] PEG2, L491C: TTGTATTCAAGGAGATGCCCAAAAGGTGTAAAAC (SEQ ID NO:6)

[0112] PEG3, K496C: TTACCAAAAGGTGTATGCCATTTGAAGGATTTTC (SEQ ID NO:7)

[0113] PEG4, L504C: AAGGATTTTCCAATTTGCCCAGGAGAAATATTC (SEQ ID NO:8)

[0114] PEG5, Q468C: GATTATATTTAAGAATTGCGCAAGCAGACCATAT (SEQ ID NO:9)

[0115] PEG6, K1808C: TAGAAAAAACTTTGTCTGCCCTAATGAAACCAAAAC (SEQ ID NO:10)

[0116] PEG7, N1810C: AACTTTGTCAAGCCTTGCGAAACCAAAACTTAC (SEQ ID NO:11)

[0117] PEG8, T1812C: GTCAAGCCTAATGAATGCAAAACTTACTTTTGGA (SEQ ID NO:12)

[0118] PEG9, K1813C: CAAGCCTAATGAAACCTGCACTTACTTTTGGAAAG (SEQ ID NO:13)

[0119] PEG10, Y1815C: CTAATGAAACCAAAACTTGCTTTTGGAAAGTGCAAC (SEQ ID NO:14)

[0120] PEG11, D1795C: ATTTCTTATGAGGAATGCCAGAGGCAAGGAGCA (SEQ ID NO:15)

[0121] PEG12, Q1796C: TCTTATGAGGAAGATTGCAGGCAAGGAGCAGAA (SEQ ID NO:16)

[0122] PEG13, R1803C: CAAGGAGCAGAACCTTGCAAAAACTTTGTCAAGCCT (SEQ ID NO:17)

[0123] PEG14, K1804C: GGAGCAGAACCTAGATGCAACTTTGTCAAGCCT (SEQ ID NO:18)

[0124] PEG15, K377C: CGCTCAGTTGCCAAGTGTCATCCTAAAACTTGG (SEQ ID NO:19)

[0125] PEG16, H378C: TCAGTTGCCAAGAAGTGTCCTAAAACTTGGGTA (SEQ ID NO:20)

[0126] PEG17, K556C: CTCCTCATCTGCTACTGCGAATCTGTAGATCAA (SEQ ID NO:21)

[0127] PEG18, N41C: CAAAATCTTTTCCATTCTGCACCTCAGTCGTGTAC (SEQ ID NO:22)

[0128] PEG19, N239C: GTCAATGGTTATGTATGCAGGTCTCTGCCAGGT (SEQ ID NO:23)

[0129] PEG20, N2118C: CAGACTTATCGAGGATGTTCCACTGGAACCTTA (SEQ ID NO:24)

[0130] PEG21, Y81C: ATCCAGGCTGAGGTTTGTGATACAGTGGTCATT (SEQ ID NO:25)

[0131] PEG22, F129C: GAAGATGATAAAGTCTGTCCTGGTGGAAGCCAT (SEQ ID NO:26)

[0132] PEG23, K422C: CAGCGGATTGGTAGGTGTTACAAAAAAGTCCGA (SEQ ID NO:27)

[0133] PEG24, K523C: GAAGATGGGCCAACTTGCTCAGATCCTCGGTGC (SEQ ID NO:28)

[0134] PEG25, K570C: CAGATAATGTCAGACTGCAGGAATGTCATCCTG (SEQ ID NO:29)

[0135] PEG26, N1864C: CACACTAACACACTGTGTCCTGCTCATGGGAGA (SEQ ID NO:30)

[0136] PEG27, T1911C, CAGATGGAAGATCCCTGCTTTAAAGAGAATTAT (SEQ ID NO:31)

[0137] PEG28, Q2091C: ACCCAGGGTGCCCGTTGCAAGTTCTCCAGCCTC (SEQ ID NO:32)

[0138] PEG29, Q2284C: AAAGTAAAGGTTTTTTGCGGAAATCAAGACTCC (SEQ ID NO:33)

[0139] PEG30, C630A: TTGCAGTTGTCAGTTGCTTTGCATGAGGTGGCA (SEQ ID NO:34)

[0140] PEG31, C1899A: AATATGGAAAGAAACGCTAGGGCTCCCTGCAAT (SEQ ID NO:35)

[0141] MUTEIN EXPRESSION. After insertion in a vector that confers resistance to Hygromycin B, the PEG muteins were transfected into HKB11 cells (US Patent 6,136,599) complexed with 293 Fectin Transfection Reagent (Invitrogen Corp. Cat#12347-019) per the manufacturer's instructions. FVIII expression at three days post-transfection was assessed by Coatest chromogenic assay (Chromogenix Corp. Cat#821033, see Example 12 Chromogenic Assay) (Table 1). The transfected cells were then placed under selective pressure with 50 □g/ml of Hyg B in a growth medium supplemented with 5% FBS. When Hyg B-resistant colonies appeared, they were manually picked and screened for FVIII expression by Coatest chromogenic assay. The FVIII expressing stable cells were then adapted to a medium containing HPPS supplement. The cells were expanded and seeded at 1 X 106 cells/ml in shaking flasks with fresh media. Tissue culture fluid (TCF), harvested after 3 days, was used for purification of FVIII BDD muteins. The FVIII activity of the TCF was assayed by Coatest (Table 1).

Summary of PEG Mutein Titers

		Titer (IU/ml)	
Mutation	Mutein ID	Transient	Stable Cells
Y487C	PEG1	0.07	N/A
L491C	PEG2	0.60	1.96
K496C	PEG3	0.45	N/A
L504C	PEG4	0.38	5.57
Q468C	PEG5	0.69	8.14
K1808C	PEG6	0.54	2.73
N1810C	PEG7	0.21	0.5
T1812C	PEG8	0.16	N/A
K1813C	PEG9	0.35	7.74
Y1815C	PEG10	0.09	N/A
D1795C	PEG11	0.27	N/A
Q1796C	PEG12	0.29	N/A
R1803C	PEG13	0.11	N/A
K1804C	PEG14	0.18	1.14
L491C/K1808C	PEG2+6	0.11	2.48
L491C/K1804C	PEG2+14	0.13	7.19
K377C	PEG15	0.11	12.58
H378C	PEG16	0.15	0.97
K556C	PEG17	0.09	0.15
N41C	PEG18	0.05	N/A
N239C	PEG19	0.16	N/A
N2118C	PEG20	0.13	N/A
Y81C	PEG21	0.36	N/A
F129C	PEG22	0.25	2.55
K422C	PEG23	0.28	N/A
K523C	PEG24	<0.05	N/A
K570C	PEG25	<0.05	N/A
N1864C	PEG26	0.15	N/A
T1911C	PEG27	0.28	N/A
Q2091C	PEG28	0.20	N/A
Q2284C	PEG29	0.17	N/A
C630A	PEG30	<0.05	0.20
C1899A	PEG31	0.30	1.80

Table 1. Expression level of PEG Muteins from transient and stable transfections.

[0142] MUTEIN PURIFICATION. Upon collecting the cell culture supernatant containing the secreted mutein FVIII protein, the supernatant is filtered through a 0.2 micron membrane filter to remove any remaining cells. The supernatant is then concentrated by either ultrafiltration or anion exchange. It is then applied to an immunoaffinity column where the cell culture media components and the majority of the host cell protein impurities are removed. The immunoaffinity column eluate is then buffer exchanged by diafiltration into a formulation buffer containing sucrose and frozen. Yield and recovery of protein across a monoclonal FVIII antibody column was assessed by chromogenic assay. Samples of load, flow through, various eluate fractions, strip, and the diafiltered eluate of a chromatography run were assayed for FVIII activity (Table 2). Table 2 shows the recovery of the

PEG2 mutein from a monoclonal antibody column. The antibodies are C7F7 antibodies. The percent recovery in Table 2 is determined by the chromogenic assay. The final yield was 73%. Shown in Figure 2 is a plot of the UV absorbance at 280nm with respect to time for the PEG2 protein purified over a monoclonal FVIII antibody chromatography column. The chromatography was performed using an AKTA® Explorer 100 chromatography system from Amersham Bioscience. This instrument employs a multi-wavelength UV-Visible monitor and a 2mm flow cell. The PEG2 mutein is eluted from the column in the presence of high salt and elution peak is indicated by both the absorbance at 280nm and FVIII activity assay.

Step	%Recovery
C7F7 Load	100
C7F7 Flow through	1.1
C7F7 Wash	0.2
C7F7 Eluate	86
C7F7 Strip	0.0
Post UF/DF	73

Table 2. Recovery of PEG2 mutein from monoclonal FVIII antibody column.

[0143] PEGYLATION. Native full-length FVIII or BDD cannot be PEGylated by cysteine-specific PEGs without reduction and denaturation at over 100-fold excess PEG: protein ratio (data not shown), confirming the hypothesis based on the BDD structure model that all native cysteines form disulfides or are buried within FVIII. FVIII cysteine muteins expressed and purified using the standard protocols listed above could not be PEGylated with a cysteine-specific PEG maleimide reagent, presumably because the introduced FVIII cysteine is "capped" by reacting with sulfhydryl groups such as cysteine and β-mecaptoethanol present in the cell growth media. This issue can potentially be resolved by eliminating cysteines and β-mecaptoethanol from the culture media, but this may lead to lower FVIII production and would not prevent sulfhydryls released by the cells from blocking the introduced FVIII cysteine.

[0144] In another aspect of the invention, a three-step method was developed to allow site-specific PEGylation of FVIII (Figure 3). In step 1, the purified FVIII cysteine mutein at about 1 μM is mildly reduced with reductants such as about 0.7 mM Tris(2-carboxyethyl)phosphine (TCEP) or 0.07 mM dithiothreitol (DTT) for 30 minutes at 4°C to release the "cap." In step 2, the reductant is removed along with the "cap" by a size-exclusion chromatography (SEC) method such as running the sample through a spin column (BioRad®) to allow FVIII disulfides to reform while leaving the introduced cysteine free and reduced. In step 3, at least 30 minutes after the removal of the reductant, the freed FVIII cysteine mutein is treated with at least 10-fold molar excess of PEG-maleimide with sizes ranging from 5 to 64 kD (Nektar Therapeutics and N.O.F. Corporation) for at least 1 hour at 4°C. This method yields highly consistent product profile with reproducible data for dozens of reactions repeated by different individuals.

[0145] Because the spin column method for removal of TCEP is not scaleable, gel filtration desalting chromatography was selected. However, upon testing this method using a TCEP spike sample, it was shown that the TCEP eluted at measurable levels in the column void and not just in the salt fraction as would be expected from a molecule with its low molecular weight. Western Blot assays showed significant background PEGylation probably due to incomplete removal of TCEP. In the meantime separate experiments showed that C7F7 purified material could be significantly purified further from other protein impurities using an anion exchange chromatography media combined with a salt gradient. It was then decided to reduce the C7F7 material with TCEP as described above and then process the material over the anion exchange column. Because of charge difference the FVIII protein would be retained while the TCEP would flow through the column and not be retained. At the same time during the gradient salt elution the FVIII protein would be purified away from the majority of remaining protein impurities. This meant that the later occurring PEGylation would be theoretically more homogeneous with purer starting material. However, upon testing with a spike sample of TCEP, it was shown that measurable levels of TCEP were found eluting in the gradient with the FVIII. Therefore it was decided to implement gel filtration desalting chromatography after anion exchange chromatography so these two steps when used in sequence would result in complete removal of TCEP and elimination of non-specific PEGylation.

[0146] PEGYLATION ANALYSIS BY SDS PAGE AND WESTERN BLOT. The PEGylated product can be analyzed by electrophoresis on a reducing 6% TrisGlycine SDS polyacrylamide gel (Invitrogen). Following electrophoresis, the gel can be stained with Coomassie Blue to identify all the proteins or subjected to a standard Western Blot protocol to identify PEGylation pattern on different regions of FVIII. Staining of the blot with a mouse monoclonal R8B12 or C7F7 antibody raised against the C-terminal region of the FVIII heavy chain or the N-terminal region of the VIII light chain, respectively, should identify PEGylation of the respective chains. Staining with the 413 antibody against the 484-509 region of FVIII will determine whether PEGylation is indeed site-specific or not for muteins such as PEG1-4. Likewise, staining with the CLB-CAg A antibody that recognizes the 1801-1823 region of FVIII will determine if PEGylation is site-specific or not for muteins such as PEG6-10.

[0147] PEG2 (L491C) PEGylation was shown to be selective for the heavy chain over light chain and particularly selective for the 484-509 region (Figure 4) while PEG6 (K1808C) was shown to be selective for the light chain over the heavy chain (Figure 5).

[0148] For the study depicted in figure 4, the PEG2 mutein (lanes 1 and 8) is reduced with TCEP followed by TCEP removal (lanes 2 and 9) and treatment with 5, 12, 22, 33, or 43 kD PEG-maleimide (lanes 3-7 and 10-14). UnPEGylated FVIII runs as unprocessed (H+L) and processed heavy (H) and light (L) chain bands. All three bands are detectable on the Coomassie Blue stained gel (lower right) whereas Western Staining with chain-specific antibodies reveal only the unprocessed

and the corresponding chain. Using R8B12 staining (upper left), the heavy chain (H) band is dramatically reduced in intensity when PEG2 is treated with PEG-maleimide and a new band is created that runs higher than the parent H band proportional to the size of the PEG. Using C7F7 staining (lower left), the light chain (L) bands (multiple bands due to heterogenous glycosylation) do not change intensity. The unprocessed H+L band for both stains are shifted because the H chain is part of the unprocessed FVIII. Coomassie staining also confirms much more PEGylation of the heavy chain, i.e. reduction of H band intensity, than of the light chain. Finally, the PEGylated bands lose relatively more intensity on the 413 antibody stain (upper right) than R8B12 stain in a PEG size-dependent fashion presumably due to site-specific PEGylation of 491, which blocks the binding of 413 antibody to 484-509. Quantities of FVIII loaded per lane are about 30 ng for the two left gels, about 1000 ng for the upper right gel, and about 2000 ng for the lower right gel.

[0149] Reduction followed by removal of reductant does not change the migration of FVIII (lane 1 vs. 2 and 8 vs. 9). Addition of 22kD PEG to PEG2 blocks the binding of the 413 antibody, consistent with specific PEGylation at the 491 position (Figure 4 upper right gel). This also suggests that PEGylated PEG2 will have lower immunogenicity in man because the 413 antibody has been shown to share the same epitope as human A2 inhibitory antibodies (Scandella et al., 1992, Thromb. Haemost. 67, pp. 665-71).

[0150] For the study depicted in figure 5, the PEG6 mutein is reduced with TCEP followed by TCEP removal (lanes 1 and 6) and treatment with 5, 12, 22, or 33 kD PEG-maleimide (lanes 2-5 and 7-10). UnPEGylated FVIII runs as unprocessed (H+L) and processed heavy (H) and light (L) chain bands. Because the PEG6 (K1808) mutation resides on the light chain, PEGylation was detected only on the light chain and not the heavy chain. Amount of FVIII loaded per lane is about 100 ng for the left gel and about 30 ng for the right gel.

[0151] The BDD that was run as a control did not show any significant PEGylation upon treatment with greater than100-fold molar excess of PEG-maleimide even after the reduction and reductant removal procedure described above (Figure 6a). The same method was also applied to PEG4 and PEG5 (Figure 6a). Compared to PEG2, these muteins were not PEGylated as efficiently, but they were selective for the heavy chain similar to PEG2 (L491C). PEG6 (K1808C) PEGylation efficiency is relatively low, perhaps because it is very close to the N-linked glycosylation site at N1810, which may block PEGylation at position 1808. Thus, we designed PEG7 (N1810C) to remove the native glycosylation site at 1810. PEG7 shows improved PEGylation efficiency compared to PEG6 in a head-to-head comparison (Figure 6b). Similarly PEG15 shows slightly better PEGylation efficiency than PEG2. PEG2+6, a double mutant of BDD, can be PEGylated on both heavy and light chains since PEG2 is a heavy chain cysteine mutation while PEG6 is a light chain mutation (Figure 6c). This method was also applied to wildtype full-length FVIII (Figure 6d). PEGylation was detected for the

largest fragment of heavy chain that includes A1, A2, and most of the B domain. The PEGylation pattern suggests monoPEGylation and that there is only a single cysteine PEGylated.

[0152] PEGYLATION ANALYSIS BY THROMBIN CLEAVAGE AND WESTERN BLOT. The PEGylated product can be treated with thrombin (40 IU/ug FVIII) at 37° C for 30 minutes. The thrombin used also contains APC as a contaminant. Thrombin cleavage will generate the 50 kD A1 and 43 kD A2 domains from the heavy chain while the APC cleavage will split the A2 domain further into the 21 and 22 kD fragments (Figure 7). Staining with the R8B12 antibody, which recognizes the C-terminus of the heavy chain, will identify only the intact A2 domain and the 21 kD C-terminal fragment (FVIII 562-740). Thus, if PEG2 PEGylation was specific for position 491, the 43 kD A2 domain should be PEGylated but not the 21 kD C-terminal fragment. This was indeed confirmed by the Western blot for the 22 kD PEGylated PEG2 shown on Figure 7. Thus, by elimination, PEG2 PEGylation has been localized to the N-terminal 22 kD fragment (FVIII 373-561) of A2 domain. Since PEG-maleimide is completely selective for cysteines at pH 6.8 and the only native FVIII cysteines within 373-561 come from a buried disulfide between 528 and 554, PEG2 is very likely PEGylated on the introduced cysteine at position 491. Western staining of thrombin-treated PEGylated PEG2 with a FVIII heavy chain N-terminal antibody showed no PEGylation of the A1 domain (data not shown). Selective PEGylation of PEG2 using thrombin cleavage method has also been confirmed for PEGs of 5, 12, 33, and 43 kDs (data not shown). Thrombin cleavage of PEGylated wildtype full-length FVIII shows that only B domain is PEGylated (Figure 8)

[0153] PEGYLATION ANALYSIS BY IODINE STAINING. To confirm that the newly created bands on Coomassie Blue and Western staining were indeed PEGylated bands, barium-iodine staining, which is specific for PEG, was used (Figure 9). PEGylated PEG2 was run on a 6% TrisGlycine gel (Invitrogen) and stained with the R8B12 heavy chain antibody or a barium-iodine solution (Lee et al, Pharm Dev Technol. 1999 4:269-275). The PEGylated bands matched between the two stains using the molecular weight marker to line them up, thus confirming FVIII heavy chain PEGylation.

[0154] PEGYLATION ANALYSIS BY MALDI-MASS SPEC. To confirm the PEGylation of the A2 domain in the heavy chain, the rFVIII sample, before and after PEGylation was analyzed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. The samples were mixed and crystallized on the MALDI target plate with a sinapinic acid matrix in 30 % acetonitrile, 0.1% TFA. They were then analyzed in a Voyager DE-PRO spectrometer in positive, linear mode. The results, shown in Figure 10, showed the light chain of PEG2 centered at 83 kD and the heavy chain (HC) at 89 kD. The spectrum acquired for the PEGylated sample showed a drop in the HC peak and a new peak, centered at 111 kD, to form. This confirms PEGylation of the heavy chain. No PEGylated light chain (at 105 kD) was observed above detection limit.

[0155] The samples were then both subjected to thrombin digestion at 20 units of thrombin/mg FVIII at 37°C for 30 minutes, following FVIII concentration determination by amino acid analysis (Commonwealth Biotechnologies, Inc). The heavy chain was cleaved into a 46 kD (A1) N-terminal fraction and a 43 kD (A2) fraction. The MALDI spectrum acquired for the PEGylated sample (Figure 11) shows the loss of the 43 kD peak and the development of a new 65 kD peak, due to the PEGylated A2 domain. PEGylation of the LC is again not observed above the detection limit. These results again confirm PEGylation of the A2 domain of FVIII. The same analysis was applied to PEGylated PEG6, confirming PEGylation of the light chain A3C1C2 fragment (Figure 12).

[0156] ACTIVITY MEASUREMENT

[0157] COAGULATION ASSAY. The clotting FVIII:C test method is a one-stage assay based upon the activated partial thromboplastin time (aPTT). FVIII acts as a cofactor in the presence of Factor IXa, calcium, and phospholipid in the enzymatic conversion of Factor X to Xa. In this assay, the diluted test samples are incubated at 37°C with a mixture of FVIII deficient plasma substrate and aPTT reagent. Calcium chloride is added to the incubated mixture and clotting is initiated. An inverse relationship exists between the time (seconds) it takes for a clot to form and logarithm of the concentration of FVIII:C. Activity levels for unknown samples are interpolated by comparing the clotting times of various dilutions of test material with a curve constructed from a series of dilutions of standard material of known activity and are reported in International Units per mL (IU/mL).

[0158] CHROMOGENIC ASSAY. The chromogenic assay method consists of two consecutive steps where the intensity of color is proportional to the FVIII activity. In the first step, Factor X is activated to FXa by FIXa with its cofactor, FVIIIa, in the presence of optimal amounts of calcium ions and phospholipids. Excess amounts of Factor X are present such that the rate of activation of Factor X is solely dependent on the amount of FVIII. In the second step, Factor Xa hydrolyzes the chromogenic substrate to yield a chromophore and the color intensity is read photometrically at 405 nm. Potency of an unknown is calculated and the validity of the assay is checked with the slope-ratio statistical method. Activity is reported in International Units per mL (IU/mL).

[0159] The 1811-1818 loop is involved in binding to FIX, but the importance of individual positions within this loop has not been determined. PEG7-10 muteins display nearly identical specific chromogenic activity relative to native FVIII (Table 3). Table 3 shows the percent specific activity (S.A.) of PEG muteins and PEGylated PEG2 or PEG6 relative to BDD. S.A. was determined by dividing the chromogenic, coagulation, or vWF binding activity by the total antigen ELISA (TAE) value. The S.A. of PEGylated muteins was then divided by the S.A. of BDD (8 IU/ug chromogenic, 5 IU/ug

coagulation, and 1 vWF/TAE) and multiplied by 100 to obtain the percent S.A. listed in Table 3 under the headings chromogenic, coagulation and vWF/TAE.

	Mutation	Chromogenic	Coagulation	vWF/TAE
BDD		100	100	100
PEG1	Y487C			
PEG2	L491C	125	130	138
PEG2 red	L491C	137	141	98
PEG2-5 kD PEG	L491C	124	93	125
PEG2-12 kD PEG	L491C	118	25	71
PEG2-22 kD PEG	L491C	103	13	87
PEG2-33 kD PEG	L491C	130	17	59
PEG2-43 kD PEG	L491C	91	9	57
PEG3	K496C			
PEG4	L504C			
PEG5	Q468C	92		
PEG6	K1808C	83	60	100
PEG6-33 kD PEG	K1808C	42	6	90
PEG7	N1810C	100		
PEG8	T1812C	100		
PEG9	K1813C	83		
PEG10	Y1815C	75		
PEG11	D1795C			
PEG12	Q1796C			
PEG13	R1803C			
PEG14	K1804C			
PEG2+6	491C/1808C			
PEG15	K377C	82		
PEG16	H378C	126		
PEG17	K556C	43		
PEG18	N41C	80		
PEG19	N239C			

PEG20	N2118C	127		
PEG21	Y81C			
PEG22	F129C	83		
PEG23	K422C			
PEG24	K523C			
PEG25	K570C			
PEG26	N1864C			
PEG27	T1911C			
PEG28	Q2091C			
PEG29	Q2284C			

Table 3. Percent specific activity (S.A.) of PEG muteins and PEGylated PEG2 and PEG6 relative to BDD.

[0160] As used in Table 3, "PEG2 red" is PEG2 mutein that has been treated with reductant followed by the removal of reductant. This reduction procedure did not significantly alter the three functional activities of FVIII. PEG2 mutein conjugated to PEGs ranging from 5 kD (PEG2-5kD) to 43 kD (PEG2-43kD) did not lose a significant amount of chromogenic activity, but had greatly lower coagulation activity as the PEG size increases beyond 5 kD. There may be a modest reduction in vWF binding for larger size PEGylated PEG2 also.

[0161] TOTAL ANTIGEN ELISA (TAE). FVIII is captured on a microtiter plate that has been coated with a polyclonal FVIII antibody. The FVIII bound is detected with a biotinylated polyclonal rFVIII antibody and streptavidin horseradish peroxidase (HRP) conjugate. The peroxidase-streptavidin complex produces a color reaction upon addition of the tetramethylbenzidine (TMB) substrate. Sample concentrations are interpolated from a standard curve using four parameter fit models. FVIII results are reported in µg/mL.

[0162] vWF BINDING ELISA. FVIII is allowed to bind to vWf in Severe Hemophilic Plasma in solution. The FVIII-vWf complex is then captured on a microtiter plate that has been coated with a vWf-specific monoclonal antibody. The FVIII bound to the vWf is detected with a FVIII polyclonal antibody and a horseradish peroxidase-anti-rabbit conjugate. The peroxidase-conjugated antibody complex produces a color reaction upon addition of the substrate. Sample concentrations are interpolated from a standard curve using four parameter fit model. FVIII binding results are reported in µg/mL. There was no significant impact on any of the activities upon PEGylation, which would be consistent with PEGylation at the B domain.

Sample	TAE	Coagulation Assay			Chromogenic Assay			vWF ELISA		
	ug/mL	IU/mL	IU/ug	%Start	IU/mL	IU/ug	%Start	ug/mL	vWF/TAE	%Start
KG-2 start	1.31	4.8	3.6	**100**	5.60	4.3	**100**	0.42	0.32	**100**
Reduced only	0.93	3.1	3.4	**93**	4.08	4.4	**103**			
KG-2-5kD PEG	0.71	2.5	3.5	**96**	3.09	4.3	**102**			
KG-2-12kD PEG	0.59	2.3	3.9	**107**	2.99	5.0	**118**			
KG-2-22kD PEG	0.63	2.5	3.9	**108**	3.06	4.8	**113**	0.19	0.30	**94**
KG-2-30kD PEG	0.59	2.5	4.1	**114**	3.01	5.1	**119**	0.19	0.32	**100**
KG-2-43kD PEG	0.52	2.4	4.6	**128**	2.86	5.5	**129**			

Table 4. Specific activity (S. A.) of wildtype full length FVIII (KG-2) before and after PEGylation with different sizes of PEG.

[0163] PURIFICATION OF PEGylated FVIII BY ION-EXCHANGE CHROMATOGRAPHY. PEGylated FVIII is applied to an anion exchange column or cation exchange column where the protein binds to the column while any excess free PEG reagent does not bind and is removed in the flow through. The PEG mutein is then eluted from the column with a sodium chloride gradient. A barium-iodine stained 4-12% Bis-Tris gel of load, flow through, and gradient fractions was used to confirm that the column elution fractions have PEGylated mutein.

[0164] PURIFICATION OF PEGylated FVIII BY SIZE-EXCLUSION CHROMATOGRAPHY. The anion exchange fractions containing the majority of PEG2 mutein are pooled and concentrated by ultrafiltration then applied to a size exclusion column. The column is then eluted using the formulation buffer. Because of the difference in the size and shape of the protein depends on whether PEG is bound to the protein, this column separates the PEGylated PEG2 mutein from that of any remaining PEG2, which is not PEGylated. The PEGylated mutein FVIII fractions are pooled based on having the most FVIII activity then frozen for subsequent animal studies and molecular characterization. Figure 13 compares the elution of non-PEGylated PEG2 mutein versus that of the 43 kD PEGylated PEG2 mutein. The PEGylated PEG2 elutes significantly earlier, which indicates an increase in its size and shape from the covalently attached PEG.

[0165] With muteins such as PEG6 that show lower efficiencies of PEGylation, i.e. less than 50%, the most effective purification scheme to yield highly pure mono-PEGylated product is to use a combination of cation exchange chromatography followed by size exclusion chromatography. For example, with PEG6, the cation exchange chromatography purifies the PEGylated PEG6 (earlier eluting fraction, Fig 14) away from the majority of un-PEGylated PEG6 (later eluting fraction, Fig 15). The size exclusion chromatography then polishes the PEGylated protein (earlier eluting fraction, Fig 15) from the remainder of un-PEGylated protein (later eluting fraction Fig 15).

[0166] EFFECT OF PEG SIZE ON ACTIVITY. To test whether PEG sizes have an effect on both coagulation and chromogenic activities of FVIII upon PEGylation, purified full-length FVIII, PEG2, PEG6, and PEG14 were reduced by TCEP followed by reductant removal and reaction with a buffer control or PEGs ranging from 6 kD to 64 kD. The resulting PEGylated FVIII was directly assayed without removal of excess PEG or unPEGylated FVIII. Control experiments showed that the excess PEG has no effect on FVIII activity.

[0167] Fig. 16 shows the results of this study. Purified full-length FVIII is represented as KG-2 in Fig. 16. The percent activity reported in Fig. 16 was determined by dividing the value of sample treated with PEG after reduction and reductant removal by that of the sample treated with buffer control taking into consideration the PEGylation yield. PEGylation yields were comparable across all PEGs for any given FVIII construct. They are about 80% for KG-2, PEG2, and PEG14 and about 40% for PEG6. For example, PEG14 buffer control treated has a coagulation activity of 6.8 IU/mL vs. 3.2 IU/mL for the 12 kD PEGylated PEG14 sample. However, the PEGylation efficiency was about 80%, meaning the 3.2 IU/mL represents the aggregate activity of about 80% PEGylated and about 20% unPEGylated. Assuming the unPEGylated sample has the same activity as the buffer control treated PEG14, the percent activity of unPEGylated for the PEGylated PEG14 works out to be 34% = (3.2-6.8 times 20%)/(6.8 times 80%).

[0168] PEGylation within the A2 or A3 domain at PEG2, PEG6, or PEG14 position of BDD led to dramatic losses of coagulation activity when PEG size increases beyond 6 kD. However, PEGylation within the B domain at a native B-domain cysteine of the full-length FVIII had no effect on the coagulation activity. Interestingly, the chromogenic activity is not affected for all PEGylated constructs. This may be due to assay differences. It is possible that the small chromogenic peptide substrate has an easier access to a PEGylated FVIII/FIX/FX complex than the larger protein substrate used in the coagulation assay. Alternatively, PEG may affect activation of the mutein. This would be more readily detected by the one-stage coagulation assay than the two-stage chromogenic assay.

[0169] To confirm the observation of PEG effects on the coagulation activity of PEG2, 6, and 14, several PEGylated contructs were purified away from excess PEG and unPEGylated. Since PEG does not have any effect on the chromogenic activity, the chromogenic to coagulation activity ratio is a good estimate on the relative effect of PEG on coagulation activity (Table 5). Larger PEGs at a given position such as PEG2 and a higher number of PEGs as in the case with the PEG2+6 construct induce a greater loss of coagulation activity.

PEGylated BDD		Chromogenic IU/mL / Coagulation IU/mL	
Sample ID	PEG	Raw Ratio	Ratio relative to BDD
BDD	no PEG	1.7	1
PEG2 (pool2)	22kD 491	9	5
PEG2	43kD* 491	25	15
PEG6	12kD 1808	5	3
PEG6 (old)	33kD 1808	13	7
PEG6 (new)	33kD 1808	8	5
PEG2+6 (LSP25)	33kD at 491, Mono	10	6
PEG2+6 (LSP22)	33kD at 491/1808, Di	24	14
PEG2+6 (ESP)	33kD at 491/1808/A3, Tri	60	35
PEG22	64kD* 129	14	8
PEG14	12kD 1804	3.2	1.9
PEG14	20kD* 1804	4.2	2.5
PEG14	33kD 1804	5	2.9
PEG2+14 (ESP19)	33kD at 491/1804, Di	21	12

Table 5. Ratio of Chromogenic to Coagulation for Purified PEGylated BDD.

*branched PEG

[0170] RABBIT PK STUDY. To understand the effects of PEGylation on the pharmacokinetics (PK) of FVIII, PK studies were performed in a number of species. NZW SPF rabbits were used for the study: 10 females, 5 rabbits per group, 2 groups (PEG2 FVIII and 22kD PEGylated PEG2). Samples were diluted into sterile PBS with a final concentration of 100 IU/mL (chromogenic units). Each rabbit received a dose of 1 ml/kg (100 IU/kg) of the diluted test or control substance via marginal ear vein. At various times post-injection, blood samples (1 mL) were drawn into a 1 mL syringe (charged with 100 µL of 3.8% Na-Citrate) from the central ear artery at defined time points after dosing. Plasma samples were incubated with R8B12 heavy chain antibody coated on a 96-well plate to specifically capture the dosed human FVIII. The activity of the captured FVIII was determined by the chromogenic assay (Figure 17). PEGylated PEG2 and PEGylated PEG6 were also compared with BDD (Figures 18 and 19), with PEGylated muteins showing an improvement in plasma recovery compared to BDD. PEGylated wildtype full-length FVIII did not appear to show much improvement (Figure 20).

[0171] MOUSE PK STUDY. As a second species, ICR normal or hemophilic, FVIII deficient, mice (Taconic, Hudson, NY) were used in PK studies. Normal mice were used for the study, 5 mice per group per time point. Test materials were diluted into formulation buffer to a nominal final concentration of 25 IU/mL. Each mouse can be administered 4 mL/kg (~0.1 mL total volume) of the dilute test material via tail vein. Blood samples (0.45 or 0.3 mL for normal or hemophilic mouse study, respectively) are drawn into a 1 mL syringe (charged with 50 or 30 µL of 3.8% Na-Citrate for normal or hemophilic mouse study, respectively) from the inferior vena cava at the indicated time point (one animal per sample). Plasma samples are assayed for FVIII concentration using the chromogenic assay method described above. PEGylated PEG6 shows greater plasma

recovery compared to BDD or PEG6 (Figure 21). PEGylated PEG2 shows greater plasma recovery compared to BDD (Figures 22 and 23).

Construct	Half-life, hr	Species
BDD	6.6	Normal Rabbit
PEG2	4.8	Normal Rabbit
PEG2-22kD PEG	7.5	Normal Rabbit
PEG2-43kD PEG	8.0	Normal Rabbit
PEG6-12kD PEG	8.2	Normal Rabbit
PEG6-33kD PEG*	9.6	Normal Rabbit
PEG6-33kD PEG	17.4	Normal Rabbit
BDD	4.5	Normal Mouse
PEG2-22kD PEG	7.3	Normal Mouse
PEG6-12kD	5.3	Normal Mouse
PEG14-33kD PEG	7.3	Normal Mouse
PEG14-12kD PEG	5.5	Normal Mouse
PEG22-64kD	9.2	Normal Mouse

Table 6. PK study summary of PEGylated FVIII showing plasma half-lives in hours. *Initial prep of 33kD PEGylated PEG6 with half-life of 9.6 hr in rabbits was not as pure as a later prep that yielded 17.4 hr.

Mutein	PEG	Fold
PEG 6	12 kD	2.9
PEG 6	33 kD	2.9
PEG 2+6	33 kD	3.3
PEG 14	33 kD	2.5
PEG 2+6	33 kD	4.4
PEG 2+14	33 kD	2.1
PEG22	64 kD	3.2

Table 7. Plasma recovery of PEGylated PEG muteins in hemophilic mice. Fold-improvement in plasma recovery at 16 hours post-injection compared to the BDD control performed on the same date is reported.

[0172] HEMOPHILIC MOUSE (BDD) FACTOR VIII RECOVERY. The Hemophilic Mouse (BDD) Factor VIII recovery histogram shown in Figure 24 depicts a pharmacokinetic (PK) assessment of the half-life of two species of BDD Factor VIII in a hemophilic mouse assay. This assay was designed to measure plasma concentrations of both BDD Factor VIII (referred to in Figure 24 as "wt"

or wild type BDD Factor VIII) and the PEG 2+6 double PEGylated variant of BDD Factor VIII (and identified elsewhere herein as the L491C, K1808C double variant of BDD Factor VIII) at three time points post intravenous administration in a mouse model. While the PK assessments at both the 0.8 and 4 hour time points were comparable, the 16 hour assessment is particularly note worthy. At 16 hours, approximately four times (400%) as much of the doubly PEGylated BDD Factor VIII variant (PEG 2+6) remained in the mouse plasma 16 hours after administration as compared to the un-PEGylated molecule.

[0173] KIDNEY LACERATION MODEL. To determine if PEGylated FVIII muteins were efficacious at stopping a bleed in a hemophilic mouse, the kidney laceration model was employed. Hemophilic mice (C57/BL6 with a disrupted FVIII gene) are anesthetized under isofluorane and weighed. The inferior vena cava was exposed and 100 ul of either saline or FVIII were injected using a 31 gauge needle. The needle was carefully removed and pressure applied at the sight of injection for 30-45 seconds to prevent bleeding. After two minutes, the right kidney was exposed and held between the forceps along the vertical axis. Using a #15 scalpel, the kidney was cut horizontally to a depth of 3 mm. To insure a uniform depth of the lesion, kidney was lightly held in the middle to expose equal tissue on either side of the forceps. The exposed surface of the kidney was cut to the depth of the forceps. Blood loss was quantified as described above. Different doses of FVIII were tested on mice to characterize the dose response relationship of FVIII on kidney bleeding. PEGylated PEG2 shows comparable potency to BDD in reducing blood loss after mouse kidney injury (Figure 25). Thus, although the coagulation activity of PEGylated PEG2 is lower than that of BDD, this kidney laceration model shows that the in vivo efficacy of PEGylated PEG2 was not measurably reduced compared to BDD, consistent with the chromogenic assay data.

[0174] ANTIBODY INHIBITION ASSAY. Adding a high molecular weight polymer such as polyethylene glycol (PEG) specifically at position 491 (i.e. PEG2) should reduce binding and sensitivity to mAB 413, and by extension to a large proportion of patient inhibitory antibodies since many patients develop inhibitor antibodies against the same mAB 413 epitope. To test this, increasing amounts of mAB 413 was incubated with non-saturating amounts (0.003 IU/mL) of BDD or 43 kD PEGylated PEG2 and tested for functional activity in a chromogenic assay (Figure 26). R8B12, a non-inhibitory antibody, and ESH4, an inhibitory antibody that targets the C2 domain were used as controls. PEGylated PEG2 is indeed more resistant to mAB 413 inhibition than BDD and shows a similar inhibition pattern in the presence of the control antibodies that do not bind near the 491 position. Furthermore, the protection effect of PEG against mAB 413 inhibition is dependent on PEG size, with larger PEGs having a greater effect (Figure 27). To test whether PEGylated FVIII is more resistant to inhibitor antibodies from patients, chromogenic activity was measured in the presence of a panel of plasma derived from hemophilia A patients who have developed inhibitors to FVIII. Of the 8 patient plasma tested, 43kD PEGylated PEG2 was more resistant to patient plasma inhibition than

BDD in 4 patient plasma samples. For example, PEGylated PEG2, PEG6, or PEG2+6 showed greater residual activity than BDD in one patient plasma but not in another plasma (Figure 28). The diPEGylated PEG2+6 appears to be more resistant than monoPEGylated PEG2 or PEG6. These results suggest that PEGylated PEG muteins can be more effective in treating patients that develop inhibitors to FVIII.

[0175] HIGH THROUGHPUT PEGYLATION SCREENING. PEGylation efficiency of a particular PEG mutein is unpredictable, especially since there is no direct structural information of BDD. For example, based on the structure model of BDD, one would predict the PEGylation efficiency of PEG4 and PEG5 should be very high, similar to that of PEG2 and PEG15 since all three positions are surface exposed and point outwardly according to the structure. Thus, to use PEG to search for novel clearance mechanism via systematic PEGylation will require a large number of muteins to be screened.

[0176] To rapidly screen a large number of PEG muteins, a novel high throughput method has been developed that can test PEGylation efficiency and functional activity of PEGylated products from transiently transfected muteins. As little as 5-10 mL of transiently expressed PEG muteins with an FVIII chromogenic value of as low as 0.1-0.2 IU/mL is concentrated by about 50-fold using Amicon-centra Ultra device MWCO 30K so that the concentration of FVIII reaches above 1 nM, near the affinity range of antibody to FVIII interaction. The concentrated PEG mutein (~300 uL) is incubated with ~30 uL of C7F7 FVIII antibody resin overnight at 4°C, washed, eluted, dialyzed, and reduced. The reductant is removed and the reduced PEG muteins is PEGylated and run on a Western analysis as described above (Figures 29 and 30). Relative PEGylation efficiency of transiently expressed PEG muteins matches exactly to that of purified PEG muteins.

[0177] Dozens of PEG muteins can be screened by this method in one to two months. For example, PEG14 (K1804C BDD) had at least about 80% PEGylation of light chain with a 12 kD PEG and no PEGylation of heavy chain (data not shown), consistent with the K1804C mutation located on the light chain. The C□ to C□ distance between K1804 and K1808 (PEG6 position) is only 8.4 angstrom based on the BDD structure, suggesting that the introduction of a 43 kD PEG at this position will have similar improvement in PK as the 33 kD PEGylated PEG6, with the advantage of much higher PEGylation yield. Relative PEGylation yield for all PEG muteins tested are summarized in Table 8. PEGylation was highly selective for the particular FVIII chain where the cysteine mutation was introduced, in that every mutein with the cysteine in the heavy chain only gets PEGylated on the heavy chain while every mutein with the cysteine in the light chain gets PEGylated on the light chain. Mutein numbers 2 to 31 represent cysteine mutations of BDD replacing the native amino acid at the position listed with a cysteine. PEG2+6 is a double mutein of BDD where position 491 and 1808 were substituted with cysteines. A1 and A2, (and B domain for KG-2, the full-length FVIII) belong to the

heavy chain while A3, C1, and C2 belong to the light chain. PEGylation efficiency was estimated from running the PEGylated products on a SDS PAGE comparing the intensities of the PEGylated band with unPEGylated band: +++ ~ >80% PEGylation yield, ++ ~30-70% yield, + ~10-30% yield, and - ~<10% yield.

PEG Mutein	Position	Domain	H-PEG	L-PEG
2	491	A2	+++	-
4	504	A2	+	-
5	468	A2	+	-
6	1808	A3	-	++
7	1810	A3	-	++
8	1812	A3	-	-
9	1815	A3	-	-
11	1795	A3	-	+
12	1796	A3	-	+
13	1803	A3	-	++
14	1804	A3	-	+++
15	377	A2	+++	-
16	378	A2	+++	-
17	556	A2	++	-
20	2118	A3	-	+
21	81	A1	++	-
22	129	A1	++	-
23	422	A2	-	-
25	570	A2	-	-
26	1864	A3	-	++
27	1911	A3	-	+++
28	2091	C1	-	++
29	2284	C2	-	+
30	711	A2	+	-
31	1903	A3	-	++
2+6	490/1808	A2/A3	+++	++
2+14	490/1804	A2/A3	+++	+++
KG-2		B	+++	-

Table 8 PEGylation efficiency for various PEGylated FVIII.

[0178] MASS SPECTROMETRY ANALYSIS OF REDUCED PEG MUTEINS. To determine the identity of the "cap" that prevents direct PEGylation of PEG muteins or full-length FVIII, PEG2+14 was reduced with TCEP at concentrations ranging from 67 uM to 670 uM. PEGylation yield increased in proportion to increasing amounts of TCEP (Figure 31). The same samples were also analyzed by mass spectrometry prior to PEGylation (Figure 32). In order to have a protein domain that could be directly studied, the samples were digested with thrombin at a ratio of 20 units/mg FVIII for 30 minutes at 37 °C. Thrombin cleavage produces an A2 fragment that includes residues 372 to 740 and no occupied glycosylation sites. The digested sample was injected onto a C4 reversed phase liquid chromatography system and the eluent from the column was introduced directly into the quadrupole time-of-flight mass spectrometer via an electrospray interface. The mass spectrum from under the chromatographic peak corresponding to the A2 domain was deconvoluted to provide a protein intact

mass value. Prior to reduction, the A2 domain of PEG2+14 yields a mass that is 118 daltons larger than theoretically predicted. As the TCEP concentration is increased, a new peak that has the precise predicted mass of A2 domain appears. The proportion of this new peak increases as the TCEP concentration is increased. The 118 dalton difference can be accounted for by cysteinylation at residue Cys 491 via disulfide formation with a cystine (119 Da) and instrumental accuracy. Thus this shows that the PEG muteins are capped by a cysteine, which prevents direct PEGylation.

[0179] All of the references disclosed herein are hereby incorporated herein in their entireties.

We claim:

1. A conjugate having factor VIII procoagulant activity comprising a functional factor VIII polypeptide covalently attached at one or more predefined sites on the polypeptide to one or more biocompatible polymers, wherein the predefined site is a not an N-terminal amine.

2. The conjugate of claim 1, wherein the biocompatible polymer comprises polyethylene glycol.

3. The conjugate of claim 2, wherein the polyethylene glycol comprises methoxypolyethylene glycol.

4. The conjugate of claim 3, wherein the methoxypolyethylene glycol has a size range from 5 kD to 64 kD.

5. The conjugate of claim 1, wherein the biocompatible polymer is covalently attached to the functional factor VIII polypeptide at an amino acid residue in or near (a) a binding site for a factor VIII clearance receptor, (b) a binding site for a protease capable of degradation of factor VIII and/or (c) a binding site for factor VIII inhibitory antibodies.

6. The conjugate of claim 1, wherein the biocompatible polymer is covalently attached at the predefined site on the functional factor VIII polypeptide such that binding of low-density lipoprotein receptor related protein to the polypeptide is less than to the polypeptide when it is not conjugated.

7. The conjugate of claim 6, wherein the binding of low-density lipoprotein receptor related protein to the conjugate is more than twofold less than the binding to the polypeptide when it is not conjugated.

8. The conjugate of claim 1, wherein the biocompatible polymer is covalently attached at the predefined site on the functional factor VIII polypeptide such that binding of heparan sulphate proteoglycans to the polypeptide is less than to the polypeptide when it is not conjugated.

9. The conjugate of claim 8, wherein the binding of heparin sulphate proteoglycans to the conjugate is more than twofold less than the binding to the polypeptide when it is not conjugated.

10. The conjugate of claim 1, wherein the biocompatible polymer is covalently attached at the predefined site on the functional factor VIII polypeptide such that binding of factor VIII inhibitory antibodies to the polypeptide is less than to the polypeptide when it is not conjugated.

11. The conjugate of claim 10, wherein the binding of factor VIII inhibitory antibodies to the conjugate is more than twofold less than the binding to the polypeptide when it is not conjugated.

12. The conjugate of claim 1, wherein the biocompatible polymer is covalently attached at the predefined site on the functional factor VIII polypeptide such that binding of low density lipoprotein receptor to the polypeptide is less than to the polypeptide when it is not conjugated.

13. The conjugate of claim 12, wherein the binding of low density lipoprotein receptor to the conjugate is more than twofold less than the binding to the polypeptide when it is not conjugated.

14. The conjugate of claim 1, wherein the biocompatible polymer is covalently attached at the predefined site on the functional factor VIII polypeptide such that a plasma protease degrades the polypeptide less than when the polypeptide is not conjugated.

15. The conjugate of claim 14, wherein the degradation of the polypeptide by the plasma protease is more than twofold less than the degradation of the polypeptide when it is not conjugated as measured under the same conditions over the same time period.

16. The conjugate of claim 1, wherein the biocompatible polymer is covalently attached to the polypeptide at one of the factor VIII amino acid positions 81, 129, 377, 378, 468, 487, 491, 504, 556, 570, 711, 1648, 1795, 1796, 1803, 1804, 1808, 1810, 1864, 1903, 1911, 2091, 2118 and 2284.

17. The conjugate of claim 1, wherein the biocompatible polymer is covalently attached to the polypeptide at one or more of factor VIII amino acid positions 377, 378, 468, 491, 504, 556, 1795, 1796, 1803, 1804, 1808, 1810, 1864, 1903, 1911 and 2284 and further wherein (1) the binding of the conjugate to low-density lipoprotein receptor related protein is less than the binding of the unconjugated polypeptide to the low-density lipoprotein receptor related protein; (2) the binding of the conjugate to low-density lipoprotein receptor is less than the binding of the unconjugated polypeptide to the low-density lipoprotein receptor; or (3) the binding of the conjugate to both low-density lipoprotein receptor related protein and low-density lipoprotein receptor is less than the binding of the unconjugated polypeptide to the low-density lipoprotein receptor related protein and the low-density lipoprotein receptor.

18. The conjugate of claim 1, wherein the biocompatible polymer is covalently attached to the polypeptide at one or more of factor VIII amino acid positions 377, 378, 468, 491, 504, 556 and 711 and further wherein the binding of the conjugate to heparin sulphate proteoglycan is less than the binding of the unconjugated polypeptide to heparin sulphate proteoglycan.

19. The conjugate of claim 1, wherein the biocompatible polymer is covalently attached to the polypeptide at one or more of factor VIII amino acid positions 81, 129, 377, 378, 468, 487, 491, 504, 556, 570, 711, 1648, 1795, 1796, 1803, 1804, 1808, 1810, 1864, 1903, 1911, 2091, 2118 and 2284 and the conjugate has less binding to factor VIII inhibitory antibodies than the unconjugated polypeptide.

20. The conjugate of claim 1, wherein the biocompatible polymer is covalently attached to the polypeptide at one or more of factor VIII amino acid positions 81, 129, 377, 378, 468, 487, 491, 504, 556, 570, 711, 1648, 1795, 1796, 1803, 1804, 1808, 1810, 1864, 1903, 1911, 2091, 2118 and 2284 and the conjugate has less degradation from a plasma protease capable of factor VIII degradation than does the unconjugated polypeptide.

21. The conjugate of claim 20, wherein the plasma protease is activated protein C.

22. The conjugate of claim 1, wherein the functional factor VIII polypeptide is B-domain deleted factor VIII.

23. The conjugate of claim 22, wherein the biocompatible polymer is covalently attached to B-domain deleted factor VIII at amino acid position 129, 491, 1804, and/or 1808.

24. The conjugate of claim 1, wherein the biocompatible polymer is attached to the polypeptide at factor VIII amino acid position 1804 and comprises polyethylene glycol.

25. The conjugate of claim 1, wherein the one or more predefined sites for biocompatible polymer attachment are controlled by site specific cysteine mutation.

26. A method for the preparation of the conjugate of claim 1 comprising:

mutating a nucleotide sequence that encodes for the functional factor VIII polypeptide to substitute a coding sequence for a cysteine residue at a pre-defined site;

expressing the mutated nucleotide sequence to produce a cysteine enhanced mutein;

purifying the mutein;

reacting the mutein with the biocompatible polymer that has been activated to react with polypeptides at substantially only reduced cysteine residues such that the conjugate is formed; and

purifying the conjugate.

27. The method of claim 26, wherein the biocompatible polymer comprises polyethylene glycol.

28. The method of claim 27, wherein polyethylene glycol is activated by the addition of a maleimide group that can react specifically to cysteines in proteins.

29. A pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of the conjugate of claim 1 and a pharmaceutically acceptable adjuvant.

30. A method of treating hemophilia comprising administering to a patient in need thereof an effective amount of the composition of claim 29.

31. A method for site-directed PEGylation of a factor VIII mutein comprising:

(a) expressing a site-directed factor VIII mutein wherein the mutein has a cysteine replacement for an amino acid residue on the exposed surface of the factor VIII mutein and that cysteine is capped;

(b) contacting the cysteine mutein with a reductant under conditions to mildly reduce the cysteine mutein and to release the cap;

(c) removing the cap and the reductant from the cysteine mutein; and

(d) at least about 5 minutes after the removal of the reductant, treating the cysteine mutein with PEG comprising a sulfhydryl coupling moiety under conditions such that PEGylated factor VIII mutein is produced.

32. The method of claim 31, wherein in step (c) the cap and the reductant are removed from the cysteine mutein by size-exclusion or ion exchange chromatography.

33. The method of claim 31, wherein the factor VIII mutein is a mutein of B-domain deleted factor VIII.

34. The method of claim 31, wherein the PEG-maleimide has a size range from 5 kD to 64 kD.

35. The method of claim 31, wherein the sulfhydryl moiety of the PEG is selected from the group consisting of thiol, triflate, tresylate, aziridine, oxirane, S-pyridyl and maleimide moieties.