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(54) Title: THROMBIN CLEAVABLE LINKER

(57) Abstract: The present invention provides a chimeric molecule comprising a VWF protein fused to a heterologous moiety via a VWF linker. The invention provides an efficient VWF linker that can be cleaved in the presence of thrombin. The chimeric molecule can further comprise a polypeptide chain comprising a FVIII protein and a second heterologous moiety, wherein the chain comprising the VWF protein and the chain comprising the FVIII protein are associated with each other. The invention also includes nucleotides, vectors, host cells, methods of using the VWF fragment, or the chimeric proteins.

THROMBIN CLEAVABLE LINKER

BACKGROUND OF THE INVENTION

[0001] Haemophilia A is a bleeding disorder caused by defects in the gene encoding coagulation factor VIII (FVIII) and affects 1-2 in 10,000 male births. Graw et al., Nat. Rev. Genet. 6(6): 488-501 (2005). Patients affected with hemophilia A can be treated with infusion of purified or recombinantly produced FVIII. All commercially available FVIII products, however, are known to have a half-life of about 8-12 hours, requiring frequent intravenous administration to the patients. See Weiner M.A. and Cairo, M.S., Pediatric Hematology Secrets, Lee, M.T., 12. Disorders of Coagulation, Elsevier Health Sciences, 2001; Lillicrap, D. Thromb. Res. 122 Suppl 4:S2-8 (2008). In addition, a number of approaches have been tried in order to extend the FVIII half-life. For example, the approaches in development to extend the half-life of clotting factors include pegylation, glycopegylation, and conjugation with albumin. See Dumont et al., Blood. 119(13): 3024-3030 (Published online Jan. 13, 2012). Regardless of the protein engineering used, however, the long acting FVIII products currently under development have improved half-lives, but the half-lives are reported to be limited – only to about 1.5 to 2 fold improvement in preclinical animal models. *See Id.* Consistent results have been demonstrated in humans, for example, rFVIIIFc was reported to improve half-life up to ~ 1.7 fold compared with ADVATE® in hemophilia A patients. See Id. Therefore, the half-life increases, despite minor improvements, may indicate the presence of other $t_{1/2}$ limiting factors.

[0002] Due to the frequent dosing and inconvenience caused by the dosing schedule, there is still a need to develop FVIII products requiring less frequent administration, *i.e.*, a FVIII product that has a half-life longer than the 1.5 to 2 fold half-life limitation.

BRIEF SUMMARY OF THE INVENTION

[0003] The present invention provides a chimeric molecule comprising a Von Willebrand Factor (VWF) protein, a heterologous moiety (H1), and a VWF linker connecting the VWF protein with the heterologous moiety, wherein the VWF linker comprises a polypeptide selected from: (a) an a2 region from Factor VIII ("FVIII"); (b)an a1 region from FVIII; (c) an a3 region from FVIII; (d) a thrombin cleavage site which comprises X-

V-P-R (SEQ ID NO: 3) and a PAR1 exosite interaction motif, wherein X is an aliphatic amino acid; or (e) any combination thereof.

[0004] In one embodiment, the VWF linker comprises an a2 region which comprises an amino acid sequence at least about 80%, about 85%, about 90%, about 95%, or 100% identical to Glu720 to Arg740 corresponding to full-length mature FVIII, wherein the a2 region is capable of being cleaved by thrombin.

[0005] In another embodiment, the VWF linker comprises an a1 region which comprises an amino acid sequence at least about 80%, about 85%, about 90%, about 95%, or 100% identical to Met337 to Arg372 corresponding to full-length mature FVIII, wherein the a1 region is capable of being cleaved by thrombin.

[0006] In other embodiments, the VWF linker comprises an a3 region which comprises an amino acid sequence at least about 80%, about 85%, about 90%, about 95%, or 100% identical to Glu1649 to Arg1689 corresponding to full-length mature FVIII, wherein the a3 region is capable of being cleaved by thrombin.

[0007] In certain embodiments, the VWF linker comprises a thrombin cleavage site which comprises X-V-P-R (SEQ ID NO: 3) and a PAR1 exosite interaction motif, wherein the PAR1 exosite interaction motif comprises S-F-L-L-R-N (SEQ ID NO: 4). In some embodiments, the PAR1 exosite interaction motif further comprises an amino acid sequence selected from P, P-N, P-N-D, P-N-D-K (SEQ ID NO: 5), P-N-D-K-Y (SEQ ID NO: 6), P-N-D-K-Y-E (SEQ ID NO: 7), P-N-D-K-Y-E-P (SEQ ID NO: 8), P-N-D-K-Y-E-P-F (SEQ ID NO: 9), P-N-D-K-Y-E-P-F-W (SEQ ID NO: 10), P-N-D-K-Y-E-P-F-W-E (SEQ ID NO: 11), P-N-D-K-Y-E-P-F-W-E-D (SEQ ID NO: 12), P-N-D-K-Y-E-P-F-W-E-D-E (SEQ ID NO: 13), P-N-D-K-Y-E-P-F-W-E-D-E-E (SEQ ID NO: 14), P-N-D-K-Y-E-P-F-W-E-D-E-E-S (SEQ ID NO: 20), or any combination thereof. In other embodiments, the aliphatic amino acid is selected from Glycine, Alanine, Valine, Leucine, or Isoleucine.

[0008] In other embodiments, thrombin cleaves the VWF linker at least about 10 times, at least about 20 times, at least about 30 times, at least about 40 times, at least about 50 times, at least about 60 times, at least about 70 times, at least about 80 times, at least about 90 times or at least about 100 times faster than thrombin would cleave the thrombin cleavage site if the thrombin cleavage site were substituted for the VWF linker in the chimeric molecule. In still other embodiments, the VWF linker further comprises one or

more amino acids having a length of at least about 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1200, 1400, 1600, 1800, or 2000 amino acids.

[0009] In one embodiment, the VWF protein comprises a D' domain and a D3 domain of VWF, wherein the D' domain and the D3 domain are capable of binding to FVIII. In another embodiment, the VWF protein further comprises the D1 domain, the D2 domain, or the D1 and D2 domains of VWF.

[0010] In certain embodiments, a heterologous moiety (H1) fused to a VWF protein via a VWF linker is capable of extending the half-life of the chimeric molecule. Examples of the heterologous moiety (H1) include, but are not limited to, an immunoglobulin constant region or a portion thereof, albumin, albumin-binding moiety, PAS, HAP, transferrin or a fragment thereof, PSA, the C-terminal peptide (CTP) of the β subunit of human chorionic gonadotropin, polyethylene glycol (PEG), hydroxyethyl starch (HES), or any combination thereof. In a specific embodiment, the heterologous moiety is an FcRn binding partner or an Fc region.

[0011] In some embodiments, a chimeric molecule further comprises a second polypeptide chain comprising a FVIII protein and a second heterologous moiety (H2), wherein the FVIII protein is associated with the VWF protein. In other embodiments, the second heterologous moiety is selected from an immunoglobulin constant region or a portion thereof, albumin, albumin-binding moiety, PAS, HAP, transferrin or a fragment thereof, PSA, the C-terminal peptide (CTP) of the β subunit of human chorionic gonadotropin, polyethylene glycol (PEG), hydroxyethyl starch (HES), or any combination thereof. In a particular embodiment, the second heterologous moiety (H2) comprises an FcRn binding partner or an Fc regin. In certain embodiments, the first heterologous moiety and the second heterologous moiety are identical or different. In other embodiments, the first heterologous moiety and the second heterologous moiety are associated with each other. The association between the first heterologous moiety and the second heterologous moiety can be a disulfide bond.

[0012] In certain embodiments, a chimeric molecule comprises a formula selected from: (a) V-L1-H1: H2-L2-C, or (b) C-L2-H2:H1-L1-V; wherein V is a VWF protein; L1 is a VWF linker; L2 is an optional FVIII linker; H1 is a first heterologous moiety; H2 is a

second heterologous moiety; C is a FVIII protein; (-) is a peptide bond or one or more amino acids; and (:) is a covalent bond between the H1 and the H2.

[0013] In some embodiments, a chimeric molecule comprises a formula selected from: (i) V-L1-H1-L3-C-L2-H2, (ii) H2-L2-C-L3-H1-L1-V, (iii) C-L2-H2-L3-V-L1-H1, (iv) H1-L1-V-L3-H2-L2-C, (v) H1-L1-V-L3-C-L2-H2, (vi) H2-L2-C-L3-V-L1-H1, (vii) V-L1-H1-L3-H2-L2-C, or (viii) C-L2-H2-L3-H1-L1-V, wherein V comprises a VWF protein; L1 is a VWF linker; L2 is an optional FVIII linker; L3 is a processable linker that is processed by a protease, H1 is a first heterologous moiety; H2 is a second heterologous moiety; C comprises a FVIII protein; and (-) is a peptide bond or one or more amino acids.

[0014] In some embodiments, the protease is a proprotein convertase, *e.g.*, PC5, PC7, PACE, furin, or any combination thereof.

[0015] The invention also includes a polynucleotide or a set of polynucleotides encoding the chimeric molecule or any complementary sequence thereof. The invention also includes a vector or a set of vectors comprising the polynucleotide or the set of polynucleotides and one or more promoter operably linked to the polynucleotide or the set of polynucleotides. The vector or the set of vectors can further comprise an additional vector, which comprises a polynucleotide chain encoding a proprotein convertase, *e.g.*, PC5 or PC7.

[0016] The invention also provides a host cell comprising the polynucleotide or the vector or the set of vectors.

[0017] Also included is a method of reducing a frequency or degree of a bleeding episode in a subject in need thereof or a method of preventing an occurrence of a bleeding episode in a subject in need thereof comprising administering an effective amount of the chimeric molecule, the polynucleotide, the vector, the host cell, or the composition thereof.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[0018] FIG. 1 shows a schematic diagram of a chimeric molecule (FVIII/VWF heterodimer) comprising two polypeptide chains, the first chain comprising a VWF protein (*e.g.*, a D' domain and a D3 domain of VWF) fused to an Fc region via a thrombin cleavable VWF linker and the second chain comprising a FVIII protein fused to a second Fc region via a FVIII linker.

[0019] FIG. 2 shows various VWF constructs, each construct comprising a D' domain and a D3 domain fused to an Fc region via a thrombin cleavable VWF linker except control (*i.e.*, VWF-052). VWF-031 comprises a linker of 48 amino acids comprising a thrombin cleavage site of L-V-P-R (SEQ ID NO: 21). VWF-035 comprises a linker of 73 amino acids comprising a thrombin cleavage site of L-V-P-R (SEQ ID NO: 21). VWF-036 comprises a linker of 98 amino acids comprising a thrombin cleavage site of L-V-P-R (SEQ ID NO: 21). VWF-039 comprises a VWF linker of 26 amino acids comprising a thrombin cleavage site of L-V-P-R (SEQ ID NO: 21) and a PAR1 exosite interaction motif. VWF-051 comprises a linker of 54 amino acids comprising a thrombin cleavage site of A-L-R-P-R-V-V (SEQ ID NO: 22). VWF-052 comprises a linker of 48 amino acids without any thrombin cleavage site (control). VWF-054 comprises a VWF linker of 40 amino acids comprising an a1 region from FVIII. VWF-055 comprises a VWF linker of 34 amino acids comprising an a2 region from FVIII. VWF-056 comprises a VWF linker of 46 amino acids comprising an a3 region from FVIII.

[0020] FIG. 3A shows the rate of thrombin-mediated cleavage in units of resonance units per second (RU/s) as a function of capture density in units of RU for VWF-Fc fusion constructs VWF-031, VWF-036, VWF-039, VWF-051, and VWF-052. FIG. 3B shows the rate of thrombin-mediated cleavage in units of resonance units per second (RU/s) as a function of capture density in units of RU for VWF-Fc fusion constructs VWF-031, VWF-036, VWF-051, and VWF-052. In these experiements, each VWF-Fc fusion construct was captured at various densities and subsequently exposed to a fixed concentration of human alpha-thrombin. The slopes of each curve in FIG. 3A and FIG. 3B directly reflect the susceptibility to thrombin cleavage for each construct.

[0021] FIG. 4A shows the rate of thrombin-mediated cleavage in units of resonance units per second (RU/s) as a function of capture density in units of RU for VWF-Fc fusion constructs VWF-054, VWF-055, and VWF-056. FIG. 4B shows the rate of thrombin-mediated cleavage in units of resonance units per second (RU/s) as a function of capture density in units of RU for VWF-Fc fusion constructs VWF-031, VWF-039, VWF-054, VWF-055, and VWF-056. In these experiements, each VWF-Fc fusion construct was captured at various densities and subsequently exposed to a fixed concentration of human alpha-thrombin. The slopes of each curve in FIG. 4A and FIG. 4B directly reflect the susceptibility to thrombin cleavage for each construct.

[0022] FIG. 5 shows the results of a linear regression analysis to determine the susceptibility of various VWF-Fc constructs to thrombin-mediated cleavage. Values are expressed in units of inverse seconds and reflect the slopes of the curves presented in FIG. 3 and FIG. 4. The relative susceptibility of two different constructs is derived from the quotient of their respective slopes. $Slope_{VWF-039}/slope_{VWF-031}$ is 71, indicating that VWF-Fc fusion construct VWF-039 is 71-fold more susceptible to thrombin-mediated cleavage than is VWF-031. For comparison, $slope_{VWF-055}/slope_{VWF-031}$ is 65, and $slope_{VWF-051}/slope_{VWF-031}$ is 1.8.

[0023] FIG. 6 shows clotting time of various chimeric molecules in a HemA patient measured by whole blood ROTEM assay. FVII155/VWF-031 comprises two polypeptide chains, the first chain comprising BDD FVIII fused to an Fc region and the second chain comprising a D' domain and a D3 domain of VWF fused to an Fc region via a minimal thrombin cleavage site (*i.e.,* L-V-P-R (SEQ ID NO: 21)). FVII155/VWF-039 comprises two polypeptide chains, the first chain comprising BDD FVIII fused to an Fc region and the second chain comprising a D' domain and a D3 domain of VWF fused to an Fc region via a VWF linker comprising L-V-P-R (SEQ ID NO: 21) and a PAR1 exosite interaction motif. FVII155/VWF-055 comprises two polypeptide chains, the first chain comprising BDD FVIII fused to an Fc region and the second chain comprising a D' domain and a D3 domain of VWF fused to an Fc region via a VWF linker comprising an a2 region from FVIII.

DETAILED DESCRIPTION OF THE INVENTION

[0024] The present invention is directed to a chimeric molecule comprising a thrombin cleavable linker connecting a VWF protein or a FVIII protein with a heterologous moiety, *e.g.*, a half-life extending moiety. The thrombin cleavable linker (VWF linker or FVIII linker) can be cleaved efficiently by thrombin at the site of injury where thrombin is readily available. Exemplary chimeric proteins are illustrated in the instant description and figures. In some embodiments, the invention pertains to chimeric molecules having the structures set forth, for example, in FIGS. 1 to 6. In other embodiments, the invention pertains to polynucleotide encoding chimeric molecule constructs disclosed herein.

[0025] In order to provide a clear understanding of the specification and claims, the following definitions are provided below.

I. Definitions

[0026] It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, "a nucleotide sequence," is understood to represent one or more nucleotide sequences. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

[0027] The term "about" is used herein to mean approximately, roughly, around, or in the regions of. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 10 percent, up or down (higher or lower).

[0028] The term "polynucleotide" or "nucleotide" is intended to encompass a singular nucleic acid as well as plural nucleic acids, and refers to an isolated nucleic acid molecule or construct, *e.g.*, messenger RNA (mRNA) or plasmid DNA (pDNA). In certain embodiments, a polynucleotide comprises a conventional phosphodiester bond or a non-conventional bond (*e.g.*, an amide bond, such as found in peptide nucleic acids (PNA)). The term "nucleic acid" refers to any one or more nucleic acid segments, *e.g.*, DNA or RNA fragments, present in a polynucleotide. By "isolated" nucleic acid or polynucleotide is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, a recombinant polynucleotide encoding a Factor VIII polypeptide contained in a vector is considered isolated for the purposes of the present invention. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) from other polynucleotides in a solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of polynucleotides of the present invention. Isolated polynucleotides or nucleic acids according to the present invention further include such molecules produced synthetically. In addition, a polynucleotide or a nucleic acid can include regulatory elements such as promoters, enhancers, ribosome binding sites, or transcription termination signals.

[0029] As used herein, a "coding region" or “coding sequence” is a portion of polynucleotide which consists of codons translatable into amino acids. Although a "stop codon" (TAG, TGA, or TAA) is typically not translated into an amino acid, it may be considered to be part of a coding region, but any flanking sequences, for example

promoters, ribosome binding sites, transcriptional terminators, introns, and the like, are not part of a coding region. The boundaries of a coding region are typically determined by a start codon at the 5' terminus, encoding the amino terminus of the resultant polypeptide, and a translation stop codon at the 3'terminus, encoding the carboxyl terminus of the resulting polypeptide. Two or more coding regions of the present invention can be present in a single polynucleotide construct, *e.g.*, on a single vector, or in separate polynucleotide constructs, *e.g.*, on separate (different) vectors. It follows, then, that a single vector can contain just a single coding region, or comprise two or more coding regions, *e.g.*, a single vector can separately encode a first polypeptide chain and a second polypeptide chain of a chimeric molecule as described below. In addition, a vector, polynucleotide, or nucleic acid of the invention can encode heterologous coding regions, either fused or unfused to a nucleic acid encoding a chimeric molecule of the invention. Heterologous coding regions include without limitation specialized elements or motifs, such as a secretory signal peptide or a heterologous functional domain.

[0030] Certain proteins secreted by mammalian cells are associated with a secretory signal peptide which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Those of ordinary skill in the art are aware that signal peptides are generally fused to the N-terminus of the polypeptide, and are cleaved from the complete or "full-length" polypeptide to produce a secreted or "mature" form of the polypeptide. In certain embodiments, a native signal peptide, *e.g.*, a FVIII signal peptide or a VWF signal peptide is used, or a functional derivative of that sequence that retains the ability to direct the secretion of the polypeptide that is operably associated with it. Alternatively, a heterologous mammalian signal peptide, *e.g.*, a human tissue plasminogen activator (TPA) or mouse ß-glucuronidase signal peptide, or a functional derivative thereof, can be used.

[0031] The term "downstream" refers to a nucleotide sequence that is located 3' to a reference nucleotide sequence. In certain embodiments, downstream nucleotide sequences relate to sequences that follow the starting point of transcription. For example, the translation initiation codon of a gene is located downstream of the start site of transcription.

[0032] The term "upstream" refers to a nucleotide sequence that is located 5' to a reference nucleotide sequence. In certain embodiments, upstream nucleotide sequences relate to sequences that are located on the 5' side of a coding region or starting point of transcription. For example, most promoters are located upstream of the start site of transcription.

[0033] As used herein, the term "regulatory region" refers to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding region, and which influence the transcription, RNA processing, stability, or translation of the associated coding region. Regulatory regions may include promoters, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing sites, effector binding sites and stem-loop structures. If a coding region is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

[0034] A polynucleotide which encodes a gene product, *e.g.*, a polypeptide, can include a promoter and/or other transcription or translation control elements operably associated with one or more coding regions. In an operable association a coding region for a gene product, *e.g.*, a polypeptide, is associated with one or more regulatory regions in such a way as to place expression of the gene product under the influence or control of the regulatory region(s). For example, a coding region and a promoter are "operably associated" if induction of promoter function results in the transcription of mRNA encoding the gene product encoded by the coding region, and if the nature of the linkage between the promoter and the coding region does not interfere with the ability of the promoter to direct the expression of the gene product or interfere with the ability of the DNA template to be transcribed. Other transcription control elements, besides a promoter, for example enhancers, operators, repressors, and transcription termination signals, can also be operably associated with a coding region to direct gene product expression.

[0035] A variety of transcription control regions are known to those skilled in the art. These include, without limitation, transcription control regions which function in vertebrate cells, such as, but not limited to, promoter and enhancer segments from cytomegaloviruses (the immediate early promoter, in conjunction with intron-A), simian virus 40 (the early promoter), and retroviruses (such as Rous sarcoma virus). Other

transcription control regions include those derived from vertebrate genes such as actin, heat shock protein, bovine growth hormone and rabbit ß-globin, as well as other sequences capable of controlling gene expression in eukaryotic cells. Additional suitable transcription control regions include tissue-specific promoters and enhancers as well as lymphokine-inducible promoters (*e.g.*, promoters inducible by interferons or interleukins).

[0036] Similarly, a variety of translation control elements are known to those of ordinary skill in the art. These include, but are not limited to ribosome binding sites, translation initiation and termination codons, and elements derived from picornaviruses (particularly an internal ribosome entry site, or IRES, also referred to as a CITE sequence).

[0037] The term "expression" as used herein refers to a process by which a polynucleotide produces a gene product, for example, an RNA or a polypeptide. It includes without limitation transcription of the polynucleotide into messenger RNA (mRNA), transfer RNA (tRNA), small hairpin RNA (shRNA), small interfering RNA (siRNA) or any other RNA product, and the translation of an mRNA into a polypeptide. Expression produces a "gene product." As used herein, a gene product can be either a nucleic acid, *e.g.*, a messenger RNA produced by transcription of a gene, or a polypeptide which is translated from a transcript. Gene products described herein further include nucleic acids with post transcriptional modifications, *e.g.*, polyadenylation or splicing, or polypeptides with post translational modifications, *e.g.*, methylation, glycosylation, the addition of lipids, association with other protein subunits, or proteolytic cleavage.

[0038] A "vector" refers to any vehicle for the cloning of and/or transfer of a nucleic acid into a host cell. A vector may be a replicon to which another nucleic acid segment may be attached so as to bring about the replication of the attached segment. A "replicon" refers to any genetic element (*e.g.*, plasmid, phage, cosmid, chromosome, virus) that functions as an autonomous unit of replication *in vivo*, *i.e.*, capable of replication under its own control. The term "vector" includes both viral and nonviral vehicles for introducing the nucleic acid into a cell *in vitro*, *ex vivo* or *in vivo*. A large number of vectors are known and used in the art including, for example, plasmids, modified eukaryotic viruses, or modified bacterial viruses. Insertion of a polynucleotide into a suitable vector can be accomplished by ligating the appropriate polynucleotide fragments into a chosen vector that has complementary cohesive termini.

[0039] Vectors may be engineered to encode selectable markers or reporters that provide for the selection or identification of cells that have incorporated the vector. Expression of selectable markers or reporters allows identification and/or selection of host cells that incorporate and express other coding regions contained on the vector. Examples of selectable marker genes known and used in the art include: genes providing resistance to ampicillin, streptomycin, gentamycin, kanamycin, hygromycin, bialaphos herbicide, sulfonamide, and the like; and genes that are used as phenotypic markers, *i.e.*, anthocyanin regulatory genes, isopentanyl transferase gene, and the like. Examples of reporters known and used in the art include: luciferase (Luc), green fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT), -galactosidase (LacZ), -glucuronidase (Gus), and the like. Selectable markers may also be considered to be reporters.

[0040] The term "plasmid" refers to an extra-chromosomal element often carrying a gene that is not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear, circular, or supercoiled, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell.

[0041] Eukaryotic viral vectors that can be used include, but are not limited to, adenovirus vectors, retrovirus vectors, adeno-associated virus vectors, poxvirus, *e.g.*, vaccinia virus vectors, baculovirus vectors, or herpesvirus vectors. Non-viral vectors include plasmids, liposomes, electrically charged lipids (cytofectins), DNA-protein complexes, and biopolymers.

[0042] A "cloning vector" refers to a "replicon," which is a unit length of a nucleic acid that replicates sequentially and which comprises an origin of replication, such as a plasmid, phage or cosmid, to which another nucleic acid segment may be attached so as to bring about the replication of the attached segment. Certain cloning vectors are capable of replication in one cell type, *e.g.*, bacteria and expression in another, *e.g.*, eukaryotic cells. Cloning vectors typically comprise one or more sequences that can be used for selection of cells comprising the vector and/or one or more multiple cloning sites for insertion of nucleic acid sequences of interest.

[0043] The term "expression vector" refers to a vehicle designed to enable the expression of an inserted nucleic acid sequence following insertion into a host cell. The inserted nucleic acid sequence is placed in operable association with regulatory regions as described above.

[0044] Vectors are introduced into host cells by methods well known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter.

[0045] "Culture," "to culture" and "culturing," as used herein, means to incubate cells under *in vitro* conditions that allow for cell growth or division or to maintain cells in a living state. "Cultured cells," as used herein, means cells that are propagated *in vitro*.

[0046] As used herein, the term "polypeptide" is intended to encompass a singular "polypeptide" as well as plural "polypeptides," and refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term "polypeptide" refers to any chain or chains of two or more amino acids, and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides, "protein," "amino acid chain," or any other term used to refer to a chain or chains of two or more amino acids, are included within the definition of "polypeptide," and the term "polypeptide" can be used instead of, or interchangeably with any of these terms. The term "polypeptide" is also intended to refer to the products of post-expression modifications of the polypeptide, including without limitation glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, or modification by non-naturally occurring amino acids. A polypeptide can be derived from a natural biological source or produced recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. It can be generated in any manner, including by chemical synthesis.

[0047] An "isolated" polypeptide or a fragment, variant, or derivative thereof refers to a polypeptide that is not in its natural milieu. No particular level of purification is required. For example, an isolated polypeptide can simply be removed from its native or natural environment. Recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for the purpose of the invention, as are native or recombinant

polypeptides which have been separated, fractionated, or partially or substantially purified by any suitable technique.

[0048] Also included in the present invention are fragments or variants of polypeptides, and any combination thereof. The term "fragment" or "variant" when referring to polypeptide binding domains or binding molecules of the present invention include any polypeptides which retain at least some of the properties (*e.g.*, FcRn binding affinity for an FcRn binding domain or Fc variant, coagulation activity for an FVIII variant, or FVIII binding activity for the VWF protein) of the reference polypeptide. Fragments of polypeptides include proteolytic fragments, as well as deletion fragments, in addition to specific antibody fragments discussed elsewhere herein, but do not include the naturally occurring full-length polypeptide (or mature polypeptide). Variants of polypeptide binding domains or binding molecules of the present invention include fragments as described above, and also polypeptides with altered amino acid sequences due to amino acid substitutions, deletions, or insertions. Variants can be naturally or non-naturally occurring. Non-naturally occurring variants can be produced using art-known mutagenesis techniques. Variant polypeptides can comprise conservative or non-conservative amino acid substitutions, deletions or additions.

[0049] The term “VWF fragment” or “VWF fragments” used herein means any VWF fragments that interact with FVIII and retain at least one or more properties that are normally provided to FVIII by full-length VWF, *e.g.*, preventing premature activation to FVIIIa, preventing premature proteolysis, preventing association with phospholipid membranes that could lead to premature clearance, preventing binding to FVIII clearance receptors that can bind naked FVIII but not VWF-bound FVIII, and/or stabilizing the FVIII heavy chain and light chain interactions. In a particular embodiment, the “VWF fragment” as used herein comprises a D’ domain and a D3 domain of the VWF protein, but does not include the A1 domain, the A2 domain, the A3 domain, the D4 domain, the B1 domain, the B2 domain, the B3 domain, the C1 domain, the C2 domain, and the CK domain of the VWF protein.

[0050] The term "half-life limiting factor" or "FVIII half-life limiting factor" as used herein indicates a factor that prevents the half-life of a FVIII protein from being longer than 1.5 fold or 2 fold compared to wild-type FVIII (*e.g.*, ADVATE® or REFACTO®). For example, full length or mature VWF can act as a FVIII half-life limiting factor by

inducing the FVIII and VWF complex to be cleared from system by one or more VWF clearance pathways. In one example, endogenous VWF is a FVIII half-life limiting factor. In another example, a full-length recombinant VWF molecule non-covalently bound to a FVIII protein is a FVIII-half-life limiting factor.

[0051] The term "endogenous VWF" as used herein indicates VWF molecules naturally present in plasma. The endogenous VWF molecule can be multimer, but can be a monomer or a dimer. Endogenous VWF in plasma binds to FVIII and forms a non-covalent complex with FVIII.

[0052] A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, if an amino acid in a polypeptide is replaced with another amino acid from the same side chain family, the substitution is considered to be conservative. In another embodiment, a string of amino acids can be conservatively replaced with a structurally similar string that differs in order and/or composition of side chain family members.

[0053] As known in the art, "sequence identity" between two polypeptides is determined by comparing the amino acid sequence of one polypeptide to the sequence of a second polypeptide. When discussed herein, whether any particular polypeptide is at least about 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% identical to another polypeptide can be determined using methods and computer programs/software known in the art such as, but not limited to, the BESTFIT program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). BESTFIT uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981), to find the best segment of homology between two sequences. When using BESTFIT or any other sequence alignment program to determine whether a particular sequence is, for example, 95% identical to a reference sequence according to the present invention, the parameters

are set, of course, such that the percentage of identity is calculated over the full-length of the reference polypeptide sequence and that gaps in homology of up to 5% of the total number of amino acids in the reference sequence are allowed.

[0054] As used herein, an "amino acid corresponding to" or an "equivalent amino acid" in a VWF sequence or a FVIII protein sequence is identified by alignment to maximize the identity or similarity between a first VWF or FVIII sequence and a second VWF or FVIII sequence. The number used to identify an equivalent amino acid in a second VWF or FVIII sequence is based on the number used to identify the corresponding amino acid in the first VWF or FVIII sequence.

[0055] A "fusion" or "chimeric" molecule comprises a first amino acid sequence linked to a second amino acid sequence with which it is not naturally linked in nature. The amino acid sequences which normally exist in separate proteins can be brought together in the fusion polypeptide, or the amino acid sequences which normally exist in the same protein can be placed in a new arrangement in the fusion polypeptide, *e.g.*, fusion of a Factor VIII domain of the invention with an immunoglobulin Fc domain. A fusion protein is created, for example, by chemical synthesis, or by creating and translating a polynucleotide in which the peptide regions are encoded in the desired relationship. A chimeric protein can further comprises a second amino acid sequence associated with the first amino acid sequence by a covalent, non-peptide bond or a non-covalent bond.

[0056] As used herein, the term "half-life" refers to a biological half-life of a particular polypeptide *in vivo*. Half-life may be represented by the time required for half the quantity administered to a subject to be cleared from the circulation and/or other tissues in the animal. When a clearance curve of a given polypeptide is constructed as a function of time, the curve is usually biphasic with a rapid α-phase and longer β-phase. The α-phase typically represents an equilibration of the administered polypeptide between the intra- and extra-vascular space and is, in part, determined by the size of the polypeptide. The β-phase typically represents the catabolism of the polypeptide in the intravascular space. In some embodiments, chimeric molecule of the invention are monophasic, and thus do not have an alpha phase, but just the single beta phase. Therefore, in certain embodiments, the term half-life as used herein refers to the half-life of the polypeptide in the β-phase. The typical β phase half-life of a human antibody in humans is 21 days.

[0057] The term "heterologous" as applied to a polynucleotide or a polypeptide, means that the polynucleotide or polypeptide is derived from a distinct entity from that of the entity to which it is being compared. Therefore, a heterologous polypeptide linked to a VWF protein means a polypeptide chain that is linked to a VWF protein and is not a naturally occurring part of the VWF protein. For instance, a heterologous polynucleotide or antigen can be derived from a different species, different cell type of an individual, or the same or different type of cell of distinct individuals.

[0058] The term "linked," "fused," or "connected" as used herein refers to a first amino acid sequence or nucleotide sequence joined to a second amino acid sequence or nucleotide sequence (*e.g.*, via a peptide bond or a phosphodiester bond, respectively). The term "covalently linked" or "covalent linkage" refers to a covalent bond, *e.g.*, a disulfide bond, a peptide bond, or one or more amino acids, *e.g.*, a linker, between the two moieties that are linked together. The first amino acid or nucleotide sequence can be directly joined to the second amino acid or nucleotide sequence or alternatively an intervening sequence can join the first sequence to the second sequence. The term "linked," "fused," or "connected" means not only a fusion of a first amino acid sequence to a second amino acid sequence at the C-terminus or the N-terminus, but also includes insertion of the whole first amino acid sequence (or the second amino acid sequence) into any two amino acids in the second amino acid sequence (or the first amino acid sequence, respectively). In one embodiment, the first amino acid sequence can be joined to a second amino acid sequence by a peptide bond or a linker. The first nucleotide sequence can be joined to a second nucleotide sequence by a phosphodiester bond or a linker. The linker can be a peptide or a polypeptide (for polypeptide chains) or a nucleotide or a nucleotide chain (for nucleotide chains) or any chemical moiety (for both polypeptide and polynucleotide chains). The covalent linkage is sometimes indicated as (-) or hyphen.

[0059] As used herein the term "associated with" refers to a covalent or non-covalent bond formed between a first amino acid chain and a second amino acid chain. In one embodiment, the term "associated with" means a covalent, non-peptide bond or a non-covalent bond. In some embodiments this association is indicated by a colon, *i.e.*, (:). In another embodiment, it means a covalent bond except a peptide bond. In other embodiments, the term "covalently associated" as used herein means an association between two moieties by a covalent bond, *e.g.*, a disulfide bond, a peptide bond, or one or

more amino acids (*e.g.*, a linker). For example, the amino acid cysteine comprises a thiol group that can form a disulfide bond or bridge with a thiol group on a second cysteine residue. In most naturally occurring IgG molecules, the CH1 and CL regions are associated by a disulfide bond and the two heavy chains are associated by two disulfide bonds at positions corresponding to 239 and 242 using the Kabat numbering system (position 226 or 229, EU numbering system). Examples of covalent bonds include, but are not limited to, a peptide bond, a metal bond, a hydrogen bond, a disulfide bond, a sigma bond, a pi bond, a delta bond, a glycosidic bond, an agnostic bond, a bent bond, a dipolar bond, a Pi backbond, a double bond, a triple bond, a quadruple bond, a quintuple bond, a sextuple bond, conjugation, hyperconjugation, aromaticity, hapticity, or antibonding. Non-limiting examples of non-covalent bond include an ionic bond (*e.g.*, cation-pi bond or salt bond), a metal bond, an hydrogen bond (*e.g.*, dihydrogen bond, dihydrogen complex, low-barrier hydrogen bond, or symmetric hydrogen bond), van der Walls force, London dispersion force, a mechanical bond, a halogen bond, aurophilicity, intercalation, stacking, entropic force, or chemical polarity.

[0060] As used herein, the term "cleavage site" or "enzymatic cleavage site" refers to a site recognized by an enzyme. In one embodiment, a polypeptide has an enzymatic cleavage site cleaved by an enzyme that is activated during the clotting cascade, such that cleavage of such sites occurs at the site of clot formation. In another embodiment, a FVIII linker connecting a FVIII protein and a second heterologous moiety can comprise a cleavage site. Exemplary such sites include *e.g.*, those recognized by thrombin, Factor XIa or Factor Xa. Exemplary FXIa cleavage sites include, *e.g.* TQSFNDFTR (SEQ ID NO: 23) and SVSQTSKLTR (SEQ ID NO: 24). Exemplary thrombin cleavage sites include, *e.g.* DFLAEGGGVR (SEQ ID NO: 25), TTKIKPR (SEQ ID NO: 26), LVPRG (SEQ ID NO: 27) and ALRPR (SEQ ID NO: 50). Other enzymatic cleavage sites are known in the art. A cleavage site that can be cleaved by thrombin is referred to herein as "thrombin cleavage site."

[0061] As used herein, the term "processing site" or "intracellular processing site" refers to a type of enzymatic cleavage site in a polypeptide which is the target for enzymes that function after translation of the polypeptide. In one embodiment, such enzymes function during transport from the Golgi lumen to the trans-Golgi compartment. Intracellular processing enzymes cleave polypeptides prior to secretion of the protein from the cell.

Examples of such processing sites include, *e.g.*, those targeted by the PACE/furin (where PACE is an acronym for Paired basic Amino acid Cleaving Enzyme) family of endopeptidases. These enzymes are localized to the Golgi membrane and cleave proteins on the carboxy terminal side of the sequence motif Arg-[any residue]-(Lys or Arg)-Arg. As used herein the "furin" family of enzymes includes, *e.g.*, PCSK1 (also known as PC1/Pc3), PCSK2 (also known as PC2), PCSK3 (also known as furin or PACE), PCSK4 (also known as PC4), PCSK5 (also known as PC5 or PC6), PCSK6 (also known as PACE4), or PCSK7 (also known as PC7/LPC, PC8, or SPC7). Other processing sites are known in the art. The term "processable linker" referred to herein means a linker comprising an intracellular processing site.

[0062] The term "Furin" refers to the enzymes corresponding to EC No. 3.4.21.75. Furin is subtilisin-like proprotein convertase, which is also known as PACE (Paired basic Amino acid Cleaving Enzyme). Furin deletes sections of inactive precursor proteins to convert them into biologically active proteins. During its intracellular transport, pro-peptide is cleaved from mature VWF molecule by a Furin enzyme in the Golgi.

[0063] In constructs that include more than one processing or cleavage site, it will be understood that such sites may be the same or different.

[0064] Hemostatic disorder, as used herein, means a genetically inherited or acquired condition characterized by a tendency to hemorrhage, either spontaneously or as a result of trauma, due to an impaired ability or inability to form a fibrin clot. Examples of such disorders include the hemophilias. The three main forms are hemophilia A (factor VIII deficiency), hemophilia B (factor IX deficiency or "Christmas disease") and hemophilia C (factor XI deficiency, mild bleeding tendency). Other hemostatic disorders include, *e.g.*,Von Willebrand disease, Factor XI deficiency (PTA deficiency), Factor XII deficiency, deficiencies or structural abnormalities in fibrinogen, prothrombin, Factor V, Factor VII, Factor X or factor XIII, Bernard-Soulier syndrome, which is a defect or deficiency in GPIb. GPIb, the receptor for VWF, can be defective and lead to lack of primary clot formation (primary hemostasis) and increased bleeding tendency), and thrombasthenia of Glanzman and Naegeli (Glanzmann thrombasthenia). In liver failure (acute and chronic forms), there is insufficient production of coagulation factors by the liver; this may increase bleeding risk.

[0065] The chimeric molecules of the invention can be used prophylactically. As used herein the term "prophylactic treatment" refers to the administration of a molecule prior to a bleeding episode. In one embodiment, the subject in need of a general hemostatic agent is undergoing, or is about to undergo, surgery. The chimeric protein of the invention can be administered prior to or after surgery as a prophylactic. The chimeric protein of the invention can be administered during or after surgery to control an acute bleeding episode. The surgery can include, but is not limited to, liver transplantation, liver resection, dental procedures, or stem cell transplantation.

[0066] The chimeric molecule of the invention is also used for on-demand (also referred to as "episodic") treatment. The term "on-demand treatment" or "episodic treatment" refers to the administration of a chimeric molecule in response to symptoms of a bleeding episode or before an activity that may cause bleeding. In one aspect, the on-demand (episodic) treatment can be given to a subject when bleeding starts, such as after an injury, or when bleeding is expected, such as before surgery. In another aspect, the on-demand treatment can be given prior to activities that increase the risk of bleeding, such as contact sports.

[0067] As used herein the term "acute bleeding" refers to a bleeding episode regardless of the underlying cause. For example, a subject may have trauma, uremia, a hereditary bleeding disorder (*e.g.*, factor VII deficiency) a platelet disorder, or resistance owing to the development of antibodies to clotting factors.

[0068] Treat, treatment, treating, as used herein refers to, *e.g.*, the reduction in severity of a disease or condition; the reduction in the duration of a disease course; the amelioration of one or more symptoms associated with a disease or condition; the provision of beneficial effects to a subject with a disease or condition, without necessarily curing the disease or condition, or the prophylaxis of one or more symptoms associated with a disease or condition. In one embodiment, the term "treating" or "treatment" means maintaining a FVIII trough level at least about 1 IU/dL, 2 IU/dL, 3 IU/dL, 4 IU/dL, 5 IU/dL, 6 IU/dL, 7 IU/dL, 8 IU/dL, 9 IU/dL, 10 IU/dL, 11 IU/dL, 12 IU/dL, 13 IU/dL, 14 IU/dL, 15 IU/dL, 16 IU/dL, 17 IU/dL, 18 IU/dL, 19 IU/dL, or 20 IU/dL in a subject by administering a chimeric molecule of the invention. In another embodiment, treating or treatment means maintaining a FVIII trough level between about 1 and about 20 IU/dL, about 2 and about 20 IU/dL, about 3 and about 20 IU/dL, about 4 and about 20 IU/dL,

about 5 and about 20 IU/dL, about 6 and about 20 IU/dL, about 7 and about 20 IU/dL, about 8 and about 20 IU/dL, about 9 and about 20 IU/dL, or about 10 and about 20 IU/dL. Treatment or treating of a disease or condition can also include maintaining FVIII activity in a subject at a level comparable to at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20% of the FVIII activity in a non-hemophiliac subject. The minimum trough level required for treatment can be measured by one or more known methods and can be adjusted (increased or decreased) for each person.

II. Chimeric Molecules

[0069] A chimeric molecule of the invention is designed to improve release of a VWF protein or FVIII protein from another moiety that the VWF protein or FVIII protein is fused to. The invention provides a thrombin cleavable linker that can be cleaved fast and efficient at the site of injury. Examples of the heterologous moieties that a VWF protein (or FVIII protein) can be fused to include, *e.g.*, a FVIII protein (VWF protein), an immunoglobulin constant region or a portion thereof, transferrin or a fragment thereof, albumin or a fragment thereof, an albumin binding moiety, the C-terminal peptide (CTP) of the β subunit of human chorionic gonadotropin, a HAP sequence, a PAS sequence, or any combination thereof. Non-limiting examples of the non-polypeptide moiety includes polyethylene glycol (PEG), polysialic acid, hydroxyethyl starch (HES), a derivative thereof, or any combination thereof. Other such moieties useful in present invention are known in the art.

II.A. VWF Linker or FVIII Linker

[0070] The present invention provides a thrombin cleavable VWF linker or FVIII linker useful for fusing a VWF protein with a heterologous moiety or a FVIII protein with a heterologous moiety, respectively, wherein the linker comprises an a1 region of FVIII. The present invention also provides a thrombin cleavable VWF linker or FVIII linker useful for fusing a VWF protein or FVIII protein with a heterologous moiety, respectively, wherein the linker comprises an a2 region of FVIII. Also provided is a thrombin cleavable VWF linker or FVIII linker useful for fusing a VWF protein or a FVIII protein with a heterologous moiety, respectively, wherein the linker comprises an a3 region of FVIII. The instant disclosure also includes a thrombin cleavable VWF linker

or FVIII linker useful for fusing a VWF protein or a FVII protein with a heterologous moiety, respectively, wherein the linker comprises a thrombin cleavage site which comprises X-V-P-R (SEQ ID NO: 3) and a PAR1 exosite interaction motif, wherein X is an aliphatic amino acid.

[0071] In one embodiment, the VWF linker or FVIII linker comprises an a1 region which comprises an amino acid sequence at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% identical to Met337 to Arg372 corresponding to full-length mature FVIII, wherein the a1 region is capable of being cleaved by thrombin. In another embodiment, the VWF linker or FVIII linker comprises an a1 region which comprises an amino acid sequence at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% identical to amino acids 337 to 374 corresponding to full-length mature FVIII, wherein the a1 region is capable of being cleaved by thrombin. In other embodiments, the VWF linker or FVIII linker further comprises additional amino acids, *e.g.*, one, two, three, four, five, ten, or more. In a particular embodiment, the VWF linker or FVIII linker comprises ISMKNNEEAEDYDDDLTDSEMDVVRFDDDNSPSFIQIRSV (SEQ ID NO: 28).

[0072] In some embodiments, the VWF linker or FVIII linker comprises an a2 region which comprises an amino acid sequence at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% identical to Glu720 to Arg740 corresponding to full-length mature FVIII, wherein the a2 region is capable of being cleaved by thrombin. In other embodiments, the VWF lilnker or FVIII linker comprises an a2 region which comprises an amino acid sequence at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% identical to amino acids 712 to 743 corresponding to full-length mature FVIII. In still other embodiments, the VWF linker or FVIII linker further comprises additional amino acids, *e.g.*, one, two, three, four, five, ten, or more. In a particular embodiment, the VWF linker or FVIII linker comprises ISDKNTGDYYEDSYEDISAYLLSKNNAIEPRSFS (SEQ ID NO: 29).

[0073] In certain embodiments, the VWF linker or FVIII linker comprises an a3 region which comprises an amino acid sequence at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% identical to Glu1649 to Arg1689 corresponding to full-length mature FVIII, wherein the a3 region is capable of

being cleaved by thrombin. In some embodiments, the VWF linker or FVIII linker comprises an a3 region which comprises an amino acid sequence at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% identical to amino acids 1649 to 1692 corresponding to full-length mature FVIII, wherein the a3 region is capable of being cleaved by thrombin. In other embodiments, the VWF linker or FVIII linker further comprises additional amino acids, *e.g.*, one, two, three, four, five, ten, or more. In a specific embodiment, a VWF linker or FVIII linker comprises ISEITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQSP**R**SFQ (SEQ ID NO: 30).

[0074] In other embodiments, the VWF linker or FVIII linker comprises a thrombin cleavage site comprising X-V-P-R (SEQ ID NO: 3) and a PAR1 exosite interaction motif and wherein the PAR1 exosite interaction motif comprises S-F-L-L-R-N (SEQ ID NO: 4). In some embodiments, the PAR1 exosite interaction motif further comprises an amino acid sequence selected from P, P-N, P-N-D, P-N-D-K (SEQ ID NO: 5), P-N-D-K-Y (SEQ ID NO: 6), P-N-D-K-Y-E (SEQ ID NO: 7), P-N-D-K-Y-E-P (SEQ ID NO: 8), P-N-D-K-Y-E-P-F (SEQ ID NO: 9), P-N-D-K-Y-E-P-F-W (SEQ ID NO: 10), P-N-D-K-Y-E-P-F-W-E (SEQ ID NO: 11), P-N-D-K-Y-E-P-F-W-E-D (SEQ ID NO: 12), P-N-D-K-Y-E-P-F-W-E-D-E (SEQ ID NO: 13), P-N-D-K-Y-E-P-F-W-E-D-E-E (SEQ ID NO: 14), P-N-D-K-Y-E-P-F-W-E-D-E-E-S (SEQ ID NO: 20), or any combination thereof. In other embodiments, the aliphatic amino acid for the thrombin cleavage site comprising X-V-P-R is selected from Glycine, Alanine, Valine, Leucine, or Isoleucine. In a specific embodiment, the thrombin cleavage site comprises L-V-P-R. In some embodiments, thrombin cleaves the VWF linker or FVIII linker faster than thrombin would cleave the thrombin cleavage site (*e.g.*, L-V-P-R) if the thrombin cleavage site (L-V-P-R) were substituted for the VWF linker or FVIII linker (*i.e.*, without the PAR1 exosite interaction motif). In some embodiments, thrombin cleaves the VWF linker or FVIII linker at least about 10 times, at least about 20 times, at least about 30 times, at least about 40 times, at least about 50 times, at least about 60 times, at least about 70 times, at least about 80 times, at least about 90 times or at least about 100 times faster than thrombin would cleave the thrombin cleavage site (*e.g.*, L-V-P-R) if the thrombin cleavage site (*e.g.*, L-V-P-R) were substituted for the VWF linker or FVIII linker.

[0075] In some embodiments, a VWF linker or FVIII linker comprising (i) an a1 region, (ii) an a2 region, (iii) an a3 region or (iv) a thrombin cleavage site X-V-P-R and a PAR1 exosite interaction motif further comprises one or more amino acids having a length of at least about 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1200, 1400, 1600, 1800, or 2000 amino acids. In one embodiment, the one or more amino acids comprise a gly peptide. In another embodiment, the one or more amino acids comprise GlyGly. In other embodiments, the one or more amino acids comprise IleSer. In still other embodiments, the one or more amino acids comprise a gly/ser peptide. In yet other embodiments, the one or more amino acids comprise a gly/ser peptide having a formula of $(Gly_4Ser)n$ or $S(Gly_4Ser)n$, wherein n is a positive integer selected from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, or 100. In some embodiments, the one or more amino acids comprise $(Gly_4Ser)_3$ (SEQ ID NO: 48) or $(Gly_4Ser)_4$ (SEQ ID NO: 49).

[0076] In one embodiment, either one of the FVIII linker and the VWF linker is present in the chimeric molecule. In another embodiment, both of the FVIII linker and the VWF linker present and are the same. In other embodiments, both of the FVIII linker and the VWF linker are present, but are different.

II.B. VWF Protein

[0077] VWF (also known as F8VWF) is a large multimeric glycoprotein present in blood plasma and produced constitutively in endothelium (in the Weibel-Palade bodies), megakaryocytes (α-granules of platelets), and subendothelian connective tissue. The basic VWF monomer is a 2813 amino acid protein. Every monomer contains a number of specific domains with a specific function, the D'/D3 domain (which binds to Factor VIII), the A1 domain (which binds to platelet GPIb-receptor, heparin, and/or possibly collagen), the A3 domain (which binds to collagen), the C1 domain (in which the RGD domain binds to platelet integrin αIIbβ3 when this is activated), and the "cysteine knot" domain at the C-terminal end of the protein (which VWF shares with platelet-derived growth factor (PDGF), transforming growth factor-β (TGFβ) and β-human chorionic gonadotropin (βHCG)).

[0078] The term "a VWF protein" as used herein includes, but is not limited to, full-length VWF protein or functional VWF fragments comprising a D' domain and a D3 domain, which are capable of inhibiting binding of endogenous VWF to FVIII. In one embodiment, a VWF protein binds to FVIII. In another embodiment, the VWF protein blocks the VWF binding site on FVIII, thereby inhibiting interaction of FVIII with endogenous VWF. In other embodiments, a VWF protein is not cleared by a VWF clearance pathway. The VWF proteins include derivatives, variants, mutants, or analogues that retain these activities of VWF.

[0079] The 2813 monomer amino acid sequence for human VWF is reported as Accession Number _NP_000543.2__ in Genbank. The nucleotide sequence encoding the human VWF is reported as Accession Number __NM_000552.3_ in Genbank. The nucleotide sequence of human VWF is designated as SEQ ID NO: 1. SEQ ID NO: 2 is the amino acid sequence encoded by SEQ ID NO: 1. Each domain of VWF is listed in Table 1.

TABLE 1. VWF Sequences

VWF domains	Amino acid Sequence
VWF Signal Peptide (Amino acids 1 to 22 of SEQ ID NO: 2)	1 MIPARFAGVL LALALILPGT LC 22
VWF D1D2 region (Amino acids 23 to 763 of SEQ ID NO: 2)	23 AEGTRGRS STARCSLFGS DFVNTFDGSM 51 YSFAGYCSYL LAGGCQKRSF SIIGDFQNGK RVSLSVYLGE FFDIHLFVNG 101 TVTQGDQRVS MPYASKGLYL ETEAGYYKLS GEAYGFVARI DGSGNFQVLL 151 SDRYFNKTCG LCGNFNIFAE DDFMTQEGTL TSDPYDFANS WALSSGEQWC 201 ERASPPSSSC NISSGEMQKG LWEQCQLLKS TSVFARCHPL VDPEPFVALC 251 EKTLCECAGG LECACPALLE YARTCAQEGM VLYGWTDHSA CSPVCPAGME 301 YRQCVSPCAR TCQSLHINEM CQERCVDGCS CPEGQLLDEG LCVESTECPC 351 VHSGKRYPPG TSLSRDCNTC ICRNSQWICS NEECPGECLV TGQSHFKSFD 401 NRYFTFSGIC QYLLARDCQD HSFSIVIETV QCADDRDAVC TRSVTVRLPG 451 LHNSLVKLKH GAGVAMDGQD IQLPLLKGDL RIQHTVTASV RLSYGEDLQM 501 DWDGRGRLLV KLSPVYAGKT CGLCGNYNGN QGDDFLTPSG LAEPRVEDFG 551 NAWKLHGDCQ DLQKQHSDPC ALNPRMTRFS EEACAVLTSP TFEACHRAVS

	601 PLPYLRNCRY DVCSCSDGRE CLCGALASYA AACAGRGVRV AWREPGRCEL 651 NCPKGQVYLQ CGTPCNLTCR SLSYPDEECN EACLEGCFCP PGLYMDERGD 701 CVPKAQCPCY YDGEIFQPED IFSDHHTMCY CEDGFMHCTM SGVPGSLLPD 751 AVLSSPLSHR SKR 763
VWF D' Domain	764 SLSCRPP MVKLVCPADN LRAEGLECTK TCQNYDLECM 801 SMGCVSGCLC PPGMVRHENR CVALERCPCF HQGKEYAPGE TVKIGCNTCV 851 CRDRKWNCTD HVCDAT 866
VWF D3 Domain	867 CSTI GMAHYLTFDG LKYLFPGECQ YVLVQDYCGS 901 NPGTFRILVG NKGCSHPSVK CKKRVTILVE GGEIELFDGE VNVKRPMKDE 951 THFEVVESGR YIILLLGKAL SVVWDRHLSI SVVLKQTYQE KVCGLCGNFD 1001 GIQNNDLTSS NLQVEEDPVD FGNSWKVSSQ CADTRKVPLD SSPATCHNNI 1051 MKQTMVDSSC RILTSDVFQD CNKLVDPEPY LDVCIYDTCS CESIGDCACF 1101 CDTIAAYAHV CAQHGKVVTW RTATLCPQSC EERNLRENGY ECEWRYNSCA 1151 PACQVTCQHP EPLACPVQCV EGCHAHCPPG KILDELLQTC VDPEDCPVCE 1201 VAGRRFASGK KVTLNPSDPE HCQICHCDVV NLTCEACQEP 1240
VWF A1 Domain	1241 GGLVVPPTDA 1251 PVSPTTLYVE DISEPPLHDF YCSRLLDLVF LLDGSSRLSE AEFEVLKAFV 1301 VDMMERLRIS QKWVRVAVVE YHDGSHAYIG LKDRKRPSEL RRIASQVKYA 1351 GSQVASTSEV LKYTLFQIFS KIDRPEASRI ALLLMASQEP QRMSRNFVRY 1401 VQGLKKKKVI VIPVGIGPHA NLKQIRLIEK QAPENKAFVL SSVDELEQQR 1451 DEIVSYLCDL APEAPPPTLP PDMAQVTVG 1479
	1480 P GLLGVSTLGP KRNSMVLDVA 1501 FVLEGSDKIG EADFNRSKEF MEEVIQRMDV GQDSIHVTVL QYSYMVTVEY 1551 PFSEAQSKGD ILQRVREIRY QGGNRTNTGL ALRYLSDHSF LVSQGDREQA 1600 1601 PNLVYMVTGN PASDEIKRLP GDIQVVPIGV GPNANVQELE RIGWPNAPIL 1651 IQDFETLPRE APDLVLQRCC SGEGLQIPTL SPAPDCSQPL DVILLLDGSS 1701 SFPASYFDEM KSFAKAFISK ANIGPRLTQV SVLQYGSITT IDVPWNVVPE 1751 KAHLLSLVDV MQREGGPSQI GDALGFAVRY LTSEMHGARP GASKAVVILV 1801 TDVSVDSVDA AADAARSNRV TVFPIGIGDR YDAAQLRILA GPAGDSNVVK 1851 LQRIEDLPTM VTLGNSFLHK LCSGFVRICM DEDGNEKRPG DVWTLPDQCH 1901 TVTCQPDGQT LLKSHRVNCD RGLRPSCPNS QSPVKVEETC

	GCRWTCPCVC 1951 TGSSTRHIVT FDGQNFKLTG SCSYVLFQNK EQDLEVILHN GACSPGARQG 2001 CMKSIEVKHS ALSVEXHSDM EVTVNGRLVS VPYVGGNMEV NVYGAIMHEV 2051 RFNHLGHIFT FTPQNNEFQL QLSPKTFASK TYGLCGICDE NGANDFMLRD 2101 GTVTTDWKTL VQEWTVQRPG QTCQPILEEQ CLVPDSSHCQ VLLLPLFAEC 2151 HKVLAPATFY AICQQDSCHQ EQVCEVIASY AHLCRTNGVC VDWRTPDFCA 2201 MSCPPSLVYN HCEHGCPRHC DGNVSSCGDH PSEGCFCPPD KVMLEGSCVP 2251 EEACTQCIGE DGVQHQFLEA WVPDHQPCQI CTCLSGRKVN CTTQPCPTAK 2301 APTCGLCEVA RLRQNADQCC PEYECVCDPV SCDLPPVPHC ERGLQPTLTN 2351 PGECRPNFTC ACRKEECKRV SPPSCPPHRL PTLRKTQCCD EYECACNCVN 2401 STVSCPLGYL ASTATNDCGC TTTTCLPDKV CVHRSTIYPV GQFWEEGCDV 2451 CTCTDMEDAV MGLRVAQCSQ KPCEDSCRSG FTYVLHEGEC CGRCLPSACE 2501 VVTGSPRGDS QSSWKSVGSQ WASPENPCLI NECVRVKEEV FIQQRNVSCP 2551 QLEVPVCPSG FQLSCKTSAC CPSCRCERME ACMLNGTVIG PGKTVMIDVC 2601 TTCRCMVQVG VISGFKLECR KTTCNPCPLG YKEENNTGEC CGRCLPTACT 2651 IQLRGGQIMT LKRDETLQDG CDTHFCKVNE RGEYFWEKRV TGCPPFDEHK 2701 CLAEGGKIMK IPGTCCDTCE EPECNDITAR LQYVKVGSCK SEVEVDIHYC 2751 QGKCASKAMY SIDINDVQDQ CSCCSPTRTE PMQVALHCTN GSVVYHEVLN 2801 AMECKCSPRK CSK
	Nucleotide Sequence (SEQ ID NO: 1)
Full-length VWF	1 ATGATTCCTG CCAGATTTGC CGGGGTGCTG CTTGCTCTGG CCCTCATTTT 51 GCCAGGGACC CTTTGTGCAG AAGGAACTCG CGGCAGGTCA TCCACGGCCC 101 GATGCAGCCT TTTCGGAAGT GACTTCGTCA ACACCTTTGA TGGGAGCATG 151 TACAGCTTTG CGGGATACTG CAGTTACCTC CTGGCAGGGG GCTGCCAGAA 201 ACGCTCCTTC TCGATTATTG GGGACTTCCA GAATGGCAAG AGAGTGAGCC 251 TCTCCGTGTA TCTTGGGGAA TTTTTTGACA TCCATTTGTT TGTCAATGGT 301 ACCGTGACAC AGGGGGACCA AAGAGTCTCC ATGCCCTATG CCTCCAAAGG 351 GCTGTATCTA GAAACTGAGG CTGGGTACTA CAAGCTGTCC GGTGAGGCCT 401 ATGGCTTTGT GGCCAGGATC GATGGCAGCG GCAACTTTCA AGTCCTGCTG 451 TCAGACAGAT ACTTCAACAA GACCTGCGGG CTGTGTGGCA ACTTTAACAT 501 CTTTGCTGAA GATGACTTTA TGACCCAAGA

AGGGACCTTG ACCTCGGACC
551 CTTATGACTT TGCCAACTCA TGGGCTCTGA
GCAGTGGAGA ACAGTGGTGT
601 GAACGGGCAT CTCCTCCCAG CAGCTCATGC
AACATCTCCT CTGGGGAAAT
651 GCAGAAGGGC CTGTGGGAGC AGTGCCAGCT
TCTGAAGAGC ACCTCGGTGT
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751 GAGAAGACTT TGTGTGAGTG TGCTGGGGGG
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TGTGCCCTGC TGGTATGGAG
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[0080] The VWF protein as used herein can comprise a D' domain and a D3 domain of VWF, wherein the VWF protein binds to FVIII and inhibits binding of endogenous VWF (full-length VWF) to FVIII. The VWF protein comprising the D' domain and the D3 domain can further comprise a VWF domain selected from an A1 domain, an A2 domain, an A3 domain, a D1 domain, a D2 domain, a D4 domain, a B1 domain, a B2 domain, a B3 domain, a C1 domain, a C2 domain, a CK domain, one or more fragments thereof, or any combination thereof. In one embodiment, a VWF protein comprises, consists essentially of, or consists of: (1) the D' and D3 domains of VWF or fragments thereof; (2) the D1, D', and D3 domains of VWF or fragments thereof; (3) the D2, D', and D3 domains of VWF or fragments thereof; (4) the D1, D2, D', and D3 domains of VWF or fragments thereof; or (5) the D1, D2, D', D3, or A1 domains of VWF or fragments thereof. The VWF protein described herein does not contain a VWF clearance receptor binding site. The VWF protein of the present invention can comprise any other sequences linked to or fused to the VWF protein. For example, a VWF protein described herein can further comprise a signal peptide.

[0081] In one embodiment, a VWF protein binds to or is associated with a FVIII protein. By binding to or being associated with a FVIII protein, the VWF protein of the invention can protect FVIII from protease cleavage and FVIII activation, stabilizes the heavy chain

and light chain of FVIII, and prevents clearance of FVIII by scavenger receptors. In another embodiment, the VWF protein binds to or associates with a FVIII protein and blocks or prevents binding of the FVIII protein to phospholipid and activated Protein C. By preventing or inhibiting binding of the FVIII protein with endogenous, full-length VWF, the VWF protein of the invention reduces the clearance of FVIII by endogenous VWF clearance receptors and thus extends half-life of the FVIII protein. The half-life extension of a FVIII protein is thus due to the association of the FVIII protein with a VWF protein lacking a VWF clearance receptor binding site and thereby shielding and/or protecting of the FVIII protein from endogenous VWF which contains the VWF clearance receptor binding site. The FVIII protein bound to or protected by the VWF protein can also allow recycling of a FVIII protein. By eliminating the VWF clearance pathway receptor binding sites in the full length VWF molecule, the FVIII/VWF heterodimers of the invention are shielded from the VWF clearance pathway, further extending FVIII half-life.

[0082] In one embodiment, a VWF protein of the present invention comprises a D' domain and a D3 domain of VWF, wherein the D' domain is at least about 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 764 to 866 of SEQ ID NO: 2, wherein the VWF protein prevents binding of endogenous VWF to FVIII. In another embodiment, a VWF protein comprises a D' domain and a D3 domain of VWF, wherein the D3 domain is at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 867 to 1240 of SEQ ID NO: 2, wherein the VWF protein prevents binding of endogenous VWF to FVIII. In some embodiments, a VWF protein described herein comprises, consists essentially of, or consists of a D' domain and a D3 domain of VWF, which are at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 764 to 1240 of SEQ ID NO: 2, wherein the VWF protein prevents binding of endogenous VWF to FVIII. In other embodiments, a VWF protein comprises, consists essentially of, or consists of the D1, D2, D', and D3 domains at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 23 to 1240 of SEQ ID NO: 2, wherein the VWF protein prevents binding of endogenous VWF to FVIII. In still other embodiments, the VWF protein further comprises a signal peptide operably linked thereto.

[0083] In some embodiments, a VWF protein of the invention consists essentially of or consists of (1) the D'D3 domain, the D1D'D3 domain, D2D'D3 domain, or D1D2D'D3 domain and (2) an additional VWF sequence up to about 10 amino acids (*e.g.*, any sequences from amino acids 764 to 1240 of SEQ ID NO: 2 to amino acids 764 to 1250 of SEQ ID NO: 2), up to about 15 amino acids (*e.g.*, any sequences from amino acids 764 to 1240 of SEQ ID NO: 2 to amino acids 764 to 1255 of SEQ ID NO: 2), up to about 20 amino acids (*e.g.*, any sequences from amino acids 764 to 1240 of SEQ ID NO: 2 to amino acids 764 to 1260 of SEQ ID NO: 2), up to about 25 amino acids (*e.g.*, any sequences from amino acids 764 to 1240 of SEQ ID NO: 2 to amino acids 764 to 1265 of SEQ ID NO: 2), or up to about 30 amino acids (*e.g.*, any sequences from amino acids 764 to 1240 of SEQ ID NO: 2 to amino acids 764 to 1260 of SEQ ID NO: 2). In a particular embodiment, the VWF protein comprising or consisting essentially of a D' domain and a D3 domain is neither amino acids 764 to 1274 of SEQ ID NO: 2 nor the full-length mature VWF. In some embodiments, the D1D2 domain is expressed in trans with the D'D3 domain. In some embodiments, the D1D2 domain is expressed in cis with the D'D3 domain.

[0084] In other embodiments, a VWF protein comprising D'D3 domains linked to D1D2 domains further comprises an intracellular processing site, *e.g.*, (a processing site by PACE (furin) or PC5), allowing cleavage of the D1D2 domains from the D'D3 domains upon expression. Non-limiting examples of the intracellular processing sites are disclosed elsewhere herein.

[0085] In yet other embodiments, a VWF protein comprises a D' domain and a D3 domain, but does not comprise an amino acid sequence selected from (1) amino acids 1241 to 2813 of SEQ ID NO: 2, (2) amino acids 1270 to amino acids 2813 of SEQ ID NO: 2, (3) amino acids 1271 to amino acids 2813 of SEQ ID NO: 2, (4) amino acids 1272 to amino acids 2813 of SEQ ID NO: 2, (5) amino acids 1273 to amino acids 2813 of SEQ ID NO: 2, (6) amino acids 1274 to amino acids 2813 of SEQ ID NO: 2, or any combination thereof.

[0086] In still other embodiments, a VWF protein of the present invention comprises, consists essentially of, or consists of an amino acid sequence corresponding to a D' domain, D3 domain, and A1 domain, wherein the amino acid sequence is at least 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino

acid 764 to 1479 of SEQ ID NO: 2, wherein the VWF protein prevents binding of endogenous VWF to FVIII. In a particular embodiment, the VWF protein is not amino acids 764 to 1274 of SEQ ID NO: 2.

[0087] In some embodiments, a VWF protein of the invention comprises a D' domain and a D3 domain, but does not comprise at least one VWF domain selected from (1) an A1 domain, (2) an A2 domain, (3) an A3 domain, (4) a D4 domain, (5) a B1 domain, (6) a B2 domain, (7) a B3 domain, (8) a C1 domain, (9) a C2 domain, (10) a CK domain, (11) a CK domain and C2 domain, (12) a CK domain, a C2 domain, and a C1 domain, (13) a CK domain, a C2 domain, a C1 domain, a B3 domain, (14) a CK domain, a C2 domain, a C1 domain, a B3 domain, a B2 domain, (15) a CK domain, a C2 domain, a C1 domain, a B3 domain, a B2 domain, and a B1 domain, (16) a CK domain, a C2 domain, a C1 domain, a B3 domain, a B2 domain, a B1 domain, and a D4 domain, (17) a CK domain, a C2 domain, a C1 domain, a B3 domain, a B2 domain, a B1 domain, a D4 domain, and an A3 domain, (18) a CK domain, a C2 domain, a C1 domain, a B3 domain, a B2 domain, a B1 domain, a D4 domain, an A3 domain, and an A2 domain, (19) a CK domain, a C2 domain, a C1 domain, a B3 domain, a B2 domain, a B1 domain, a D4 domain, an A3 domain, an A2 domain, and an A1 domain, or (20) any combination thereof.

[0088] In yet other embodiments, a VWF protein comprises D'D3 domains and one or more domains or modules. Examples of such domains or modules include, but are not limited to, the domains and modules disclosed in Zhour et al., Blood published online April 6, 2012: DOI 10.1182/blood-2012-01-405134. For example, the VWF protein can comprise D'D3 domain and one or more domains or modules selected from A1 domain, A2 domain, A3 domain, D4N module, VWD4 module, C8-4 module, TIL-4 module, C1 module, C2 module, C3 module, C4 module, C5 module, C5 module, C6 module, or any combination thereof.

[0089] In certain embodiments, a VWF protein of the invention forms a multimer, *e.g.*, dimer, trimer, tetramer, pentamer, hexamer, heptamer, or the higher order multimers. In other embodiments, the VWF protein is a monomer having only one VWF protein. In some embodiments, the VWF protein of the present invention can have one or more amino acid substitutions, deletions, additions, or modifications. In one embodiment, the VWF protein can include amino acid substitutions, deletions, additions, or modifications such that the VWF protein is not capable of forming a disulfide bond or forming a dimer

or a multimer. In another embodiment, the amino acid substitution is within the D' domain and the D3 domain. In a particular embodiment, a VWF protein of the invention contains at least one amino acid substitution at a residue corresponding to residue 1099, residue 1142, or both residues 1099 and 1142 of SEQ ID NO: 2. The at least one amino acid substitution can be any amino acids that are not occurring naturally in the wild type VWF. For example, the amino acid substitution can be any amino acids other than cysteine, *e.g.*, Isoleucine, Alanine, Leucine, Asparagine, Lysine, Aspartic acid, Methionine, Phenylalanine, Glutamic acid, Threonine, Glutamine, Tryptophan, Glycine, Valine, Proline, Serine, Tyrosine, Arginine, or Histidine. In another example, the amino acid substitution has one or more amino acids that prevent or inhibit the VWF proteins from forming multimers.

[0090] In certain embodiments, the VWF protein useful herein can be further modified to improve its interaction with FVIII, *e.g.*, to improve binding affinity to FVIII. As a non-limiting example, the VWF protein comprises a serine residue at the residue corresponding to amino acid 764 of SEQ ID NO: 2 and a lysine residue at the residue corresponding to amino acid 773 of SEQ ID NO: 2. Residues 764 and/or 773 can contribute to the binding affinity of the VWF proteins to FVIII. In other embodiments, the VWF proteisn useful for the invention can have other modifications, *e.g.*, the protein can be pegylated, glycosylated, hesylated, or polysialylated.

II.C. Heterologous Moiety

[0091] A heterologous moiety that can be fused to a VWF protein via a VWF linker or a FVIII protein via a FVIII linker can be a heterologous polypeptide or a heterologous non-polypeptide moiety. In certain embodiments, the heterologous moiety is a half-life extending molecule which is known in the art and comprises a polypeptide, a non-polypeptide moiety, or the combination of both. A heterologous polypeptide moiety can comprise an immunoglobulin constant region or a portion thereof, albumin or a fragment thereof, an albumin binding moiety, transferrin or a fragment thereof, a PAS sequence, a HAP sequence, a derivative or variant thereof, or any combination thereof. In some embodiments, the non-polypeptide binding moiety comprises polyethylene glycol (PEG), polysialic acid, hydroxyethyl starch (HES), a derivative thereof, or any combination thereof. In certain embodiments, there can be one, two, three or more heterologous moieties, which can each be the same or different molecules.

II.C.1. Immunoglobulin Constant Region or Portion Thereof

[0092] An immunoglobulin constant region is comprised of domains denoted CH (constant heavy) domains (CH1, CH2, etc.). Depending on the isotype, (*i.e.* IgG, IgM, IgA IgD, or IgE), the constant region can be comprised of three or four CH domains. Some isotypes (*e.g.* IgG) constant regions also contain a hinge region. *See* Janeway *et al.* 2001, *Immunobiology*, Garland Publishing, N.Y., N.Y.

[0093] An immunoglobulin constant region or a portion thereof for producing the chimeric protein of the present invention may be obtained from a number of different sources. In some embodiments, an immunoglobulin constant region or a portion thereof is derived from a human immunoglobulin. It is understood, however, that the immunoglobulin constant region or a portion thereof may be derived from an immunoglobulin of another mammalian species, including for example, a rodent (*e.g.,* a mouse, rat, rabbit, guinea pig) or non-human primate (*e.g.* chimpanzee, macaque) species. Moreover, the immunoglobulin constant region or a portion thereof may be derived from any immunoglobulin class, including IgM, IgG, IgD, IgA and IgE, and any immunoglobulin isotype, including IgGl, IgG2, IgG3 and IgG4. In one embodiment, the human isotype IgG1 is used.

[0094] A variety of the immunoglobulin constant region gene sequences (*e.g.* human constant region gene sequences) are available in the form of publicly accessible deposits. Constant region domains sequence can be selected having a particular effector function (or lacking a particular effector function) or with a particular modification to reduce immunogenicity. Many sequences of antibodies and antibody-encoding genes have been published and suitable Ig constant region sequences (*e.g.* hinge, CH2, and/or CH3 sequences, or portions thereof) can be derived from these sequences using art recognized techniques. The genetic material obtained using any of the foregoing methods may then be altered or synthesized to obtain polypeptides of the present invention. It will further be appreciated that the scope of this invention encompasses alleles, variants and mutations of constant region DNA sequences.

[0095] The sequences of the immunoglobulin constant region or a portion thereof can be cloned, *e.g.*, using the polymerase chain reaction and primers which are selected to amplify the domain of interest. To clone a sequence of the immunoglobulin constant region or a portion thereof from an antibody, mRNA can be isolated from hybridoma,

spleen, or lymph cells, reverse transcribed into DNA, and antibody genes amplified by PCR. PCR amplification methods are described in detail in U.S. Pat. Nos. 4,683,195; 4,683,202; 4,800,159; 4,965,188; and in, *e.g.*, "PCR Protocols: A Guide to Methods and Applications" Innis et al. eds., Academic Press, San Diego, CA (1990); Ho et al. 1989. Gene 77:51; Horton et al. 1993. Methods Enzymol. 217:270).

[0096] An immunoglobulin constant region used herein can include all domains and the hinge region or portions thereof. In one embodiment, the immunoglobulin constant region or a portion thereof comprises CH2 domain, CH3 domain, and a hinge region, *i.e.*, an Fc region or an FcRn binding partner.

[0097] As used herein, the term "Fc region" is defined as the portion of a polypeptide which corresponds to the Fc region of native immunoglobulin, *i.e.*, as formed by the dimeric association of the respective Fc domains of its two heavy chains. A native Fc region forms a homodimer with another Fc region.

[0098] In one embodiment, the "Fc region" refers to the portion of a single immunoglobulin heavy chain beginning in the hinge region just upstream of the papain cleavage site (*i.e.* residue 216 in IgG, taking the first residue of heavy chain constant region to be 114) and ending at the C-terminus of the antibody. Accordingly, a complete Fc domain comprises at least a hinge domain, a CH2 domain, and a CH3 domain.

[0099] The Fc region of an immunoglobulin constant region, depending on the immunoglobulin isotype can include the CH2, CH3, and CH4 domains, as well as the hinge region. Chimeric proteins comprising an Fc region of an immunoglobulin bestow several desirable properties on a chimeric protein including increased stability, increased serum half-life (see Capon *et al.*, 1989, *Nature* 337:525) as well as binding to Fc receptors such as the neonatal Fc receptor (FcRn) (U.S. Pat. Nos. 6,086,875, 6,485,726, 6,030,613; WO 03/077834; US2003-0235536A1), which are incorporated herein by reference in their entireties.

[00100] An immunoglobulin constant region or a portion thereof can be an FcRn binding partner. FcRn is active in adult epithelial tissues and expressed in the lumen of the intestines, pulmonary airways, nasal surfaces, vaginal surfaces, colon and rectal surfaces (U.S. Pat. No. 6,485,726). An FcRn binding partner is a portion of an immunoglobulin that binds to FcRn.

[0100] The FcRn receptor has been isolated from several mammalian species including humans. The sequences of the human FcRn, monkey FcRn, rat FcRn, and mouse FcRn are known (Story et al. 1994, J. Exp. Med. 180:2377). The FcRn receptor binds IgG (but not other immunoglobulin classes such as IgA, IgM, IgD, and IgE) at relatively low pH, actively transports the IgG transcellularly in a luminal to serosal direction, and then releases the IgG at relatively higher pH found in the interstitial fluids. It is expressed in adult epithelial tissue (U.S. Pat. Nos. 6,485,726, 6,030,613, 6,086,875; WO 03/077834; US2003-0235536A1) including lung and intestinal epithelium (Israel et al. 1997, Immunology 92:69) renal proximal tubular epithelium (Kobayashi et al. 2002, Am. J. Physiol. Renal Physiol. 282:F358) as well as nasal epithelium, vaginal surfaces, and biliary tree surfaces.

[0101] FcRn binding partners useful in the present invention encompass molecules that can be specifically bound by the FcRn receptor including whole IgG, the Fc fragment of IgG, and other fragments that include the complete binding region of the FcRn receptor. The region of the Fc portion of IgG that binds to the FcRn receptor has been described based on X-ray crystallography (Burmeister et al. 1994, Nature 372:379). The major contact area of the Fc with the FcRn is near the junction of the CH2 and CH3 domains. Fc-FcRn contacts are all within a single Ig heavy chain. The FcRn binding partners include whole IgG, the Fc fragment of IgG, and other fragments of IgG that include the complete binding region of FcRn. The major contact sites include amino acid residues 248, 250-257, 272, 285, 288, 290-291, 308-311, and 314 of the CH2 domain and amino acid residues 385-387, 428, and 433-436 of the CH3 domain. References made to amino acid numbering of immunoglobulins or immunoglobulin fragments, or regions, are all based on Kabat et al. 1991, Sequences of Proteins of Immunological Interest, U.S. Department of Public Health, Bethesda, Md.

[0102] Fc regions or FcRn binding partners bound to FcRn can be effectively shuttled across epithelial barriers by FcRn, thus providing a non-invasive means to systemically administer a desired therapeutic molecule. Additionally, fusion proteins comprising an Fc region or an FcRn binding partner are endocytosed by cells expressing the FcRn. But instead of being marked for degradation, these fusion proteins are recycled out into circulation again, thus increasing the *in vivo* half-life of these proteins. In certain embodiments, the portions of immunoglobulin constant regions are an Fc region or an

FcRn binding partner that typically associates, via disulfide bonds and other non-specific interactions, with another Fc region or another FcRn binding partner to form dimers and higher order multimers.

[0103] An FcRn binding partner region is a molecule or a portion thereof that can be specifically bound by the FcRn receptor with consequent active transport by the FcRn receptor of the Fc region. Specifically bound refers to two molecules forming a complex that is relatively stable under physiologic conditions. Specific binding is characterized by a high affinity and a low to moderate capacity as distinguished from nonspecific binding which usually has a low affinity with a moderate to high capacity. Typically, binding is considered specific when the affinity constant KA is higher than 10^6 M^{-1}, or higher than 10^8 M^{-1}. If necessary, non-specific binding can be reduced without substantially affecting specific binding by varying the binding conditions. The appropriate binding conditions such as concentration of the molecules, ionic strength of the solution, temperature, time allowed for binding, concentration of a blocking agent (*e.g.* serum albumin, milk casein), etc., may be optimized by a skilled artisan using routine techniques.

[0104] Myriad mutants, fragments, variants, and derivatives are described, *e.g.*, in PCT Publication Nos. WO 2011/069164 A2, WO 2012/006623 A2, WO 2012/006635 A2 , or WO 2012/006633 A2, all of which are incorporated herein by reference in their entireties.

II.C.2. Albumin or fragment, or variant thereof

[0105] In certain embodiments, a heterologous moiety linked to the VWF protein via a VWF linker or linked to a FVIII protein via a FVIII linker as a heterologous moiety is albumin or a functional fragment thereof. In some embodiments, the albumin fused to the VWF protein is covalently associated with an albumin fused to a FVIII protein.

[0106] Human serum albumin (HSA, or HA), a protein of 609 amino acids in its full-length form, is responsible for a significant proportion of the osmotic pressure of serum and also functions as a carrier of endogenous and exogenous ligands. The term "albumin" as used herein includes full-length albumin or a functional fragment, variant, derivative, or analog thereof. Examples of albumin or the fragments or variants thereof are disclosed in US Pat. Publ. Nos. 2008/0194481A1, 2008/0004206 A1, 2008/0161243 A1, 2008/0261877 A1, or 2008/0153751 A1 or PCT Appl. Publ. Nos. 2008/033413 A2, 2009/058322 A1, or 2007/021494 A2, which are incorporated herein by references in their entireties.

II.C.3. Albumin Binding Moiety

[0107] In certain embodiments, a heterologous moiety linked to a VWF protein via a VWF linker or to a FVIII protein via a FVIII linker is an albumin binding moiety, which comprises an albumin binding peptide, a bacterial albumin binding domain, an albumin-binding antibody fragment, or any combination thereof. For example, the albumin binding protein can be a bacterial albumin binding protein, an antibody or an antibody fragment including domain antibodies (see U.S. Pat. No. 6,696,245). An albumin binding protein, for example, can be a bacterial albumin binding domain, such as the one of streptococcal protein G (Konig, T. and Skerra, A. (1998) *J. Immunol. Methods* 218, 73-83). Other examples of albumin binding peptides that can be used as conjugation partner are, for instance, those having a Cys-Xaa $_1$ -Xaa $_2$ -Xaa $_3$ -Xaa $_4$ -Cys consensus sequence, wherein Xaa$_1$ is Asp, Asn, Ser, Thr, or Trp; Xaa$_2$ is Asn, Gln, H is, Ile, Leu, or Lys; Xaa$_3$ is Ala, Asp, Phe, Trp, or Tyr; and Xaa$_4$ is Asp, Gly, Leu, Phe, Ser, or Thr as described in US patent application 2003/0069395 or Dennis et al. (Dennis et al. (2002) *J. Biol. Chem.* 277, 35035-35043).

II.C.4. PAS Sequence

[0108] In other embodiments, a heterologous moiety linked to a VWF protein via a VWF linker or to a FVIII protein via a FVIII linker as a heterologous moiety is a PAS sequence. In one embodiment, a chimeric molecule comprises a VWF protein described herein fused to a PAS sequence via a VWF linker. In another embodiment, a chimeric molecule of the invention comprises a first chain comprising a VWF protein fused to a PAS sequence via a VWF linker and a second chain comprising a FVIII protein and an additional optional PAS sequence, wherein the PAS sequence shields or protects the VWF binding site on the FVIII protein, thereby inhibiting or preventing interaction of the FVIII protein with endogenous VWF. The two PAS sequences can be covalently associated with each other.

[0109] A PAS sequence, as used herein, means an amino acid sequence comprising mainly alanine and serine residues or comprising mainly alanine, serine, and proline residues, the amino acid sequence forming random coil conformation under physiological conditions. Accordingly, the PAS sequence is a building block, an amino acid polymer, or a sequence cassette comprising, consisting essentially of, or consisting of alanine,

serine, and proline which can be used as a part of the heterologous moiety in the chimeric protein. Yet, the skilled person is aware that an amino acid polymer also may form random coil conformation when residues other than alanine, serine, and proline are added as a minor constituent in the PAS sequence. The term "minor constituent" as used herein means that amino acids other than alanine, serine, and proline may be added in the PAS sequence to a certain degree, *e.g.*, up to about 12%, *i.e.*, about 12 of 100 amino acids of the PAS sequence, up to about 10%, *i.e.* about 10 of 100 amino acids of the PAS sequence, up to about 9%, *i.e.*, about 9 of 100 amino acids, up to about 8%, *i.e.*, about 8 of 100 amino acids, about 6%, *i.e.*, about 6 of 100 amino acids, about 5%, *i.e.*, about 5 of 100 amino acids, about 4%, *i.e.*, about 4 of 100 amino acids, about 3%, *i.e.*, about 3 of 100 amino acids, about 2%, *i.e.*, about 2 of 100 amino acids, about 1%, *i.e.*, about 1 of 100 of the amino acids. The amino acids different from alanine, serine and proline may be selected from the group consisting of Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr, and Val.

[0110] Under physiological conditions, the PAS sequence stretch forms a random coil conformation and thereby can mediate an increased *in vivo* and/or *in vitro* stability to the VWF factor or the protein of coagulation activity. Since the random coil domain does not adopt a stable structure or function by itself, the biological activity mediated by the VWF protein or the FVIII protein to which it is fused is essentially preserved. In other embodiments, the PAS sequences that form random coil domain are biologically inert, especially with respect to proteolysis in blood plasma, immunogenicity, isoelectric point/electrostatic behavior, binding to cell surface receptors or internalization, but are still biodegradable, which provides clear advantages over synthetic polymers such as PEG.

[0111] Non-limiting examples of the PAS sequences forming random coil conformation comprise an amino acid sequence selected from the group consisting of ASPAAPAPASPAAPAPSAPA (SEQ ID NO: 32), AAPASPAPAAPSAPAPAAPS (SEQ ID NO: 33), APSSPSPSAPSSPSPASPSS (SEQ ID NO: 34), APSSPSPSAPSSPSPASPS (SEQ ID NO: 35), SSPSAPSPSSPASPSPSSPA (SEQ ID NO: 36), AASPAAPSAPPAAASPAAPSAPPA (SEQ ID NO: 37) and ASAAAPAAASAAASAPSAAA (SEQ ID NO: 38) or any combination thereof.

Additional examples of PAS sequences are known from, *e.g.*, US Pat. Publ. No. 2010/0292130 A1 and PCT Appl. Publ. No. WO 2008/155134 A1.

II.C.5. HAP Sequence

[0112] In certain embodiments, a heterologous moiety linked to a VWF protein via a VWF linker or to a FVIII protein via a FVIII linker as a heterologous moiety is a glycine-rich homo-amino-acid polymer (HAP). The HAP sequence can comprise a repetitive sequence of glycine, which has at least 50 amino acids, at least 100 amino acids, 120 amino acids, 140 amino acids, 160 amino acids, 180 amino acids, 200 amino acids, 250 amino acids, 300 amino acids, 350 amino acids, 400 amino acids, 450 amino acids, or 500 amino acids in length. In one embodiment, the HAP sequence is capable of extending half-life of a moiety fused to or linked to the HAP sequence. Non-limiting examples of the HAP sequence includes, but are not limited to $(Gly)_n$, $(Gly_4Ser)_n$ or $S(Gly_4Ser)_n$, wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20. In one embodiment, n is 20, 21, 22, 23, 24, 25, 26, 26, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40. In another embodiment, n is 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200. See, *e.g.*, Schlapschy M *et al.*, Protein Eng. Design Selection, 20: 273-284 (2007).

II.C.6. Transferrin or Fragment thereof

[0113] In certain embodiments, a heterologous moiety linked to a VWF protein via a VWF linker or to a FVIII protein via a FVIII linker as a heterologous moiety is transferrin or a fragment thereof. Any transferrin may be used to make chimeric molecules of the invention. As an example, wild-type human Tf (Tf) is a 679 amino acid protein, of approximately 75 KDa (not accounting for glycosylation), with two main domains, N (about 330 amino acids) and C (about 340 amino acids), which appear to originate from a gene duplication. See GenBank accession numbers NM001063, XM002793, M12530, XM039845, XM 039847 and S95936 (www.ncbi.nlm.nih.gov/), all of which are herein incorporated by reference in their entirety. Transferrin comprises two domains, N domain and C domain. N domain comprises two subdomains, N1 domain and N2 domain, and C domain comprises two subdomains, C1 domain and C2 domain.

[0114] In one embodiment, the transferrin portion of the chimeric molecule includes a transferrin splice variant. In one example, a transferrin splice variant can be a splice

variant of human transferrin, *e.g.*, Genbank Accession AAA61140. In another embodiment, the transferrin portion of the chimeric molecule includes one or more domains of the transferrin sequence, *e.g.*, N domain, C domain, N1 domain, N2 domain, C1 domain, C2 domain or any combination thereof.

II.C.7. Polymer, *e.g.*, Polyethylene Glycol (PEG)

[0115] In other embodiments, a heterologous moiety attached to a VWF protein via a VWF linker or to a FVIII protein via a FVIII linker as a heterologous moiety is a soluble polymer known in the art, including, but not limited to, polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, or polyvinyl alcohol. The heterologous moiety such as soluble polymer can be attached to any positions within the chimeric molecule.

[0116] In certain embodiments, a chimeric molecule comprises a VWF protein fused to a heterologous moiety (*e.g.*, an Fc region) via a VWF linker, wherein the VWF protein is further linked to PEG. In another embodiment, a chimeric molecule comprises a VWF protein fused to an Fc region via a VWF linker and a FVIII protein, which are associated with each other, wherein the FVIII protein is linked to PEG.

[0117] Also provided by the invention are chemically modified derivatives of the chimeric molecule of the invention which may provide additional advantages such as increased solubility, stability and circulating time of the polypeptide, or decreased immunogenicity (see U.S. Pat. No. 4,179,337). The chemical moieties for modification can be selected from water soluble polymers including, but not limited to, polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, or polyvinyl alcohol. A chimeric molecule may be modified at random positions within the molecule or at the N- or C- terminus, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties.

[0118] The polymer can be of any molecular weight, and can be branched or unbranched. For polyethylene glycol, in one embodiment, the molecular weight is between about 1 kDa and about 100 kDa for ease in handling and manufacturing. Other sizes may be used, depending on the desired profile (*e.g.*, the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a protein or analog). For example, the polyethylene glycol may have an average molecular weight of about

200, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10,000, 10,500, 11,000, 11,500, 12,000, 12,500, 13,000, 13,500, 14,000, 14,500, 15,000, 15,500, 16,000, 16,500, 17,000, 17,500, 18,000, 18,500, 19,000, 19,500, 20,000, 25,000, 30,000, 35,000, 40,000, 45,000, 50,000, 55,000, 60,000, 65,000, 70,000, 75,000, 80,000, 85,000, 90,000, 95,000, or 100,000 kDa.

[0119] In some embodiments, the polyethylene glycol may have a branched structure. Branched polyethylene glycols are described, for example, in U.S. Pat. No. 5,643,575; Morpurgo *et al., Appl. Biochem. Biotechnol.* 56:59-72 (1996); Vorobjev *et al., Nucleosides Nucleotides* 18:2745-2750 (1999); and Caliceti *et al., Bioconjug. Chem.* 10:638-646 (1999), each of which is incorporated herein by reference in its entirety.

[0120] The number of polyethylene glycol moieties attached to each chimeric molecule (*i.e.*, the degree of substitution) may also vary. For example, the pegylated proteins of the invention may be linked, on average, to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 17, 20, or more polyethylene glycol molecules. Similarly, the average degree of substitution within ranges such as 1-3, 2-4, 3-5, 4-6, 5-7, 6-8, 7-9, 8-10, 9-11, 10-12, 11-13, 12-14, 13-15, 14-16, 15-17, 16-18, 17-19, or 18-20 polyethylene glycol moieties per protein molecule. Methods for determining the degree of substitution are discussed, for example, in Delgado *et al., Crit. Rev. Thera. Drug Carrier Sys.* 9:249-304 (1992).

[0121] In other embodiments, a FVIII protein used in the invention is conjugated to one or more polymers. The polymer can be water-soluble and covalently or non-covalently attached to Factor VIII or other moieties conjugated to Factor VIII. Non-limiting examples of the polymer can be poly(alkylene oxide), poly(vinyl pyrrolidone), poly(vinyl alcohol), polyoxazoline, or poly(acryloylmorpholine). Additional types of polymer-conjugated FVIII are disclosed in U.S. Patent No. 7,199,223.

II.C.8. Hydroxyethyl Starch (HES)

[0122] In certain embodiments, the heterologous moiety linked to a VWF protein via a VWF linker or a FVIII protein via a FVIII linker as a heterologous moiety is a polymer, *e.g.*, hydroxyethyl starch (HES) or a derivative thereof.

[0123] Hydroxyethyl starch (HES) is a derivative of naturally occurring amylopectin and is degraded by alpha-amylase in the body. HES is a substituted derivative of the carbohydrate polymer amylopectin, which is present in corn starch at a concentration of up to 95% by weight. HES exhibits advantageous biological properties and is used as a

blood volume replacement agent and in hemodilution therapy in the clinics (Sommermeyer *et al.*, *Krankenhauspharmazie*, 8(8), 271-278 (1987); and Weidler *et al.*, *Arzneim.-Forschung/Drug Res.*, 41, 494-498 (1991)).

[0124] Amylopectin contains glucose moieties, wherein in the main chain alpha-1,4-glycosidic bonds are present and at the branching sites alpha-1,6-glycosidic bonds are found. The physical-chemical properties of this molecule are mainly determined by the type of glycosidic bonds. Due to the nicked alpha-1,4-glycosidic bond, helical structures with about six glucose-monomers per turn are produced. The physico-chemical as well as the biochemical properties of the polymer can be modified via substitution. The introduction of a hydroxyethyl group can be achieved via alkaline hydroxyethylation. By adapting the reaction conditions it is possible to exploit the different reactivity of the respective hydroxy group in the unsubstituted glucose monomer with respect to a hydroxyethylation. Owing to this fact, the skilled person is able to influence the substitution pattern to a limited extent.

[0125] HES is mainly characterized by the molecular weight distribution and the degree of substitution. The degree of substitution, denoted as DS, relates to the molar substitution, is known to the skilled people. See Sommermeyer *et al.*, *Krankenhauspharmazie*, 8(8), 271-278 (1987), as cited above, in particular p. 273.

[0126] In one embodiment, hydroxyethyl starch has a mean molecular weight (weight mean) of from 1 to 300 kD, from 2 to 200kD, from 3 to 100 kD, or from 4 to 70kD. hydroxyethyl starch can further exhibit a molar degree of substitution of from 0.1 to 3, preferably 0.1 to 2, more preferred, 0.1 to 0.9, preferably 0.1 to 0.8, and a ratio between C2:C6 substitution in the range of from 2 to 20 with respect to the hydroxyethyl groups. A non-limiting example of HES having a mean molecular weight of about 130 kD is a HES with a degree of substitution of 0.2 to 0.8 such as 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, or 0.8, preferably of 0.4 to 0.7 such as 0.4, 0.5, 0.6, or 0.7. In a specific embodiment, HES with a mean molecular weight of about 130 kD is VOLUVEN® from Fresenius. VOLUVEN® is an artificial colloid, employed, *e.g.*, for volume replacement used in the therapeutic indication for therapy and prophylaxis of hypovolaemia. The characteristics of VOLUVEN® are a mean molecular weight of 130,000+/−20,000 D, a molar substitution of 0.4 and a C2:C6 ratio of about 9:1. In other embodiments, ranges of the mean molecular weight of hydroxyethyl starch are, *e.g.*, 4 to 70 kD or 10 to 70 kD or 12 to 70

kD or 18 to 70 kD or 50 to 70 kD or 4 to 50 kD or 10 to 50 kD or 12 to 50 kD or 18 to 50 kD or 4 to 18 kD or 10 to 18 kD or 12 to 18 kD or 4 to 12 kD or 10 to 12 kD or 4 to 10 kD. In still other embodiments, the mean molecular weight of hydroxyethyl starch employed is in the range of from more than 4 kD and below 70 kD, such as about 10 kD, or in the range of from 9 to 10 kD or from 10 to 11 kD or from 9 to 11 kD, or about 12 kD, or in the range of from 11 to 12 kD) or from 12 to 13 kD or from 1 1 to 13 kD, or about 18 kD, or in the range of from 17 to 18 kD or from 18 to 19 kD or from 17 to 19 kD, or about 30 kD, or in the range of from 29 to 30, or from 30 to 31 kD, or about 50 kD, or in the range of from 49 to 50 kD or from 50 to 51 kD or from 49 to 51 kD.

[0127] In certain embodiments, the heterologous moiety can be mixtures of hydroxyethyl starches having different mean molecular weights and/or different degrees of substitution and/or different ratios of C2: C6 substitution. Therefore, mixtures of hydroxyethyl starches may be employed having different mean molecular weights and different degrees of substitution and different ratios of C2: C6 substitution, or having different mean molecular weights and different degrees of substitution and the same or about the same ratio of C2:C6 substitution, or having different mean molecular weights and the same or about the same degree of substitution and different ratios of C2:C6 substitution, or having the same or about the same mean molecular weight and different degrees of substitution and different ratios of C2:C6 substitution, or having different mean molecular weights and the same or about the same degree of substitution and the same or about the same ratio of C2:C6 substitution, or having the same or about the same mean molecular weights and different degrees of substitution and the same or about the same ratio of C2:C6 substitution, or having the same or about the same mean molecular weight and the same or about the same degree of substitution and different ratios of C2: C6 substitution, or having about the same mean molecular weight and about the same degree of substitution and about the same ratio of C2:C6 substitution.

II.C.9. Polysialic Acids (PSA)

[0128] In certain embodiments, the non-polypeptide heterologous moiety linked to a VWF protein via a VWF linker or to a FVIII protein via a FVIII linker as a heterologous moiety is a polymer, *e.g.*, polysialic acids (PSAs) or a derivative thereof. Polysialic acids (PSAs) are naturally occurring unbranched polymers of sialic acid produced by certain bacterial strains and in mammals in certain cells. Roth J., et al. (1993) in *Polysialic Acid:*

From Microbes to Man, eds. Roth J., Rutishauser U., Troy F. A. (Birkhäuser Verlag, Basel, Switzerland), pp 335–348. They can be produced in various degrees of polymerization from n=about 80 or more sialic acid residues down to n=2 by limited acid hydrolysis or by digestion with neuraminidases, or by fractionation of the natural, bacterially derived forms of the polymer. The composition of different polysialic acids also varies such that there are homopolymeric forms *i.e.* the alpha-2,8-linked polysialic acid comprising the capsular polysaccharide of *E. coli* strain K1 and the group-B meningococci, which is also found on the embryonic form of the neuronal cell adhesion molecule (N-CAM). Heteropolymeric forms also exist—such as the alternating alpha-2,8 alpha-2,9 polysialic acid of *E. coli* strain K92 and group C polysaccharides of *N. meningitidis*. Sialic acid may also be found in alternating copolymers with monomers other than sialic acid such as group W135 or group Y of *N. meningitidis*. Polysialic acids have important biological functions including the evasion of the immune and complement systems by pathogenic bacteria and the regulation of glial adhesiveness of immature neurons during foetal development (wherein the polymer has an anti-adhesive function) Cho and Troy, *P.N.A.S., USA*, 91 (1994) 11427-11431, although there are no known receptors for polysialic acids in mammals. The alpha-2,8-linked polysialic acid of *E. coli* strain K1 is also known as 'colominic acid' and is used (in various lengths) to exemplify the present invention. Various methods of attaching or conjugating polysialic acids to a polypeptide have been described (for example, see U.S. Pat. No. 5,846,951; WO-A-0187922, and US 2007/0191597 A1, which are incorporated herein by reference in their entireties.

II.D. Chimeric Molecule Comprising Linker

[0129] The present invention includes a chimeric molecule comprising two polypeptide chains, a first chain comprising a VWF protein fused to a heterologous moiety (H1) via a VWF linker and a second chain comprising a FVIII protein, which are associated with the VWF protein, and a second heterologous moiety (H2), which is connected to the FVIII protein. The first heterologous moiety and the second heterologous moiety can be associated with each other by a bond stronger than the association between the FVIII protein and the VWF protein. The VWF linker connecting the first heterologous moiety and the VWF protein can thus be cleaved by thrombin at the site of injury, allowing FVIII to be dissociated from the VWF protein.

[0130] In one embodiment, a chimeric molecule comprises a formula selected from: (a) V-L1-H1: H2-L2-C, or (b) C-L2-H2:H1-L1-V;

wherein V is a VWF protein; L1 is a VWF linker; L2 is an optional FVIII linker; H1 is a first heterologous moiety; H2 is a second heterologous moiety; C is a FVIII protein; (-) is a peptide bond or one or more amino acids; and (:) is a covalent bond between the H1 and the H2.

[0131] In another embodiment, a chimeric molecule can be a single polypeptide chain comprising a VWF protein, a VWF linker, a first heterologous moiety, a processable linker, a FVIII protein, an optional linker, and a second heterologous moiety. In other embodiments, the processable linker comprises one or more site that can be cleaved by an intracellular protease, *e.g.*, proprotein convertase. Therefore, in some embodiments, the single chain chimeric molecule can be cleaved into two polypeptide chains by a proprotein convertase (*e.g.*, PC5, PC7, or PACE) upon expression in cells.

[0132] In some embodiments, a chimeric molecule comprises a formula selected from: (i) V-L1-H1-L3-C-L2-H2, (ii) H2-L2-C-L3-H1-L1-V, (iii) C-L2-H2-L3-V-L1-H1, (iv) H1-L1-V-L3-H2-L2-C, (v) H1-L1-V-L3-C-L2-H2, (vi) H2-L2-C-L3-V-L1-H1, (vii) V-L1-H1-L3-H2-L2-C, or (viii) C-L2-H2-L3-H1-L1-V, wherein V comprises a VWF protein; L1 is a VWF linker; L2 is an optional FVIII linker; L3 is a processable linker that is processed by a protease, H1 is a first heterologous moiety; H2 is a second heterologous moiety; C comprises a FVIII protein; and (-) is a peptide bond or one or more amino acids.

[0133] In some embodiments, the protease that can cleave the single chain chimeric molecule into two chain molecule is a proprotein convertase. Examples of the proprotein convertase are described elsewhere herein.

[0134] In other embodiments, the second heterologous moiety that is fused to the FVIII protein is selected from the heterologous moieties listed in Section II.C. described above. In other embodiments, the second heterologous moiety that is fused to the FVIII protein is the same as or different from the first heterologous moiety fused to the VWF protein via a VWF linker. In yet other embodiments, the second heterologous moiety (H2) is capable of extending half-life of the chimeric molecule. In certain embodiments, the first heterologous moiety and the second heterologous moiety are associated with each other.

In some embodiments, the association between the first polypeptide chain and the second polypeptide chain is a covalent bond, *e.g.*, a disulfide bond.

[0135] In certain embodiments, the the second heterologous moiety is fused to the FVIII protein by a FVIII linker. The FVIII linker can a cleavable linker comprising a cleavage site, *e.g.,* a thrombin cleavable linker. For example, the FVIII linker can be identical to the VWF linker. In some embodiments, the FVIII linker is different from the VWF linker.

[0136] In some embodiments, a chimeric molecule comprises two polypeptide chains, the first chain comprising a FVIII protein fused to a first heterologous moiety via a FVIII linker, and a VWF protein (*e.g.*, a D' domain and a D3 domain of VWF) fused to a second heterologous moiety, wherein the rVIII linker in the first polypeptide chain comprises: (i) an a2 region from FVIII; (ii) an a1 region from FVIII; (iii) an a3 region from FVIII; (iv) a thrombin cleavage site which comprises X-V-P-R (SEQ ID NO: 3) and a PAR1 exosite interaction motif, wherein X is an aliphatic amino acid; or (v) any combination thereof, and wherein the first polypeptide chain and the second polypeptide chain are associated with each other. In a specific embodiment the linker in the first polypeptide chain comprises an a2 region from FVIII.

[0137] In certain embodiments, a chimeric molecule comprises a formula selected from: (i) V-L2-H2: H1-L1-C or (ii) C-L1-H1: H2-L2-V, wherein V is a VWF protein; L1 is a FVIII linker; L2 is an optional VWF linker; H1 is a first heterologous moiety; H2 is a second heterologous moiety; C is a FVIII protein; (-) is a peptide bond or one or more amino acids; and (:) is a covalent bond between the H1 and the H2.

[0138] In some embodiments, a chimeric molecule of the invention can comprise a third heterologous moiety (H3), a fourth heterologous moiety (H4), a fifth heterologous moiety (H5), or the sixth heterologous moiety (H6). One or more of the third heterologous moiety (H3), the fourth heterologous moiety (H4), the fifth heterologous moiety (H5), and the sixth heterologous moiety (H6) can be capable of extending the half-life of the chimeric molecule. In one embodiment, the additional heterologous moiety can be fused to the FVIII protein, the second heterologous moiety, the VWF protein, the VWF linker, or the first heterologous moiety. In other embodiments, one or more of the third heterologous moiety (H3), the fourth heterologous moiety (H4), the fifth heterologous

moiety (H5), and the sixth heterologous moiety (H6) are selected from the heterologous moieties listed in Section II.C. described above.

II.D.1. FVIII protein.

[0139] "A FVIII protein" as used herein means a functional FVIII polypeptide in its normal role in coagulation, unless otherwise specified. The term a FVIII protein includes a functional fragment, variant, analog, or derivative thereof that retains the function of full-length wild-type Factor VIII in the coagulation pathway. "A FVIII protein" is used interchangeably with FVIII polypeptide (or protein) or FVIII. Examples of the FVIII functions include, but not limited to, an ability to activate coagulation, an ability to act as a cofactor for factor IX, or an ability to form a tenase complex with factor IX in the presence of Ca^{2+} and phospholipids, which then converts Factor X to the activated form Xa. The FVIII protein can be the human, porcine, canine, rat, or murine FVIII protein. In addition, comparisons between FVIII from humans and other species have identified conserved residues that are likely to be required for function (Cameron *et al., Thromb. Haemost.* 79:317-22 (1998); US 6,251,632).

[0140] A number of tests are available to assess the function of the coagulation system: activated partial thromboplastin time (aPTT) test, chromogenic assay, ROTEM assay, prothrombin time (PT) test (also used to determine INR), fibrinogen testing (often by the Clauss method), platelet count, platelet function testing (often by PFA-100), TCT, bleeding time, mixing test (whether an abnormality corrects if the patient's plasma is mixed with normal plasma), coagulation factor assays, antiphosholipid antibodies, D-dimer, genetic tests (*e.g.* factor V Leiden, prothrombin mutation G20210A), dilute Russell's viper venom time (dRVVT), miscellaneous platelet function tests, thromboelastography (TEG or Sonoclot), thromboelastometry (TEM®, e.g, ROTEM®), or euglobulin lysis time (ELT).

[0141] The aPTT test is a performance indicator measuring the efficacy of both the "intrinsic" (also referred to the contact activation pathway) and the common coagulation pathways. This test is commonly used to measure clotting activity of commercially available recombinant clotting factors, *e.g.*, FVIII or FIX. It is used in conjunction with prothrombin time (PT), which measures the extrinsic pathway.

[0142] ROTEM analysis provides information on the whole kinetics of haemostasis: clotting time, clot formation, clot stability and lysis. The different parameters in

thromboelastometry are dependent on the activity of the plasmatic coagulation system, platelet function, fibrinolysis, or many factors which influence these interactions. This assay can provide a complete view of secondary haemostasis.

[0143] The FVIII polypeptide and polynucleotide sequences are known, as are many functional fragments, mutants and modified versions. Examples of human FVIII sequences (full-length) are shown as subsequences in SEQ ID NO: 16 or 18.

Table 2. Full-length FVIII (FVIII signal peptide underlined; FVIII heavy chain is double underlined; B domain is italicized; and FVIII light chain is in plain text)

Signal Peptide: (SEQ ID NO: 15)
MQIELSTCFFLCLLRFCFS

Mature Factor VIII (SEQ ID NO: 16)*
ATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPKSFPFNTSVVYKKTLFVEFTDHLFNIAKPRPPWMGLL
GPTIQAEVYDTVVITLKNMASHPVSLHAVGVSYWKASEGAEYDDQTSQREKEDDKVFPGGSHTYVWQVLKEN
GPMASDPLCLTYSYLSHVDLVKDLNSGLIGALLVCREGSLAKEKTQTLHKFILLFAVFDEGKSWHSETKNSL
MQDRDAASARAWPKMHTVNGYVNRSLPGLIGCHRKSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEI
SPITFLTAQTLLMDLGQFLLFCHISSHQHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDLTDSEMDVVRF
DDDNSPSFIQIRSVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYKSQYLNNGPQRIGRKYKKVRFMAYT
DETFKTREAIQHESGILGPLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVRPLYSRRLPKGVKHLKDFPIL
PGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFVNMERDLASGLIGPLLICYKESVDQRGNQIMSDKRNVILF
SVFDENRSWYLTENIQRFLPNPAGVQLEDPEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDF
LSVFFSGYTFKHKMVYEDTLTLFPFSGETVFMSMENPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYYE
DSYEDISAYLLSKNNAIEPRSFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTPMPKIQNVSSSDLLM
LLRQSPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESGLQLRLNEKLG
TTAATELKKLDFKVSSTSNNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTTLFGKKSSPLTESGGPL
SLSEENNDSKLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHGPALLTKDNALFKVSISLLKTNKTSNNSA
TNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTPLIHDRMLMDKNATALRLNHMSNKTTSSKNMEMVQQK
KEGPIPPDAQNPDMSFFKMLFLPESARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVVV
GKGEFTKDVGLKEMVFPSSRNLFLTNLDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGTKNFM
KNLFLLSTRQNVEGSYDGAYAPVLQDFRSLNDSTNRTKKHTAHFSKKGEEENLEGLGNQTKQIVEKYACTTR
ISPNTSQQNFVTQRSKRALKQFRLPLEETELEKRIIVDDTSTQWSKNMKHLTPSTLTQIDYNEKEKGAITQS
PLSDCLTRSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHLPAASYRKKDSGVQESSHFLQGAKK
NNLSLAILTLEMTGDQREVGSLGTSATNSVTYKKVENTVLPKPDLPKTSGKVELLPKVHIYQKDLFPTETSN
GSPGHLDLVEGSLLQGTEGAIKWNEANRPGKVPFLRVATESSAKTPSKLLDPLAWDNHYGTQIPKEEWKSQE
KSPEKTAFKKKDTILSLNACESNHAIAAINEGQNKPEIEVTWAKQGRTERLCSQNPPVLKRHQREITRTTLQ
SDQEEIDYDDTISVEMKKEDFDIYDEDENQSPRSFQKKTRHYFIAAVERLWDYGMSSSPHVLRNRAQSGSVP
QFKKVVFQEFTDGSFTQPLYRGELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFYSSLISYEEDQRQGA
EPRKNFVKPNETKTYFWKVQHHMAPTKDEFDCKAWAYFSDVDLEKDVHSGLIGPLLVCHTNTLNPAHGRQVT
VQEFALFFTIFDETKSWYFTENMERNCRAPCNIQMEDPTFKENYRFHAINGYIMDTLPGLVMAQDQRIRWYL
LSMGSNENIHSIHFSGHVFTVRKKEEYKMALYNLYPGVFETVEMLPSKAGIWRVECLIGEHLHAGMSTLFLV
YSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQG
ARQKFSSLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIRS
TLRMELMGCDLNSCSMPLGMESKAISDAQITASSYFTNMFATWSPSKARLHLQGRSNAWRPQVNNPKEWLQV
DFQKTMKVTGVTTQGVKSLLTSMYVKEFLISSSQDGHQWTLFFQNGKVKVFQGNQDSFTPVVNSLDPPLLTR
YLRIHPQSWVHQIALRMEVLGCEAQDLY

Table 3. Nucleotide Sequence Encoding Full-Length FVIII (SEQ ID NO: 17)*

```
661                                                ATG CAAATAGAGC TCTCCACCTG
721  CTTCTTTCTG TGCCTTTTGC GATTCTGCTT TAGTGCCACC AGAAGATACT ACCTGGGTGC
781  AGTGGAACTG TCATGGGACT ATATGCAAAG TGATCTCGGT GAGCTGCCTG TGGACGCAAG
841  ATTTCCTCCT AGAGTGCCAA AATCTTTTCC ATTCAACACC TCAGTCGTGT ACAAAAAGAC
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 901  TCTGTTTGTA GAATTCACGG ATCACCTTTT CAACATCGCT AAGCCAAGGC CACCCTGGAT
 961  GGGTCTGCTA GGTCCTACCA TCCAGGCTGA GGTTTATGAT ACAGTGGTCA TTACACTTAA
1021  GAACATGGCT TCCCATCCTG TCAGTCTTCA TGCTGTTGGT GTATCCTACT GGAAAGCTTC
1081  TGAGGGAGCT GAATATGATG ATCAGACCAG TCAAAGGGAG AAAGAAGATG ATAAAGTCTT
1141  CCCTGGTGGA AGCCATACAT ATGTCTGGCA GGTCCTGAAA GAGAATGGTC CAATGGCCTC
1201  TGACCCACTG TGCCTTACCT ACTCATATCT TTCTCATGTG GACCTGGTAA AAGACTTGAA
1261  TTCAGGCCTC ATTGGAGCCC TACTAGTATG TAGAGAAGGG AGTCTGGCCA AGGAAAAGAC
1321  ACAGACCTTG CACAAATTTA TACTACTTTT TGCTGTATTT GATGAAGGGA AAAGTTGGCA
1381  CTCAGAAACA AAGAACTCCT TGATGCAGGA TAGGGATGCT GCATCTGCTC GGGCCTGGCC
1441  TAAAATGCAC ACAGTCAATG GTTATGTAAA CAGGTCTCTG CCAGGTCTGA TTGGATGCCA
1501  CAGGAAATCA GTCTATTGGC ATGTGATTGG AATGGGCACC ACTCCTGAAG TGCACTCAAT
1561  ATTCCTCGAA GGTCACACAT TTCTTGTGAG GAACCATCGC CAGGCGTCCT TGGAAATCTC
1621  GCCAATAACT TTCCTTACTG CTCAAACACT CTTGATGGAC CTTGGACAGT TTCTACTGTT
1681  TTGTCATATC TCTTCCCACC AACATGATGG CATGGAAGCT TATGTCAAAG TAGACAGCTG
1741  TCCAGAGGAA CCCCAACTAC GAATGAAAAA TAATGAAGAA GCGGAAGACT ATGATGATGA
1801  TCTTACTGAT TCTGAAATGG ATGTGGTCAG GTTTGATGAT GACAACTCTC CTTCCTTTAT
1861  CCAAATTCGC TCAGTTGCCA AGAAGCATCC TAAAACTTGG GTACATTACA TTGCTGCTGA
1921  AGAGGAGGAC TGGGACTATG CTCCCTTAGT CCTCGCCCCC GATGACAGAA GTTATAAAAG
1981  TCAATATTTG AACAATGGCC CTCAGCGGAT TGGTAGGAAG TACAAAAAAG TCCGATTTAT
2041  GGCATACACA GATGAAACCT TTAAGACTCG TGAAGCTATT CAGCATGAAT CAGGAATCTT
2101  GGGACCTTTA CTTTATGGGG AAGTTGGAGA CACACTGTTG ATTATATTTA AGAATCAAGC
2161  AAGCAGACCA TATAACATCT ACCCTCACGG AATCACTGAT GTCCGTCCTT TGTATTCAAG
2221  GAGATTACCA AAAGGTGTAA AACATTTGAA GGATTTTCCA ATTCTGCCAG GAGAAATATT
2281  CAAATATAAA TGGACAGTGA CTGTAGAAGA TGGGCCAACT AAATCAGATC CTCGGTGCCT
2341  GACCCGCTAT TACTCTAGTT TCGTTAATAT GGAGAGAGAT CTAGCTTCAG GACTCATTGG
2401  CCCTCTCCTC ATCTGCTACA AAGAATCTGT AGATCAAAGA GGAAACCAGA TAATGTCAGA
2461  CAAGAGGAAT GTCATCCTGT TTTCTGTATT TGATGAGAAC CGAAGCTGGT ACCTCACAGA
2521  GAATATACAA CGCTTTCTCC CCAATCCAGC TGGAGTGCAG CTTGAGGATC CAGAGTTCCA
2581  AGCCTCCAAC ATCATGCACA GCATCAATGG CTATGTTTTT GATAGTTTGC AGTTGTCAGT
2641  TTGTTTGCAT GAGGTGGCAT ACTGGTACAT TCTAAGCATT GGAGCACAGA CTGACTTCCT
2701  TTCTGTCTTC TTCTCTGGAT ATACCTTCAA ACACAAAATG GTCTATGAAG ACACACTCAC
2761  CCTATTCCCA TTCTCAGGAG AAACTGTCTT CATGTCGATG GAAAACCCAG GTCTATGGAT
2821  TCTGGGGTGC CACAACTCAG ACTTTCGGAA CAGAGGCATG ACCGCCTTAC TGAAGGTTTC
2881  TAGTTGTGAC AAGAACACTG GTGATTATTA CGAGGACAGT TATGAAGATA TTTCAGCATA
2941  CTTGCTGAGT AAAAACAATG CCATTGAACC AAGAAGCTTC TCCCAGAATT CAAGACACCC
3001  TAGCACTAGG CAAAAGCAAT TTAATGCCAC CACAATTCCA GAAAATGACA TAGAGAAGAC
3061  TGACCCTTGG TTTGCACACA GAACACCTAT GCCTAAAATA CAAAATGTCT CCTCTAGTGA
3121  TTTGTTGATG CTCTTGCGAC AGAGTCCTAC TCCACATGGG CTATCCTTAT CTGATCTCCA
3181  AGAAGCCAAA TATGAGACTT TTTCTGATGA TCCATCACCT GGAGCAATAG ACAGTAATAA
3241  CAGCCTGTCT GAAATGACAC ACTTCAGGCC ACAGCTCCAT CACAGTGGGG ACATGGTATT
3301  TACCCCTGAG TCAGGCCTCC AATTAAGATT AAATGAGAAA CTGGGGACAA CTGCAGCAAC
3361  AGAGTTGAAG AAACTTGATT TCAAAGTTTC TAGTACATCA AATAATCTGA TTTCAACAAT
3421  TCCATCAGAC AATTTGGCAG CAGGTACTGA TAATACAAGT TCCTTAGGAC CCCCAAGTAT
3481  GCCAGTTCAT TATGATAGTC AATTAGATAC CACTCTATTT GGCAAAAAGT CATCTCCCCT
3541  TACTGAGTCT GGTGGACCTC TGAGCTTGAG TGAAGAAAAT AATGATTCAA AGTTGTTAGA
3601  ATCAGGTTTA ATGAATAGCC AAGAAAGTTC ATGGGGAAAA AATGTATCGT CAACAGAGAG
3661  TGGTAGGTTA TTTAAAGGGA AAAGAGCTCA TGGACCTGCT TTGTTGACTA AAGATAATGC
3721  CTTATTCAAA GTTAGCATCT CTTTGTTAAA GACAAACAAA ACTTCCAATA ATTCAGCAAC
3781  TAATAGAAAG ACTCACATTG ATGGCCCATC ATTATTAATT GAGAATAGTC CATCAGTCTG
3841  GCAAAATATA TTAGAAAGTG ACACTGAGTT TAAAAAAGTG ACACCTTTGA TTCATGACAG
3901  AATGCTTATG GACAAAAATG CTACAGCTTT GAGGCTAAAT CATATGTCAA ATAAAACTAC
3961  TTCATCAAAA AACATGGAAA TGGTCCAACA GAAAAAAGAG GGCCCCATTC CACCAGATGC
4021  ACAAAATCCA GATATGTCGT TCTTTAAGAT GCTATTCTTG CCAGAATCAG CAAGGTGGAT
4081  ACAAAGGACT CATGGAAAGA ACTCTCTGAA CTCTGGGCAA GGCCCCAGTC CAAAGCAATT
4141  AGTATCCTTA GGACCAGAAA AATCTGTGGA AGGTCAGAAT TTCTTGTCTG AGAAAAACAA
4201  AGTGGTAGTA GGAAAGGGTG AATTTACAAA GGACGTAGGA CTCAAAGAGA TGGTTTTTCC
4261  AAGCAGCAGA AACCTATTTC TTACTAACTT GGATAATTTA CATGAAAATA ATACACACAA
4321  TCAAGAAAAA AAAATTCAGG AAGAAATAGA AAAGAAGGAA ACATTAATCC AAGAGAATGT
4381  AGTTTTGCCT CAGATACATA CAGTGACTGG CACTAAGAAT TTCATGAAGA ACCTTTTCTT
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4441 ACTGAGCACT AGGCAAAATG TAGAAGGTTC ATATGACGGG GCATATGCTC CAGTACTTCA
4501 AGATTTTAGG TCATTAAATG ATTCAACAAA TAGAACAAAG AAACACACAG CTCATTTCTC
4561 AAAAAAAGGG GAGGAAGAAA ACTTGGAAGG CTTGGGAAAT CAAACCAAGC AAATTGTAGA
4621 GAAATATGCA TGCACCACAA GGATATCTCC TAATACAAGC CAGCAGAATT TTGTCACGCA
4681 ACGTAGTAAG AGAGCTTTGA AACAATTCAG ACTCCCACTA GAAGAAACAG AACTTGAAAA
4741 AAGGATAATT GTGGATGACA CCTCAACCCA GTGGTCCAAA AACATGAAAC ATTTGACCCC
4801 GAGCACCCTC ACACAGATAG ACTACAATGA GAAGGAGAAA GGGGCCATTA CTCAGTCTCC
4861 CTTATCAGAT TGCCTTACGA GGAGTCATAG CATCCCTCAA GCAAATAGAT CTCCATTACC
4921 CATTGCAAAG GTATCATCAT TTCCATCTAT TAGACCTATA TATCTGACCA GGGTCCTATT
4981 CCAAGACAAC TCTTCTCATC TTCCAGCAGC ATCTTATAGA AAGAAAGATT CTGGGGTCCA
5041 AGAAAGCAGT CATTTCTTAC AAGGAGCCAA AAAAAATAAC CTTTCTTTAG CCATTCTAAC
5101 CTTGGAGATG ACTGGTGATC AAAGAGAGGT TGGCTCCCTG GGGACAAGTG CCACAAATTC
5161 AGTCACATAC AAGAAAGTTG AGAACACTGT TCTCCCGAAA CCAGACTTGC CCAAAACATC
5221 TGGCAAAGTT GAATTGCTTC CAAAAGTTCA CATTTATCAG AAGGACCTAT TCCCTACGGA
5281 AACTAGCAAT GGGTCTCCTG GCCATCTGGA TCTCGTGGAA GGGAGCCTTC TTCAGGGAAC
5341 AGAGGGAGCG ATTAAGTGGA ATGAAGCAAA CAGACCTGGA AAAGTTCCCT TTCTGAGAGT
5401 AGCAACAGAA AGCTCTGCAA AGACTCCCTC CAAGCTATTG GATCCTCTTG CTTGGGATAA
5461 CCACTATGGT ACTCAGATAC CAAAAGAAGA GTGGAAATCC CAAGAGAAGT CACCAGAAAA
5521 AACAGCTTTT AAGAAAAAGG ATACCATTTT GTCCCTGAAC GCTTGTGAAA GCAATCATGC
5581 AATAGCAGCA ATAAATGAGG GACAAAATAA GCCCGAAATA GAAGTCACCT GGGCAAAGCA
5641 AGGTAGGACT GAAAGGCTGT GCTCTCAAAA CCCACCAGTC TTGAAACGCC ATCAACGGGA
5701 AATAACTCGT ACTACTCTTC AGTCAGATCA AGAGGAAATT GACTATGATG ATACCATATC
5761 AGTTGAAATG AAGAAGGAAG ATTTTGACAT TTATGATGAG GATGAAAATC AGAGCCCCCG
5821 CAGCTTTCAA AAGAAAACAC GACACTATTT TATTGCTGCA GTGGAGAGGC TCTGGGATTA
5881 TGGGATGAGT AGCTCCCCAC ATGTTCTAAG AAACAGGGCT CAGAGTGGCA GTGTCCCTCA
5941 GTTCAAGAAA GTTGTTTTCC AGGAATTTAC TGATGGCTCC TTTACTCAGC CCTTATACCG
6001 TGGAGAACTA AATGAACATT TGGGACTCCT GGGGCCATAT ATAAGAGCAG AAGTTGAAGA
6061 TAATATCATG GTAACTTTCA GAAATCAGGC CTCTCGTCCC TATTCCTTCT ATTCTAGCCT
6121 TATTTCTTAT GAGGAAGATC AGAGGCAAGG AGCAGAACCT AGAAAAAACT TTGTCAAGCC
6181 TAATGAAACC AAAACTTACT TTTGGAAAGT GCAACATCAT ATGGCACCCA CTAAAGATGA
6241 GTTTGACTGC AAAGCCTGGG CTTATTTCTC TGATGTTGAC CTGGAAAAAG ATGTGCACTC
6301 AGGCCTGATT GGACCCCTTC TGGTCTGCCA CACTAACACA CTGAACCCTG CTCATGGGAG
6361 ACAAGTGACA GTACAGGAAT TTGCTCTGTT TTTCACCATC TTTGATGAGA CCAAAAGCTG
6421 GTACTTCACT GAAAATATGG AAAGAAACTG CAGGGCTCCC TGCAATATCC AGATGGAAGA
6481 TCCCACTTTT AAAGAGAATT ATCGCTTCCA TGCAATCAAT GGCTACATAA TGGATACACT
6541 ACCTGGCTTA GTAATGGCTC AGGATCAAAG GATTCGATGG TATCTGCTCA GCATGGGCAG
6601 CAATGAAAAC ATCCATTCTA TTCATTTCAG TGGACATGTG TTCACTGTAC GAAAAAAAGA
6661 GGAGTATAAA ATGGCACTGT ACAATCTCTA TCCAGGTGTT TTTGAGACAG TGGAAATGTT
6721 ACCATCCAAA GCTGGAATTT GGCGGGTGGA ATGCCTTATT GGCGAGCATC TACATGCTGG
6781 GATGAGCACA CTTTTTCTGG TGTACAGCAA TAAGTGTCAG ACTCCCCTGG GAATGGCTTC
6841 TGGACACATT AGAGATTTTC AGATTACAGC TTCAGGACAA TATGGACAGT GGGCCCCAAA
6901 GCTGGCCAGA CTTCATTATT CCGGATCAAT CAATGCCTGG AGCACCAAGG AGCCCTTTTC
6961 TTGGATCAAG GTGGATCTGT TGGCACCAAT GATTATTCAC GGCATCAAGA CCCAGGGTGC
7021 CCGTCAGAAG TTCTCCAGCC TCTACATCTC TCAGTTTATC ATCATGTATA GTCTTGATGG
7081 GAAGAAGTGG CAGACTTATC GAGGAAATTC CACTGGAACC TTAATGGTCT TCTTTGGCAA
7141 TGTGGATTCA TCTGGGATAA AACACAATAT TTTTAACCCT CCAATTATTG CTCGATACAT
7201 CCGTTTGCAC CCAACTCATT ATAGCATTCG CAGCACTCTT CGCATGGAGT TGATGGGCTG
7261 TGATTTAAAT AGTTGCAGCA TGCCATTGGG AATGGAGAGT AAAGCAATAT CAGATGCACA
7321 GATTACTGCT TCATCCTACT TTACCAATAT GTTTGCCACC TGGTCTCCTT CAAAAGCTCG
7381 ACTTCACCTC CAAGGGAGGA GTAATGCCTG GAGACCTCAG GTGAATAATC CAAAAGAGTG
7441 GCTGCAAGTG GACTTCCAGA AGACAATGAA AGTCACAGGA GTAACTACTC AGGGAGTAAA
7501 ATCTCTGCTT ACCAGCATGT ATGTGAAGGA GTTCCTCATC TCCAGCAGTC AAGATGGCCA
7561 TCAGTGGACT CTCTTTTTTC AGAATGGCAA AGTAAAGGTT TTTCAGGGAA ATCAAGACTC
7621 CTTCACACCT GTGGTGAACT CTCTAGACCC ACCGTTACTG ACTCGCTACC TTCGAATTCA
7681 CCCCCAGAGT TGGGTGCACC AGATTGCCCT GAGGATGGAG GTTCTGGGCT GCGAGGCACA
7741 GGACCTCTAC

*The underlined nucleic acids encode a signal peptide.

[0144] FVIII polypeptides include full-length FVIII, full-length FVIII minus Met at the N-terminus, mature FVIII (minus the signal sequence), mature FVIII with an additional Met at the N-terminus, and/or FVIII with a full or partial deletion of the B domain. In certain embodiments, FVIII variants include B domain deletions, whether partial or full deletions.

[0145] The human FVIII gene was isolated and expressed in mammalian cells (Toole, J. J., *et al., Nature* 312:342-347 (1984); Gitschier, J., *et al., Nature* 312:326-330 (1984); Wood, W. I., *et al., Nature* 312:330-337 (1984); Vehar, G. A., *et al., Nature* 312:337-342 (1984); WO 87/04187; WO 88/08035; WO 88/03558; and U.S. Pat. No. 4,757,006). The FVIII amino acid sequence was deduced from cDNA as shown in U.S. Pat. No. 4,965,199. In addition, partially or fully B-domain deleted FVIII is shown in U.S. Pat. Nos. 4,994,371 and 4,868,112. In some embodiments, the human FVIII B-domain is replaced with the human Factor V B-domain as shown in U.S. Pat. No. 5,004,803. The cDNA sequence encoding human Factor VIII and amino acid sequence are shown in SEQ ID NOs: 1 and 2, respectively, of U.S Patent No. 7,211,559.

[0146] The porcine FVIII sequence is published in Toole, J. J., *et al., Proc. Natl. Acad. Sci. USA* 83:5939-5942 (1986). Further, the complete porcine cDNA sequence obtained from PCR amplification of FVIII sequences from a pig spleen cDNA library has been reported in Healey, J. F., *et al., Blood* 88:4209-4214 (1996). Hybrid human/porcine FVIII having substitutions of all domains, all subunits, and specific amino acid sequences were disclosed in U.S. Pat. No. 5,364,771 by Lollar and Runge, and in WO 93/20093. More recently, the nucleotide and corresponding amino acid sequences of the A1 and A2 domains of porcine FVIII and a chimeric FVIII with porcine A1 and/or A2 domains substituted for the corresponding human domains were reported in WO 94/11503. U.S. Pat. No. 5,859,204, Lollar, J. S., also discloses the porcine cDNA and deduced amino acid sequences. U.S. Pat. No. 6,458,563 discloses a B-domain-deleted porcine FVIII.

[0147] U.S. Pat. No. 5,859,204 to Lollar, J. S. reports functional mutants of FVIII having reduced antigenicity and reduced immunoreactivity. U.S. Pat. No. 6,376,463 to Lollar, J. S. also reports mutants of FVIII having reduced immunoreactivity. US Appl. Publ. No. 2005/0100990 to Saenko *et al.* reports functional mutations in the A2 domain of FVIII.

[0148] In one embodiment, the FVIII protein (or FVIII portion of a chimeric protein) may be at least 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to

a FVIII amino acid sequence of amino acids 1 to 1438 of SEQ ID NO: 18 or amino acids 1 to 2332 of SEQ ID NO: 16 (without a signal sequence), wherein the FVIII has a clotting activity, *e.g.*, activates Factor IX as a cofactor to convert Factor X to activated Factor X. The FVIII (or FVIII portion of a chimeric protein) may be identical to a FVIII amino acid sequence of amino acids 1 to 1438 of SEQ ID NO: 18 or amino acids 1 to 2332 of SEQ ID NO: 16 (without a signal sequence). The FVIII protein may further comprise a signal sequence.

[0149] The "B-domain" of FVIII, as used herein, is the same as the B-domain known in the art that is defined by internal amino acid sequence identity and sites of proteolytic cleavage, *e.g.*, residues Ser741-Arg1648 of full-length human FVIII. The other human FVIII domains are defined by the following amino acid residues: A1, residues Ala1-Arg372; A2, residues Ser373-Arg740; A3, residues Ser1690-Asn2019; C1, residues Lys2020-Asn2172; C2, residues Ser2173-Tyr2332. The A3-C1-C2 sequence includes residues Ser1690-Tyr2332. The remaining sequence, residues Glu1649-Arg1689, is usually referred to as the a3 acidic region. The locations of the boundaries for all of the domains, including the B-domains, for porcine, mouse and canine FVIII are also known in the art. In one embodiment, the B domain of FVIII is deleted ("B-domain-deleted factor VIII" or "BDD FVIII"). An example of a BDD FVIII is REFACTO® (recombinant BDD FVIII), which has the same sequence as the Factor VIII portion of the sequence in Table 4. (BDD FVIII heavy chain is double underlined; B domain is italicized; and BDD FVIII light chain is in plain text).

Table 4

BDD FVIII (SEQ ID NO: 18)

ATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPKSFPFNTSVVYKKTLFVEFTDHLFNIAKPRPPWMGLL
GPTIQAEVYDTVVITLKNMASHPVSLHAVGVSYWKASEGAEYDDQTSQREKEDDKVFPGGSHTYVWQVLKEN
GPMASDPLCLTYSYLSHVDLVKDLNSGLIGALLVCREGSLAKEKTQTLHKFILLFAVFDEGKSWHSETKNSL
MQDRDAASARAWPKMHTVNGYVNRSLPGLIGCHRKSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEI
SPITFLTAQTLLMDLGQFLLFCHISSHQHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDLTDSEMDVVRF
DDDNSPSFIQIRSVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYKSQYLNNGPQRIGRKYKKVRFMAYT
DETFKTREAIQHESGILGPLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVRPLYSRRLPKGVKHLKDFPIL
PGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFVNMERDLASGLIGPLLICYKESVDQRGNQIMSDKRNVILF
SVFDENRSWYLTENIQRFLPNPAGVQLEDPEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDF
LSVFFSGYTFKHKMVYEDTLTLFPFSGETVFMSMENPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYYE
DSYEDISAYLLSKNNAIEPR*SFSQNPPVLKRHQR*EITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQ
SPRSFQKKTRHYFIAAVERLWDYGMSSSPHVLRNRAQSGSVPQFKKVVFQEFTDGSFTQPLYRGELNEHLGL
LGPYIRAEVEDNIMVTFRNQASRPYSFYSSLISYEEDQRQGAEPRKNFVKPNETKTYFWKVQHHMAPTKDEF
DCKAWAYFSDVDLEKDVHSGLIGPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAP
CNIQMEDPTFKENYRFHAINGYIMDTLPGLVMAQDQRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMA
LYNLYPGVFETVEMLPSKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQW

APKLARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFSSLYISQFIIMYSLDGKKWQTYRGN
STGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIRSTLRMELMGCDLNSCSMPLGMESKAISDAQI
TASSYFTNMFATWSPSKARLHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTGVTTQGVKSLLTSMYVKEFLI
SSSQDGHQWTLFFQNGKVKVFQGNQDSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRMEVLGCEAQDLY

Table 5. Nucleotide Sequence Encoding BDD FVIII (SEQ ID NO: 19)*

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661                                   A TGCAAATAGA GCTCTCCACC TGCTTCTTTC
721  TGTGCCTTTT GCGATTCTGC TTTAGTGCCA CCAGAAGATA CTACCTGGGT GCAGTGGAAC
781  TGTCATGGGA CTATATGCAA AGTGATCTCG GTGAGCTGCC TGTGGACGCA AGATTTCCTC
841  CTAGAGTGCC AAAATCTTTT CCATTCAACA CCTCAGTCGT GTACAAAAAG ACTCTGTTTG
901  TAGAATTCAC GGATCACCTT TTCAACATCG CTAAGCCAAG GCCACCCTGG ATGGGTCTGC
961  TAGGTCCTAC CATCCAGGCT GAGGTTTATG ATACAGTGGT CATTACACTT AAGAACATGG
1021 CTTCCCATCC TGTCAGTCTT CATGCTGTTG GTGTATCCTA CTGGAAAGCT TCTGAGGGAG
1081 CTGAATATGA TGATCAGACC AGTCAAAGGG AGAAAGAAGA TGATAAAGTC TTCCCTGGTG
1141 GAAGCCATAC ATATGTCTGG CAGGTCCTGA AAGAGAATGG TCCAATGGCC TCTGACCCAC
1201 TGTGCCTTAC CTACTCATAT CTTTCTCATG TGGACCTGGT AAAAGACTTG AATTCAGGCC
1261 TCATTGGAGC CCTACTAGTA TGTAGAGAAG GGAGTCTGGC CAAGGAAAAG ACACAGACCT
1321 TGCACAAATT TATACTACTT TTTGCTGTAT TTGATGAAGG GAAAAGTTGG CACTCAGAAA
1381 CAAAGAACTC CTTGATGCAG GATAGGGATG CTGCATCTGC TCGGGCCTGG CCTAAAATGC
1441 ACACAGTCAA TGGTTATGTA AACAGGTCTC TGCCAGGTCT GATTGGATGC CACAGGAAAT
1501 CAGTCTATTG GCATGTGATT GGAATGGGCA CCACTCCTGA AGTGCACTCA ATATTCCTCG
1561 AAGGTCACAC ATTTCTTGTG AGGAACCATC GCCAGGCGTC CTTGGAAATC TCGCCAATAA
1621 CTTTCCTTAC TGCTCAAACA CTCTTGATGG ACCTTGGACA GTTTCTACTG TTTTGTCATA
1681 TCTCTTCCCA CCAACATGAT GGCATGGAAG CTTATGTCAA AGTAGACAGC TGTCCAGAGG
1741 AACCCCAACT ACGAATGAAA AATAATGAAG AAGCGGAAGA CTATGATGAT GATCTTACTG
1801 ATTCTGAAAT GGATGTGGTC AGGTTTGATG ATGACAACTC TCCTTCCTTT ATCCAAATTC
1861 GCTCAGTTGC CAAGAAGCAT CCTAAAACTT GGGTACATTA CATTGCTGCT GAAGAGGAGG
1921 ACTGGGACTA TGCTCCCTTA GTCCTCGCCC CCGATGACAG AAGTTATAAA AGTCAATATT
1981 TGAACAATGG CCCTCAGCGG ATTGGTAGGA AGTACAAAAA AGTCCGATTT ATGGCATACA
2041 CAGATGAAAC CTTTAAGACT CGTGAAGCTA TTCAGCATGA ATCAGGAATC TTGGGACCTT
2101 TACTTTATGG GGAAGTTGGA GACACACTGT TGATTATATT TAAGAATCAA GCAAGCAGAC
2161 CATATAACAT CTACCCTCAC GGAATCACTG ATGTCCGTCC TTTGTATTCA AGGAGATTAC
2221 CAAAAGGTGT AAAACATTTG AAGGATTTTC CAATTCTGCC AGGAGAAATA TTCAAATATA
2281 AATGGACAGT GACTGTAGAA GATGGGCCAA CTAAATCAGA TCCTCGGTGC CTGACCCGCT
2341 ATTACTCTAG TTTCGTTAAT ATGGAGAGAG ATCTAGCTTC AGGACTCATT GGCCCTCTCC
2401 TCATCTGCTA CAAAGAATCT GTAGATCAAA GAGGAAACCA GATAATGTCA GACAAGAGGA
2461 ATGTCATCCT GTTTTCTGTA TTTGATGAGA ACCGAAGCTG GTACCTCACA GAGAATATAC
2521 AACGCTTTCT CCCCAATCCA GCTGGAGTGC AGCTTGAGGA TCCAGAGTTC CAAGCCTCCA
2581 ACATCATGCA CAGCATCAAT GGCTATGTTT TTGATAGTTT GCAGTTGTCA GTTTGTTTGC
2641 ATGAGGTGGC ATACTGGTAC ATTCTAAGCA TTGGAGCACA GACTGACTTC CTTTCTGTCT
2701 TCTTCTCTGG ATATACCTTC AAACACAAAA TGGTCTATGA AGACACACTC ACCCTATTCC
2761 CATTCTCAGG AGAAACTGTC TTCATGTCGA TGGAAAACCC AGGTCTATGG ATTCTGGGGT
2821 GCCACAACTC AGACTTTCGG AACAGAGGCA TGACCGCCTT ACTGAAGGTT TCTAGTTGTG
2881 ACAAGAACAC TGGTGATTAT TACGAGGACA GTTATGAAGA TATTTCAGCA TACTTGCTGA
2941 GTAAAAACAA TGCCATTGAA CCAAGAAGCT TCTCTCAAAA CCCACCAGTC TTGAAACGCC
3001 ATCAACGGGA AATAACTCGT ACTACTCTTC AGTCAGATCA AGAGGAAATT GACTATGATG
3061 ATACCATATC AGTTGAAATG AAGAAGGAAG ATTTTGACAT TTATGATGAG GATGAAAATC
3121 AGAGCCCCCG CAGCTTTCAA AAGAAAACAC GACACTATTT TATTGCTGCA GTGGAGAGGC
3181 TCTGGGATTA TGGGATGAGT AGCTCCCCAC ATGTTCTAAG AAACAGGGCT CAGAGTGGCA
3241 GTGTCCCTCA GTTCAAGAAA GTTGTTTTCC AGGAATTTAC TGATGGCTCC TTTACTCAGC
3301 CCTTATACCG TGGAGAACTA AATGAACATT TGGGACTCCT GGGGCCATAT ATAAGAGCAG
3361 AAGTTGAAGA TAATATCATG GTAACTTTCA GAAATCAGGC CTCTCGTCCC TATTCCTTCT
3421 ATTCTAGCCT TATTTCTTAT GAGGAAGATC AGAGGCAAGG AGCAGAACCT AGAAAAAACT
3481 TTGTCAAGCC TAATGAAACC AAAACTTACT TTTGGAAAGT GCAACATCAT ATGGCACCCA
3541 CTAAAGATGA GTTTGACTGC AAAGCCTGGG CTTATTTCTC TGATGTTGAC CTGGAAAAAG
3601 ATGTGCACTC AGGCCTGATT GGACCCCTTC TGGTCTGCCA CACTAACACA CTGAACCCTG
3661 CTCATGGGAG ACAAGTGACA GTACAGGAAT TTGCTCTGTT TTTCACCATC TTTGATGAGA
3721 CCAAAAGCTG GTACTTCACT GAAAATATGG AAAGAAACTG CAGGGCTCCC TGCAATATCC
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3781    AGATGGAAGA TCCCACTTTT AAAGAGAATT ATCGCTTCCA TGCAATCAAT GGCTACATAA
3841    TGGATACACT ACCTGGCTTA GTAATGGCTC AGGATCAAAG GATTCGATGG TATCTGCTCA
3901    GCATGGGCAG CAATGAAAAC ATCCATTCTA TTCATTTCAG TGGACATGTG TTCACTGTAC
3961    GAAAAAAAGA GGAGTATAAA ATGGCACTGT ACAATCTCTA TCCAGGTGTT TTTGAGACAG
4021    TGGAAATGTT ACCATCCAAA GCTGGAATTT GGCGGGTGGA ATGCCTTATT GGCGAGCATC
4081    TACATGCTGG GATGAGCACA CTTTTTCTGG TGTACAGCAA TAAGTGTCAG ACTCCCCTGG
4141    GAATGGCTTC TGGACACATT AGAGATTTTC AGATTACAGC TTCAGGACAA TATGGACAGT
4201    GGGCCCCAAA GCTGGCCAGA CTTCATTATT CCGGATCAAT CAATGCCTGG AGCACCAAGG
4261    AGCCCTTTTC TTGGATCAAG GTGGATCTGT TGGCACCAAT GATTATTCAC GGCATCAAGA
4321    CCCAGGGTGC CCGTCAGAAG TTCTCCAGCC TCTACATCTC TCAGTTTATC ATCATGTATA
4381    GTCTTGATGG GAAGAAGTGG CAGACTTATC GAGGAAATTC CACTGGAACC TTAATGGTCT
4441    TCTTTGGCAA TGTGGATTCA TCTGGGATAA AACACAATAT TTTTAACCCT CCAATTATTG
4501    CTCGATACAT CCGTTTGCAC CCAACTCATT ATAGCATTCG CAGCACTCTT CGCATGGAGT
4561    TGATGGGCTG TGATTTAAAT AGTTGCAGCA TGCCATTGGG AATGGAGAGT AAAGCAATAT
4621    CAGATGCACA GATTACTGCT TCATCCTACT TTACCAATAT GTTTGCCACC TGGTCTCCTT
4681    CAAAAGCTCG ACTTCACCTC CAAGGGAGGA GTAATGCCTG GAGACCTCAG GTGAATAATC
4741    CAAAAGAGTG GCTGCAAGTG GACTTCCAGA AGACAATGAA AGTCACAGGA GTAACTACTC
4801    AGGGAGTAAA ATCTCTGCTT ACCAGCATGT ATGTGAAGGA GTTCCTCATC TCCAGCAGTC
4861    AAGATGGCCA TCAGTGGACT CTCTTTTTTC AGAATGGCAA AGTAAAGGTT TTTCAGGGAA
4921    ATCAAGACTC CTTCACACCT GTGGTGAACT CTCTAGACCC ACCGTTACTG ACTCGCTACC
4981    TTCGAATTCA CCCCCAGAGT TGGGTGCACC AGATTGCCCT GAGGATGGAG GTTCTGGGCT
5041    GCGAGGCACA GGACCTCTAC
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*The underlined nucleic acids encode a signal peptide.

[0150] A "B-domain-deleted FVIII" may have the full or partial deletions disclosed in U.S. Pat. Nos. 6,316,226, 6,346,513, 7,041,635, 5,789,203, 6,060,447, 5,595,886, 6,228,620, 5,972,885, 6,048,720, 5,543,502, 5,610,278, 5,171,844, 5,112,950, 4,868,112, and 6,458,563. In some embodiments, a B-domain-deleted FVIII sequence of the present invention comprises any one of the deletions disclosed at col. 4, line 4 to col. 5, line 28 and Examples 1-5 of U.S. Pat. No. 6,316,226 (also in US 6,346,513). In another embodiment, a B-domain deleted Factor VIII is the S743/Q1638 B-domain deleted Factor VIII (SQ BDD FVIII) (*e.g.*, Factor VIII having a deletion from amino acid 744 to amino acid 1637, *e.g.*, Factor VIII having amino acids 1-743 and amino acids 1638-2332 of SEQ ID NO: 16, *i.e.*, SEQ ID NO: 18). In some embodiments, a B-domain-deleted FVIII of the present invention has a deletion disclosed at col. 2, lines 26-51 and examples 5-8 of U.S. Patent No. 5,789,203 (also US 6,060,447, US 5,595,886, and US 6,228,620). In some embodiments, a B-domain-deleted Factor VIII has a deletion described in col. 1, lines 25 to col. 2, line 40 of US Patent No. 5,972,885; col. 6, lines 1-22 and example 1 of U.S. Patent no. 6,048,720; col. 2, lines 17-46 of U.S. Patent No. 5,543,502; col. 4, line 22 to col. 5, line 36 of U.S. Patent no. 5,171,844; col. 2, lines 55-68, figure 2, and example 1 of U.S. Patent No. 5,112,950; col. 2, line 2 to col. 19, line 21 and table 2 of U.S. Patent No. 4,868,112; col. 2, line 1 to col. 3, line 19, col. 3, line 40 to col. 4, line 67, col. 7, line

43 to col. 8, line 26, and col. 11, line 5 to col. 13, line 39 of U.S. Patent no. 7,041,635; or col. 4, lines 25-53, of U.S. Patent No. 6,458,563.

[0151] In some embodiments, a B-domain-deleted FVIII has a deletion of most of the B domain, but still contains amino-terminal sequences of the B domain that are essential for *in vivo* proteolytic processing of the primary translation product into two polypeptide chain, as disclosed in WO 91/09122. In some embodiments, a B-domain-deleted FVIII is constructed with a deletion of amino acids 747-1638, *i.e.*, virtually a complete deletion of the B domain. Hoeben R.C., *et al. J. Biol. Chem.* 265 (13): 7318-7323 (1990). A B-domain-deleted Factor VIII may also contain a deletion of amino acids 771–1666 or amino acids 868-1562 of FVIII. Meulien P., *et al. Protein Eng.* 2(4): 301-6 (1988). Additional B domain deletions that are part of the invention include: deletion of amino acids 982 through 1562 or 760 through 1639 (Toole *et al., Proc. Natl. Acad. Sci. U.S.A.* (1986) 83, 5939-5942)), 797 through 1562 (Eaton, *et al. Biochemistry* (1986) 25:8343-8347)), 741 through 1646 (Kaufman (PCT published application No. WO 87/04187)), 747-1560 (Sarver, *et al., DNA* (1987) 6:553-564)), 741 through 1648 (Pasek (PCT application No.88/00831)), or 816 through 1598 or 741 through 1648 (Lagner (Behring Inst. Mitt. (1988) No 82:16-25, EP 295597)). In other embodiments, BDD FVIII includes a FVIII polypeptide containing fragments of the B-domain that retain one or more N-linked glycosylation sites, *e.g.*, residues 757, 784, 828, 900, 963, or optionally 943, which correspond to the amino acid sequence of the full-length FVIII sequence. Examples of the B-domain fragments include 226 amino acids or 163 amino acids of the B-domain as disclosed in Miao, H.Z., *et al., Blood* 103(a): 3412-3419 (2004), Kasuda, A, *et al., J. Thromb. Haemost.* 6: 1352-1359 (2008), and Pipe, S.W., *et al., J. Thromb. Haemost.* 9: 2235-2242 (2011) (*i.e.*, the first 226 amino acids or 163 amino acids of the B domain are retained). In some embodiments, the FVIII with a partial B-domain is FVIII198. FVIII198 is a partial B-domain containing single chain FVIIIFc molecule-226N6. 226 represents the N-terminus 226 amino acid of the FVIII B-domain, and N6 represents six N-glycosylation sites in the B-domain. In still other embodiments, BDD FVIII further comprises a point mutation at residue 309 (from Phe to Ser) to improve expression of the BDD FVIII protein. *See* Miao, H.Z., et al., Blood 103(a): 3412-3419 (2004). In still other embodiments, the BDD FVIII includes a FVIII polypeptide containing a portion of the B-domain, but not containing one or more furin cleavage sites (*e.g.*, Arg1313 and Arg

1648). *See* Pipe, S.W., *et al., J. Thromb. Haemost.* 9: 2235-2242 (2011). Each of the foregoing deletions may be made in any FVIII sequence.

[0152] A FVIII protein useful in the present invention can include FVIII having one or more additional heterologous sequences or chemical or physical modifications therein, which do not affect the FVIII coagulation activity. Such heterologous sequences or chemical or physical modifications can be fused to the C-terminus or N-terminus of the FVIII protein or inserted between one or more of the two amino acid residues in the FVIII protein. Such insertions in the FVIII protein do not affect the FVIII coagulation activity or FVIII function. In one embodiment, the insertions improve pharmacokinetic properties of the FVIII protein (*e.g.*, half-life). In another embodiment, the insertions can be more than two, three, four, five, or six sites.

[0153] In one embodiment, FVIII is cleaved right after Arginine at amino acid 1648 (in full-length Factor VIII or SEQ ID NO: 16), amino acid 754 (in the S743/Q1638 B-domain deleted Factor VIII or SEQ ID NO: 16), or the corresponding Arginine residue (in other variants), thereby resulting in a heavy chain and a light chain. In another embodiment, FVIII comprises a heavy chain and a light chain, which are linked or associated by a metal ion-mediated non-covalent bond.

[0154] In other embodiments, FVIII is a single chain FVIII that has not been cleaved right after Arginine at amino acid 1648 (in full-length FVIII or SEQ ID NO: 16), amino acid 754 (in the S743/Q1638 B-domain-deleted FVIII or SEQ ID NO: 18), or the corresponding Arginine residue (in other variants). A single chain FVIII may comprise one or more amino acid substitutions. In one embodiment, the amino acid substitution is at a residue corresponding to residue 1648, residue 1645, or both of full-length mature Factor VIII polypeptide (SEQ ID NO: 16) or residue 754, residue 751, or both of SQ BDD Factor VIII (SEQ ID NO: 18). The amino acid substitution can be any amino acids other than Arginine, *e.g.*, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, selenocysteine, serine, tyrosine, histidine, ornithine, pyrrolysine, or taurine.

[0155] FVIII can further be cleaved by thrombin and then activated as FVIIIa, serving as a cofactor for activated Factor IX (FIXa). And the activated FIX together with activated FVIII forms a Xase complex and converts Factor X to activated Factor X (FXa). For

activation, FVIII is cleaved by thrombin after three Arginine residues, at amino acids 372, 740, and 1689 (corresponding to amino acids 372, 740, and 795 in the B-domain deleted FVIII sequence), the cleavage generating FVIIIa having the 50kDa A1, 43kDa A2, and 73kDa A3-C1-C2 chains. In one embodiment, the FVIII protein useful for the present invention is non-active FVIII. In another embodiment, the FVIII protein is an activated FVIII.

[0156] The protein having FVIII polypeptide linked to or associated with the VWF fragment can comprise a sequence at least 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 16 or 18, wherein the sequence has the FVIII clotting activity, *e.g.*, activating Factor IX as a cofactor to convert Factor X to activated Factor X (FXa).

[0157] In some embodiments, the FVIII protein further comprises one or more heterologous moieties that are fused to the C-terminus or N-terminus of the FVIII protein or that are inserted between two adjacent amino acids in the FVIII protein. In other embodiments, the heterologous moieties comprise an amino acid sequence of at least about 50 amino acids, at least about 100 amino acids, at least about 150 amino acids, at least about 200 amino acids, at least about 250 amino acids, at least about 300 amino acids, at least about 350 amino acids, at least about 400 amino acids, at least about 450 amino acids, at least about 500 amino acids, at least about 550 amino acids, at least about 600 amino acids, at least about 650 amino acids, at least about 700 amino acids, at least about 750 amino acids, at least about 800 amino acids, at least about 850 amino acids, at least about 900 amino acids, at least about 950 amino acids, or at least about 1000 amino acids. In some embodiments, the half-life of the chimeric molecule is extended at least about 1.5 times, at least about 2 times, at least about 2.5 times, at least about 3 times, at least about 4 times, at least about 5 times, at least about 6 times, at least about 7 times, at least about 8 times, at least about 9 times, at least about 10 times, at least about 11 times, or at least about 12 times longer than wild-type FVIII.

III. Polynucleotides, Vectors, Host cells, and Methods of Making

[0158] Also provided in the invention is a polynucleotide encoding the chimeric molecule described herein. When a VWF protein is linked to a heterologous moiety via a VWF linker and to a FVIII protein in a chimeric protein as a single polypeptide chain, the invention is drawn to a single polynucleotide encoding the single polypeptide chain.

When the chimeric protein comprises a first and a second polypeptide chains, the first polypeptide chain comprising a VWF protein and a first heterologous moiety (*e.g.*, a first Fc region) and the second polypeptide chain comprising a FVIII protein and a second heterologous moiety (*e.g.*, a second Fc region), a polynucleotide can comprise the first nucleotide region and the second nucleotide region. In one embodiment, the first nucleotide region and the second nucleotide region are on the same polynucleotide. In another embodiment, the first nucleotide region and the second nucleotide region are on two different polynucleotides (*e.g.*, different vectors). In certain embodiments, the present invention is directed to a set of polynucleotides comprising a first nucleotide chain and a second nucleotide chain, wherein the first nucleotide chain encodes a VWF protein, a VWF linker, and a heterologous moiety of the chimeric protein and the second nucleotide chain encodes a FVIII protein and a second heterologous moiety.

[0159] In some embodiments, a set of polynucleotides comprises a first nucleotide sequence encoding a VWF protein fused to a first heterologous moiety and a second nucleotide sequence encoding a FVIII protein fused to a second heterologous moiety via a FVIII linker.

[0160] In some embodiments, a set of polynucleotides comprises a first nucleotide sequence encoding a VWF protein fused to a first heterologous moiety via a VWF linker and a second nucleotide sequence encoding a FVIII protein fused to a second heterologous moiety via a FVIII linker.

[0161] In other embodiments, the set of polynucleotides further comprises an additional nucleotide chain (*e.g.*, a second nucleotide chain when the chimeric polypeptide is encoded by a single polynucleotide chain or a third nucleotide chain when the chimeric protein is encoded by two polynucleotide chains) which encodes a protein convertase. The protein convertase can be selected from proprotein convertase subtilisin/kexin type 5 (PCSK5 or PC5), proprotein convertase subtilisin/kexin type 7 (PCSK7 or PC5), a yeast Kex 2, proprotein convertase subtilisin/kexin type 3 (PACE or PCSK3), or two or more combinations thereof. In some embodiments, the protein convertase is PACE, PC5, or PC7. In a specific embodiment, the protein convertase is PC5 or PC7. See International Application no. PCT/US2011/043568, which is incorporated herein by reference. In another embodiment, the protein convertase is PACE/Furin.

[0162] In certain embodiments, the invention includes a set of the polynucleotides comprising a first nucleotide sequence encoding a VWF protein comprising a D' domain and a D3 domain of VWF fused to a first heterologous moiety via a VWF linker, a second nucleotide sequence encoding a FVIII protein and a second heterologous moiety, and a third nucleotide sequence encoding a D1 domain and D2 domain of VWF. In this embodiment, the D1 domain and D2 domain are separately expressed (not linked to the D'D3 domain of the VWF protein) in order for the proper disulfide bond formation and folding of the D'D3 domains. The D1D2 domain expression can either be in cis or trans.

[0163] As used herein, an expression vector refers to any nucleic acid construct which contains the necessary elements for the transcription and translation of an inserted coding sequence, or in the case of an RNA viral vector, the necessary elements for replication and translation, when introduced into an appropriate host cell. Expression vectors can include plasmids, phagemids, viruses, and derivatives thereof.

[0164] Expression vectors of the invention will include polynucleotides encoding the chimeric molecule.

[0165] In one embodiment, a coding sequence for the chimeric molecule is operably linked to an expression control sequence. As used herein, two nucleic acid sequences are operably linked when they are covalently linked in such a way as to permit each component nucleic acid sequence to retain its functionality. A coding sequence and a gene expression control sequence are said to be operably linked when they are covalently linked in such a way as to place the expression or transcription and/or translation of the coding sequence under the influence or control of the gene expression control sequence. Two DNA sequences are said to be operably linked if induction of a promoter in the 5' gene expression sequence results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be operably linked to a coding nucleic acid sequence if the gene expression sequence were capable of effecting transcription of that coding nucleic acid sequence such that the resulting transcript is translated into the desired protein or polypeptide.

[0166] A gene expression control sequence as used herein is any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the coding nucleic acid to which it is operably linked. The gene expression control sequence may, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPRT), adenosine deaminase, pyruvate kinase, beta-actin promoter, and other constitutive promoters. Exemplary viral promoters which function constitutively in eukaryotic cells include, for example, promoters from the cytomegalovirus (CMV), simian virus (*e.g.*, SV40), papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus, the long terminal repeats (LTR) of Moloney leukemia virus, and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters. Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein promoter is induced to promote transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

[0167] In general, the gene expression control sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined coding nucleic acid. The gene expression sequences optionally include enhancer sequences or upstream activator sequences as desired.

[0168] Viral vectors include, but are not limited to, nucleic acid sequences from the following viruses: retrovirus, such as Moloney murine leukemia virus, Harvey murine sarcoma virus, murine mammary tumor virus, and Rous sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyomaviruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors well-known in the art. Certain viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have

been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (*i.e.*, capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., Gene Transfer and Expression, A Laboratory Manual, W.H. Freeman Co., New York (1990) and Murry, E. J., Methods in Molecular Biology, Vol. 7, Humana Press, Inc., Cliffton, N.J. (1991).

[0169] In one embodiment, the virus is an adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus can be engineered to be replication-deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

[0170] Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well-known to those of skill in the art. See, *e.g.*, Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been found to be particularly advantageous for delivering genes to cells *in vivo* because of their inability to

replicate within and integrate into a host genome. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operably encoded within the plasmid. Some commonly used plasmids available from commercial suppliers include pBR322, pUC18, pUC19, various pcDNA plasmids, pRC/CMV, various pCMV plasmids, pSV40, and pBlueScript. Additional examples of specific plasmids include pcDNA3.1, catalog number V79020; pcDNA3.1/hygro, catalog number V87020; pcDNA4/myc-His, catalog number V86320; and pBudCE4.1, catalog number V53220, all from Invitrogen (Carlsbad, CA.). Other plasmids are well-known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using standard molecular biology techniques to remove and/or add specific fragments of DNA.

[0171] In one insect expression system that may be used to produce the proteins of the invention, *Autographa californica* nuclear polyhidrosis virus (AcNPV) is used as a vector to express the foreign genes. The virus grows in *Spodoptera frugiperda* cells. A coding sequence may be cloned into non-essential regions (for example, the polyhedron gene) of the virus and placed under control of an ACNPV promoter (for example, the polyhedron promoter). Successful insertion of a coding sequence will result in inactivation of the polyhedron gene and production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedron gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed. (see, *e.g.*, Smith *et al.* (1983) *J Virol* 46:584; U.S. Pat. No. 4,215,051). Further examples of this expression system may be found in Ausubel et al., eds. (1989) Current Protocols in Molecular Biology, Vol. 2, Greene Publish. Assoc. & Wiley Interscience.

[0172] Another system which can be used to express the proteins of the invention is the glutamine synthetase gene expression system, also referred to as the "GS expression system" (Lonza Biologics PLC, Berkshire UK). This expression system is described in detail in U.S. Pat. No. 5,981,216.

[0173] In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, a coding sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is

viable and capable of expressing peptide in infected hosts. See, *e.g.*, Logan & Shenk (1984) *Proc Natl Acad Sci USA* 81:3655). Alternatively, the vaccinia 7.5 K promoter may be used. See, *e.g.*, Mackett *et al.* (1982) *Proc Natl Acad Sci USA* 79:7415; Mackett *et al.* (1984) *J Virol* 49:857; Panicali *et al.* (1982) *Proc Natl Acad Sci USA* 79:4927.

[0174] To increase efficiency of production, the polynucleotides can be designed to encode multiple units of the protein of the invention separated by enzymatic cleavage sites. The resulting polypeptide can be cleaved (*e.g.*, by treatment with the appropriate enzyme) in order to recover the polypeptide units. This can increase the yield of polypeptides driven by a single promoter. When used in appropriate viral expression systems, the translation of each polypeptide encoded by the mRNA is directed internally in the transcript; *e.g.*, by an internal ribosome entry site, IRES. Thus, the polycistronic construct directs the transcription of a single, large polycistronic mRNA which, in turn, directs the translation of multiple, individual polypeptides. This approach eliminates the production and enzymatic processing of polyproteins and may significantly increase the yield of polypeptides driven by a single promoter.

[0175] Vectors used in transformation will usually contain a selectable marker used to identify transformants. In bacterial systems, this can include an antibiotic resistance gene such as ampicillin or kanamycin. Selectable markers for use in cultured mammalian cells include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. One amplifiable selectable marker is the dihydrofolate reductase (DHFR) gene. Simonsen C C *et al.* (1983) *Proc Natl Acad Sci USA* 80:2495-9. Selectable markers are reviewed by Thilly (1986) Mammalian Cell Technology, Butterworth Publishers, Stoneham, Mass., and the choice of selectable markers is well within the level of ordinary skill in the art.

[0176] Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene of interest may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, U.S. Pat. No. 4,713,339).

[0177] The expression vectors can encode for tags that permit easy purification of the recombinantly produced protein. Examples include, but are not limited to, vector pUR278

(Ruther *et al.* (1983) *EMBO J* 2:1791), in which coding sequences for the protein to be expressed may be ligated into the vector in frame with the lac z coding region so that a tagged fusion protein is produced; pGEX vectors may be used to express proteins of the invention with a glutathione S-transferase (GST) tag. These proteins are usually soluble and can easily be purified from cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The vectors include cleavage sites (thrombin or Factor Xa protease or PRESCISSION PROTEASE™ (Pharmacia, Peapack, N.J.)) for easy removal of the tag after purification.

[0178] The expression vector or vectors are then transfected or co-transfected into a suitable target cell, which will express the polypeptides. Transfection techniques known in the art include, but are not limited to, calcium phosphate precipitation (Wigler *et al.* (1978) *Cell* 14:725), electroporation (Neumann *et al.* (1982) *EMBO J* 1:841), and liposome-based reagents. A variety of host-expression vector systems may be utilized to express the proteins described herein including both prokaryotic and eukaryotic cells. These include, but are not limited to, microorganisms such as bacteria (*e.g.*, *E. coli*) transformed with recombinant bacteriophage DNA or plasmid DNA expression vectors containing an appropriate coding sequence; yeast or filamentous fungi transformed with recombinant yeast or fungi expression vectors containing an appropriate coding sequence; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing an appropriate coding sequence; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus or tobacco mosaic virus) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing an appropriate coding sequence; or animal cell systems, including mammalian cells (*e.g.*, HEK 293, CHO, Cos, HeLa, HKB11, and BHK cells).

[0179] In one embodiment, the host cell is a eukaryotic cell. As used herein, a eukaryotic cell refers to any animal or plant cell having a definitive nucleus. Eukaryotic cells of animals include cells of vertebrates, *e.g.*, mammals, and cells of invertebrates, *e.g.*, insects. Eukaryotic cells of plants specifically can include, without limitation, yeast cells. A eukaryotic cell is distinct from a prokaryotic cell, *e.g.*, bacteria.

[0180] In certain embodiments, the eukaryotic cell is a mammalian cell. A mammalian cell is any cell derived from a mammal. Mammalian cells specifically include, but are not limited to, mammalian cell lines. In one embodiment, the mammalian cell is a human

cell. In another embodiment, the mammalian cell is a HEK 293 cell, which is a human embryonic kidney cell line. HEK 293 cells are available as CRL-1533 from American Type Culture Collection, Manassas, VA, and as 293-H cells, Catalog No. 11631-017 or 293-F cells, Catalog No. 11625-019 from Invitrogen (Carlsbad, Calif.). In some embodiments, the mammalian cell is a PER.C6® cell, which is a human cell line derived from retina. PER.C6® cells are available from Crucell (Leiden, The Netherlands). In other embodiments, the mammalian cell is a Chinese hamster ovary (CHO) cell. CHO cells are available from American Type Culture Collection, Manassas, VA. (*e.g.*, CHO-K1; CCL-61). In still other embodiments, the mammalian cell is a baby hamster kidney (BHK) cell. BHK cells are available from American Type Culture Collection, Manassas, Va. (*e.g.*, CRL-1632). In some embodiments, the mammalian cell is a HKB11 cell, which is a hybrid cell line of a HEK293 cell and a human B cell line. Mei *et al., Mol. Biotechnol.* 34(2): 165-78 (2006).

[0181] In one embodiment, a plasmid encoding a VWF protein, a VWF linker, a heterologous smoiety or the chimeric protein of the invention further includes a selectable marker, *e.g.*, zeocin resistance, and is transfected into HEK 293 cells, for production of the chimeric protein.

[0182] In still other embodiments, transfected cells are stably transfected. These cells can be selected and maintained as a stable cell line, using conventional techniques known to those of skill in the art.

[0183] Host cells containing DNA constructs of the protein are grown in an appropriate growth medium. As used herein, the term "appropriate growth medium" means a medium containing nutrients required for the growth of cells. Nutrients required for cell growth may include a carbon source, a nitrogen source, essential amino acids, vitamins, minerals, and growth factors. Optionally, the media can contain one or more selection factors. Optionally the media can contain bovine calf serum or fetal calf serum (FCS). In one embodiment, the media contains substantially no IgG. The growth medium will generally select for cells containing the DNA construct by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker on the DNA construct or co-transfected with the DNA construct. Cultured mammalian cells are generally grown in commercially available serum-containing or serum-free media (*e.g.*, MEM, DMEM, DMEM/F12). In one embodiment, the medium is CD293 (Invitrogen,

Carlsbad, CA.). In another embodiment, the medium is CD17 (Invitrogen, Carlsbad, CA.). Selection of a medium appropriate for the particular cell line used is within the level of those ordinary skilled in the art.

[0184] In order to co-express two polypeptide echains of the chimeric molecule as described herein, the host cells are cultured under conditions that allow expression of both chains. As used herein, culturing refers to maintaining living cells *in vitro* for at least a definite time. Maintaining can, but need not include, an increase in population of living cells. For example, cells maintained in culture can be static in population, but still viable and capable of producing a desired product, *e.g.*, a recombinant protein or recombinant fusion protein. Suitable conditions for culturing eukaryotic cells are well known in the art and include appropriate selection of culture media, media supplements, temperature, pH, oxygen saturation, and the like. For commercial purposes, culturing can include the use of any of various types of scale-up systems including shaker flasks, roller bottles, hollow fiber bioreactors, stirred-tank bioreactors, airlift bioreactors, Wave bioreactors, and others.

[0185] The cell culture conditions are also selected to allow association of the first chain and the second chain in the chimeric molecule. Conditions that allow expression of the chimeric molecule may include the presence of a source of vitamin K. For example, in one embodiment, stably transfected HEK 293 cells are cultured in CD293 media (Invitrogen, Carlsbad, CA) or OptiCHO media (Invitrogen, Carlsbad, CA) supplemented with 4 mM glutamine.

[0186] In one aspect, the present invention is directed to a method of expressing, making, or producing the chimeric protein comprising a) transfecting a host cell with a polynucleotide encoding the chimeric molecule and b) culturing the host cell in a culture medium under a condition suitable for expressing the chimeric molecule, wherein the chimeric molecule is expressed.

[0187] In further embodiments, the protein product containing the chimeric molecule is secreted into the media. Media is separated from the cells, concentrated, filtered, and then passed over two or three affinity columns, *e.g.*, a protein A column and one or two anion exchange columns.

[0188] In certain aspects, the present invention relates to the chimeric polypeptide produced by the methods described herein.

[0189] *In vitro* production allows scale-up to give large amounts of the desired altered polypeptides of the invention. Techniques for mammalian cell cultivation under tissue culture conditions are known in the art and include homogeneous suspension culture, *e.g.* in an airlift reactor or in a continuous stirrer reactor, or immobilized or entrapped cell culture, *e.g.* in hollow fibers, microcapsules, on agarose microbeads or ceramic cartridges. If necessary and/or desired, the solutions of polypeptides can be purified by the customary chromatography methods, for example gel filtration, ion-exchange chromatography, hydrophobic interaction chromatography (HIC, chromatography over DEAE-cellulose or affinity chromatography.

[0190] The invention also includes a method of improving FVIII activity of a chimeric FVIII protein comprising a VWF protein fused to a first heterologous moiety and a FVIII protein fused to a second heterologous moiety, the method comprising inserting a VWF linker between the VWF protein and the first heterologous moiety, wherein the VWF linker comprises a polypeptide selected from: (i) an a2 region from Factor VIII (FVIII); (ii) an a1 region from FVIII; (iii) an a3 region from FVIII; (iv) a thrombin cleavage site which comprises X-V-P-R (SEQ ID NO: 3) and a PAR1 exosite interaction motif, wherein X is an aliphatic amino acid; or (v) any combination thereof. In some embodiments, the FVIII activity is measured by aPTT assay or ROTEM assay.

IV. Pharmaceutical Composition

[0191] Compositions containing the chimeric molecule of the present invention may contain a suitable pharmaceutically acceptable carrier. For example, they may contain excipients and/or auxiliaries that facilitate processing of the active compounds into preparations designed for delivery to the site of action.

[0192] The pharmaceutical composition can be formulated for parenteral administration (*i.e.,* intravenous, subcutaneous, or intramuscular) by bolus injection. Formulations for injection can be presented in unit dosage form, *e.g.*, in ampoules or in multidose containers with an added preservative. The compositions can take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient can be in powder form for constitution with a suitable vehicle, *e.g.*, pyrogen free water.

[0193] Suitable formulations for parenteral administration also include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, including, for example, sodium carboxymethyl cellulose, sorbitol and dextran. Optionally, the suspension may also contain stabilizers. Liposomes also can be used to encapsulate the molecules of the invention for delivery into cells or interstitial spaces. Exemplary pharmaceutically acceptable carriers are physiologically compatible solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like. In some embodiments, the composition comprises isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride. In other embodiments, the compositions comprise pharmaceutically acceptable substances such as wetting agents or minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the active ingredients.

[0194] Compositions of the invention may be in a variety of forms, including, for example, liquid (*e.g.*, injectable and infusible solutions), dispersions, suspensions, semi-solid and solid dosage forms. The preferred form depends on the mode of administration and therapeutic application.

[0195] The composition can be formulated as a solution, micro emulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active ingredient in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active ingredient into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered

solution. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

[0196] The active ingredient can be formulated with a controlled-release formulation or device. Examples of such formulations and devices include implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, for example, ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for the preparation of such formulations and devices are known in the art. See *e.g.*, Sustained and Controlled Release Drug Delivery Systems, J. R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

[0197] Injectable depot formulations can be made by forming microencapsulated matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the polymer employed, the rate of drug release can be controlled. Other exemplary biodegradable polymers are polyorthoesters and polyanhydrides. Depot injectable formulations also can be prepared by entrapping the drug in liposomes or microemulsions.

[0198] Supplementary active compounds can be incorporated into the compositions. In one embodiment, a chimeric molecule of the invention is formulated with another clotting factor, or a variant, fragment, analogue, or derivative thereof. For example, the clotting factor includes, but is not limited to, factor V, factor VII, factor VIII, factor IX, factor X, factor XI, factor XII, factor XIII, prothrombin, fibrinogen, von Willebrand factor or recombinant soluble tissue factor (rsTF) or activated forms of any of the preceding. The clotting factor of hemostatic agent can also include anti-fibrinolytic drugs, *e.g.*, epsilon-amino-caproic acid, tranexamic acid.

[0199] Dosage regimens may be adjusted to provide the optimum desired response. For example, a single bolus may be administered, several divided doses may be administered over time, or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. See, *e.g.*, Remington's Pharmaceutical Sciences (Mack Pub. Co., Easton, Pa. 1980).

[0200] In addition to the active compound, the liquid dosage form may contain inert ingredients such as water, ethyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils, glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols, and fatty acid esters of sorbitan.

[0201] Non-limiting examples of suitable pharmaceutical carriers are also described in Remington's Pharmaceutical Sciences by E. W. Martin. Some examples of excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol, and the like. The composition can also contain pH buffering reagents, and wetting or emulsifying agents.

[0202] For oral administration, the pharmaceutical composition can take the form of tablets or capsules prepared by conventional means. The composition can also be prepared as a liquid for example a syrup or a suspension. The liquid can include suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats), emulsifying agents (lecithin or acacia), non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol, or fractionated vegetable oils), and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations can also include flavoring, coloring and sweetening agents. Alternatively, the composition can be presented as a dry product for constitution with water or another suitable vehicle.

[0203] For buccal administration, the composition may take the form of tablets or lozenges according to conventional protocols.

[0204] For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of a nebulized aerosol with or without excipients or in the form of an aerosol spray from a pressurized pack or nebulizer, with optionally a propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoromethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit can be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator can be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[0205] The pharmaceutical composition can also be formulated for rectal administration as a suppository or retention enema, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

V. Gene Therapy

[0206] A chimeric molecule of the invention can be produced *in vivo* in a mammal, *e.g.*, a human patient, using a gene therapy approach to treatment of a bleeding disease or disorder selected from a bleeding coagulation disorder, hemarthrosis, muscle bleed, oral bleed, hemorrhage, hemorrhage into muscles, oral hemorrhage, trauma, trauma capitis, gastrointestinal bleeding, intracranial hemorrhage, intra-abdominal hemorrhage, intrathoracic hemorrhage, bone fracture, central nervous system bleeding, bleeding in the retropharyngeal space, bleeding in the retroperitoneal space, or bleeding in the illiopsoas sheath would be therapeutically beneficial. In one embodiment, the bleeding disease or disorder is hemophilia. In another embodiment, the bleeding disease or disorder is hemophilia A. This involves administration of a suitable chimeric molecule-encoding nucleic acid operably linked to suitable expression control sequences. In certain embodiment, these sequences are incorporated into a viral vector. Suitable viral vectors for such gene therapy include adenoviral vectors, lentiviral vectors, baculoviral vectors, Epstein Barr viral vectors, papovaviral vectors, vaccinia viral vectors, herpes simplex viral vectors, and adeno associated virus (AAV) vectors. The viral vector can be a replication-defective viral vector. In other embodiments, a adenoviral vector has a deletion in its E1 gene or E3 gene. When an adenoviral vector is used, the mammal may not be exposed to a nucleic acid encoding a selectable marker gene. In other embodiments, the sequences are incorporated into a non-viral vector known to those skilled in the art.

VI. Methods of Using Chimeric Protein

[0207] The present invention further provides a method for reducing a frequency or degree of a bleeding episode in a subject in need thereof using a chimeric molecule of the invention. An exemplary method comprises administering to the subject in need thereof a therapeutically effective amount of a chimric molecule of the invention. In other aspects, the invention includes a method of preventing an occurrence of a bleeding episode in a subject in need thereof using a chimeric molecule of the invention. In other aspects,

composition comprising a DNA encoding the recombinant protein of the invention can be administered to a subject in need thereof. In certain aspects of the invention, a cell expressing a recombinant FVIII protein of the invention can be administered to a subject in need thereof. In certain aspects of the invention, the pharmaceutical composition comprises (i) a chimeric molecule, (ii) an isolated nucleic acid encoding a chimeric molecule, (iii) a vector comprising a nucleic acid encoding a chimeric molecule, (iv) a cell comprising an isolated nucleic acid encoding a chimeric molecule and/or a vector comprising a nucleic encoding a chimeric molecule, or (v) a combination thereof, and the pharmaceutical compositions further comprises an acceptable excipient or carrier.

[0208] The bleeding episode can be caused by or derived from a blood coagulation disorder. A blood coagulation disorder can also be referred to as a coagulopathy. In one example, the blood coagulation disorder, which can be treated with a pharmaceutical composition of the current disclosure, is hemophilia or von Willebrand disease (vWD). In another example, the blood coagulation disorder, which can be treated with a pharmaceutical composition of the present disclosure is hemophilia A.

[0209] In some embodiments, the type of bleeding associated with the bleeding condition is selected from hemarthrosis, muscle bleed, oral bleed, hemorrhage, hemorrhage into muscles, oral hemorrhage, trauma, trauma capitis, gastrointestinal bleeding, intracranial hemorrhage, intra-abdominal hemorrhage, intrathoracic hemorrhage, bone fracture, central nervous system bleeding, bleeding in the retropharyngeal space, bleeding in the retroperitoneal space, bleeding in the illiopsoas sheath, or any combination thereof.

[0210] In other embodiments, the subject suffering from bleeding condition is in need of treatment for surgery, including, *e.g.*, surgical prophylaxis or peri-operative management. In one example, the surgery is selected from minor surgery and major surgery. Exemplary surgical procedures include tooth extraction, tonsillectomy, inguinal herniotomy, synovectomy, craniotomy, osteosynthesis, trauma surgery, intracranial surgery, intra-abdominal surgery, intrathoracic surgery, joint replacement surgery (*e.g.*, total knee replacement, hip replacement, and the like), heart surgery, and caesarean section.

[0211] In another example, the subject is concomitantly treated with Factor IX. Because the compounds of the invention are capable of activating FIXa, they could be used to pre-activate the FIXa polypeptide before administration of the FIXa to the subject.

[0212] The methods of the invention may be practiced on a subject in need of prophylactic treatment or on-demand treatment.

[0213] Pharmaceutical compositions comprising a chimeric molecule of the invention may be formulated for any appropriate manner of administration, including, for example, topical (*e.g.*, transdermal or ocular), oral, buccal, nasal, vaginal, rectal or parenteral administration.

[0214] The term parenteral as used herein includes subcutaneous, intradermal, intravascular (*e.g.*, intravenous), intramuscular, spinal, intracranial, intrathecal, intraocular, periocular, intraorbital, intrasynovial and intraperitoneal injection, as well as any similar injection or infusion technique. The composition can be also for example a suspension, emulsion, sustained release formulation, cream, gel or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides.

[0215] Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention. All patents and publications referred to herein are expressly incorporated by reference.

Examples

[0216] Throughout the examples, the following materials and methods were used unless otherwise stated.

Materials and Methods

[0217] In general, the practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, biophysics, molecular biology, recombinant DNA technology, immunology (especially, *e.g.*, antibody technology), and standard techniques in electrophoresis. See, *e.g.*, Sambrook, Fritsch and Maniatis, Molecular Cloning: Cold Spring Harbor Laboratory Press (1989); Antibody Engineering Protocols (Methods in Molecular Biology), 510, Paul, S., Humana Pr (1996); Antibody Engineering: A Practical Approach (Practical Approach Series, 169), McCafferty, Ed., Irl Pr (1996); Antibodies: A Laboratory Manual, Harlow et al., CS.H.L. Press, Pub. (1999);

and Current Protocols in Molecular Biology, eds. Ausubel et al., John Wiley & Sons (1992).

Example 1. Evaluation of the thrombin-mediated D'D3 release of various VWF constructs

[0218] This example evaluates the kinetics of thrombin-mediated D'D3 release at 37°C of various VWF constructs mentioned in Figure 2. Biocore experiments were conducted with VWF-Fc constructs which contain different thrombin cleavable linker between D'D3 domain of VWF and Fc. The ultimate goal is to apply the information gathered from VWF-Fc thrombin digestion to FVIII-VWF heterodimers as described herein. All VWF-D'D3 constructs were ran over the chip to achieve the capture densities of protein ranging from 100-700 RU. After VWF construct was captured on the chip, 5 U/ml of thrombin was injected over the surface for 5 minutes. The Fc remains bound to the chip, while the D'D3 in the cleavable constructs is released. Rate (RU/s) vs. capture density (RU) was plotted as shown in Figures 3 and 4. Cleavage rate is proportional to starting capture density while slope provided a measure of susceptibility to thrombin cleavage for each construct.

[0219] Figure 3 shows that VWF-052 (which does not have thrombin cleavage site in the linker region) as expected is not cleaved by thrombin. The rate of VWF-039 (LVPR with PAR1 site) is comparable to FVIII cleavage rate (data not shown). Thus, VWF-039 served as the bench mark for full D'D3 release from Fc. The ratio of slopes of various VWF-Fc constructs with respect to VWF-039 was used to determine the efficiency of thrombin cleavage. VWF-039 (LVPR with PAR1 site) is cleaved with thrombin approximately 70-80-fold faster than VWF-031 (LVPR). VWF-51 (ALRPRVV) is cleaved 1.8 fold faster than VWF-031 (LVPR).

[0220] VWF-Fc constructs were also made by introducing different acidic region (a1, a2 and a3) of FVIII protein in the linker region. VWF-055, which contains a2 region in between D'D3 and Fc region, displayed similar thrombin cleavage as VWF-039 construct. As shown in Figure 4, VWF-054 (a1 region) and VWF-056 (a3 region) showed ~ 5-fold reduced thrombin cleavage.

[0221] Figure 5 shows the slope values of thrombin cleavage curves for different VWF constructs. From these results acidic region 2 (a2) of FVIII appears to be highly efficient

thrombin cleavage site and was incorporated in FVIII-VWF heterodimers as described herein.

Example 2. Evaluation of the hemostasis potency of FVIII/VWFD'D3 heterodimers with HemA patient whole blood ROTEM assay

[0222] FVIII/VWFD'D3 heterodimers containing different thrombin cleavable linkers were evaluated in HemA donor whole blood ROTEM (rotational thromboelastometry) assay for their potency on hemostasis. A whole blood sample was collected from donor with severe Hemophilia A bleeding disorder with Sodium Citrate as anti-coagulant. 40 minutes after the blood sample collection, FVIII/VWFD'D3 heterodimer variants containing different thrombin cleavable linker - FVIII155/VWF031 (48aa, LVPR site), FVIII155/VWF039 (26aa, LVPR+PAR1 site), FVIII155/VWF055 (34aa, a2 from FVIII) were diluted into the whole blood sample to the final concentration at 100%, 30%, 10%, and 3% of normal as measured by FVIII chromogenic assay. Immediately after the addition of FVIII/VWFD'D3 heterodimers, ROTEM reaction was started by the addition of $CaCl_2$. Clotting time (time to reach 2mm amplitude from beginning of the test) was recorded by an instrument and plotted against FVIII concentration in the samples (Figure 6). It was hypothesized that a more potent FVIII/VWFD'D3 heterodimer will induce faster clotting process, thus resulting in a shorter clotting time compared to a less potent FVIII/VWFD'D3 heterodimer. As shown in Figure 6, the samples with the addition of FVIII/VWF039 heterodimer had the shortest clotting time at all concentrations that had been tested, and the samples with the addition of FVIII/VWF031 heterodimer had the longest clotting time at all concentrations. The clotting time for the samples with the addition of FVIII155/VWF055 heterodimer is in the middle. Therefore, the rank of the hemostasis potency is FVIII155/VWF039 > FVIII155/VWF055 > FVIII155/VWF031. Since the only difference between the three molecules is the thrombin cleavable linkers between the VWF protein and the Fc region, the result indicates that the linker containing the LVPR site and the PAR1 exosite interaction motif and the a2 region of FVIII work better than the LVPR site alone.

[0223] **pSYN VWF039 nucleotide sequence (VWF D'D3-Fc with thrombin +PAR1 site 26 amino acid long linker)** (SEQ ID NO: 40)

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 1 ATGATTCCTG CCAGATTTGC CGGGGTGCTG CTTGCTCTGG CCCTCATTTT
51 GCCAGGGACC CTTTGTGCAG AAGGAACTCG CGGCAGGTCA TCCACGGCCC
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 101 GATGCAGCCT TTTCGGAAGT GACTTCGTCA ACACCTTTGA TGGGAGCATG
 151 TACAGCTTTG CGGGATACTG CAGTTACCTC CTGGCAGGGG GCTGCCAGAA
 201 ACGCTCCTTC TCGATTATTG GGGACTTCCA GAATGGCAAG AGAGTGAGCC
 251 TCTCCGTGTA TCTTGGGGAA TTTTTTGACA TCCATTTGTT TGTCAATGGT
 301 ACCGTGACAC AGGGGGACCA AAGAGTCTCC ATGCCCTATG CCTCCAAAGG
 351 GCTGTATCTA GAAACTGAGG CTGGGTACTA CAAGCTGTCC GGTGAGGCCT
 401 ATGGCTTTGT GGCCAGGATC GATGGCAGCG GCAACTTTCA AGTCCTGCTG
 451 TCAGACAGAT ACTTCAACAA GACCTGCGGG CTGTGTGGCA ACTTTAACAT
 501 CTTTGCTGAA GATGACTTTA TGACCCAAGA AGGGACCTTG ACCTCGGACC
 551 CTTATGACTT TGCCAACTCA TGGGCTCTGA GCAGTGGAGA ACAGTGGTGT
 601 GAACGGGCAT CTCCTCCCAG CAGCTCATGC AACATCTCCT CTGGGGAAAT
 651 GCAGAAGGGC CTGTGGGAGC AGTGCCAGCT TCTGAAGAGC ACCTCGGTGT
 701 TTGCCCGCTG CCACCCTCTG GTGGACCCCG AGCCTTTTGT GGCCCTGTGT
 751 GAGAAGACTT TGTGTGAGTG TGCTGGGGGG CTGGAGTGCG CCTGCCCTGC
 801 CCTCCTGGAG TACGCCCGGA CCTGTGCCCA GGAGGGAATG GTGCTGTACG
 851 GCTGGACCGA CCACAGCGCG TGCAGCCCAG TGTGCCCTGC TGGTATGGAG
 901 TATAGGCAGT GTGTGTCCCC TTGCGCCAGG ACCTGCCAGA GCCTGCACAT
 951 CAATGAAATG TGTCAGGAGC GATGCGTGGA TGGCTGCAGC TGCCCTGAGG
1001 GACAGCTCCT GGATGAAGGC CTCTGCGTGG AGAGCACCGA GTGTCCCTGC
1051 GTGCATTCCG GAAAGCGCTA CCCTCCCGGC ACCTCCCTCT CTCGAGACTG
1101 CAACACCTGC ATTTGCCGAA ACAGCCAGTG GATCTGCAGC AATGAAGAAT
1151 GTCCAGGGGA GTGCCTTGTC ACTGGTCAAT CCCACTTCAA GAGCTTTGAC
1201 AACAGATACT TCACCTTCAG TGGGATCTGC CAGTACCTGC TGGCCCGGGA
1251 TTGCCAGGAC CACTCCTTCT CCATTGTCAT TGAGACTGTC CAGTGTGCTG
1301 ATGACCGCGA CGCTGTGTGC ACCCGCTCCG TCACCGTCCG GCTGCCTGGC
1351 CTGCACAACA GCCTTGTGAA ACTGAAGCAT GGGGCAGGAG TTGCCATGGA
1401 TGGCCAGGAC ATCCAGCTCC CCCTCCTGAA AGGTGACCTC CGCATCCAGC
1451 ATACAGTGAC GGCCTCCGTG CGCCTCAGCT ACGGGGAGGA CCTGCAGATG
1501 GACTGGGATG GCCGCGGGAG GCTGCTGGTG AAGCTGTCCC CCGTCTATGC
1551 CGGGAAGACC TGCGGCCTGT GTGGGAATTA CAATGGCAAC CAGGGCGACG
1601 ACTTCCTTAC CCCCTCTGGG CTGGCGGAGC CCCGGGTGGA GGACTTCGGG
1651 AACGCCTGGA AGCTGCACGG GGACTGCCAG GACCTGCAGA AGCAGCACAG
1701 CGATCCCTGC GCCCTCAACC CGCGCATGAC CAGGTTCTCC GAGGAGGCGT
1751 GCGCGGTCCT GACGTCCCCC ACATTCGAGG CCTGCCATCG TGCCGTCAGC
1801 CCGCTGCCCT ACCTGCGGAA CTGCCGCTAC GACGTGTGCT CCTGCTCGGA
1851 CGGCCGCGAG TGCCTGTGCG GCGCCCTGGC CAGCTATGCC GCGGCCTGCG
1901 CGGGGAGAGG CGTGCGCGTC GCGTGGCGCG AGCCAGGCCG CTGTGAGCTG
1951 AACTGCCCGA AAGGCCAGGT GTACCTGCAG TGCGGGACCC CCTGCAACCT
2001 GACCTGCCGC TCTCTCTCTT ACCCGGATGA GGAATGCAAT GAGGCCTGCC
2051 TGGAGGGCTG CTTCTGCCCC CCAGGGCTCT ACATGGATGA GAGGGGGGAC
2101 TGCGTGCCCA AGGCCCAGTG CCCCTGTTAC TATGACGGTG AGATCTTCCA
2151 GCCAGAAGAC ATCTTCTCAG ACCATCACAC CATGTGCTAC TGTGAGGATG
2201 GCTTCATGCA CTGTACCATG AGTGGAGTCC CCGGAAGCTT GCTGCCTGAC
2251 GCTGTCCTCA GCAGTCCCCT GTCTCATCGC AGCAAAAGGA GCCTATCCTG
2301 TCGGCCCCCC ATGGTCAAGC TGGTGTGTCC CGCTGACAAC CTGCGGGCTG
2351 AAGGGCTCGA GTGTACCAAA ACGTGCCAGA ACTATGACCT GGAGTGCATG
2401 AGCATGGGCT GTGTCTCTGG CTGCCTCTGC CCCCCGGGCA TGGTCCGGCA
2451 TGAGAACAGA TGTGTGGCCC TGGAAAGGTG TCCCTGCTTC CATCAGGGCA
2501 AGGAGTATGC CCCTGGAGAA ACAGTGAAGA TTGGCTGCAA CACTTGTGTC
2551 TGTCGGGACC GGAAGTGGAA CTGCACAGAC CATGTGTGTG ATGCCACGTG
2601 CTCCACGATC GGCATGGCCC ACTACCTCAC CTTCGACGGG CTCAAATACC
2651 TGTTCCCCGG GGAGTGCCAG TACGTTCTGG TGCAGGATTA CTGCGGCAGT
2701 AACCCTGGGA CCTTTCGGAT CCTAGTGGGG AATAAGGGAT GCAGCCACCC
2751 CTCAGTGAAA TGCAAGAAAC GGGTCACCAT CCTGGTGGAG GGAGGAGAGA
2801 TTGAGCTGTT TGACGGGGAG GTGAATGTGA AGAGGCCCAT GAAGGATGAG
2851 ACTCACTTTG AGGTGGTGGA GTCTGGCCGG TACATCATTC TGCTGCTGGG
2901 CAAAGCCCTC TCCGTGGTCT GGGACCGCCA CCTGAGCATC TCCGTGGTCC
2951 TGAAGCAGAC ATACCAGGAG AAAGTGTGTG GCCTGTGTGG GAATTTTGAT
3001 GGCATCCAGA ACAATGACCT CACCAGCAGC AACCTCCAAG TGGAGGAAGA
3051 CCCTGTGGAC TTTGGGAACT CCTGGAAAGT GAGCTCGCAG TGTGCTGACA
3101 CCAGAAAAGT GCCTCTGGAC TCATCCCCTG CCACCTGCCA TAACAACATC
3151 ATGAAGCAGA CGATGGTGGA TTCCTCCTGT AGAATCCTTA CCAGTGACGT
3201 CTTCCAGGAC TGCAACAAGC TGGTGGACCC CGAGCCATAT CTGGATGTCT
3251 GCATTTACGA CACCTGCTCC TGTGAGTCCA TTGGGGACTG CGCCGCATTC
3301 TGCGACACCA TTGCTGCCTA TGCCCACGTG TGTGCCCAGC ATGGCAAGGT
3351 GGTGACCTGG AGGACGGCCA CATTGTGCCC CCAGAGCTGC GAGGAGAGGA
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3401 ATCTCCGGGA GAACGGGTAT GAGGCTGAGT GGCGCTATAA CAGCTGTGCA
3451 CCTGCCTGTC AAGTCACGTG TCAGCACCCT GAGCCACTGG CCTGCCCTGT
3501 GCAGTGTGTG GAGGGCTGCC ATGCCCACTG CCCTCCAGGG AAAATCCTGG
3551 ATGAGCTTTT GCAGACCTGC GTTGACCCTG AAGACTGTCC AGTGTGTGAG
3601 GTGGCTGGCC GGCGTTTTGC CTCAGGAAAG AAAGTCACCT TGAATCCCAG
3651 TGACCCTGAG CACTGCCAGA TTTGCCACTG TGATGTTGTC AACCTCACCT
3701 GTGAAGCCTG CCAGGAGCCG GGAGGCCTGG TGCCCCGGTC ATTTCTTCTC
3751 AGGAACCCCA ATGATAAATA TGAACCATTT TGGGAGGATG AGGAGAGCGA
3801 CAAAACTCAC ACATGCCCAC CGTGCCCAGC TCCAGAACTC CTGGGCGGAC
3851 CGTCAGTCTT CCTCTTCCCC CCAAAACCCA AGGACACCCT CATGATCTCC
3901 CGGACCCCTG AGGTCACATG CGTGGTGGTG GACGTGAGCC ACGAAGACCC
3951 TGAGGTCAAG TTCAACTGGT ACGTGGACGG CGTGGAGGTG CATAATGCCA
4001 AGACAAAGCC GCGGGAGGAG CAGTACAACA GCACGTACCG TGTGGTCAGC
4051 GTCCTCACCG TCCTGCACCA GGACTGGCTG AATGGCAAGG AGTACAAGTG
4101 CAAGGTCTCC AACAAAGCCC TCCCAGCCCC CATCGAGAAA ACCATCTCCA
4151 AAGCCAAAGG GCAGCCCCGA GAACCACAGG TGTACACCCT GCCCCCATCC
4201 CGGGATGAGC TGACCAAGAA CCAGGTCAGC CTGACCTGCC TGGTCAAAGG
4251 CTTCTATCCC AGCGACATCG CCGTGGAGTG GGAGAGCAAT GGGCAGCCGG
4301 AGAACAACTA CAAGACCACG CCTCCCGTGT TGGACTCCGA CGGCTCCTTC
4351 TTCCTCTACA GCAAGCTCAC CGTGGACAAG AGCAGGTGGC AGCAGGGGAA
4401 CGTCTTCTCA TGCTCCGTGA TGCATGAGGC TCTGCACAAC CACTACACGC
4451 AGAAGAGCCT CTCCCTGTCT CCGGGTAAAT GA
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[0224] **pSYN VWF039 protein sequence (VWF D'D3-Fc with thrombin +PAR1 site 26 amino acid long linker)** (SEQ ID NO: 41)

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  1 MIPARFAGVL LALALILPGT LCAEGTRGRS STARCSLFGS DFVNTFDGSM
 51 YSFAGYCSYL LAGGCQKRSF SIIGDFQNGK RVSLSVYLGE FFDIHLFVNG
101 TVTQGDQRVS MPYASKGLYL ETEAGYYKLS GEAYGFVARI DGSGNFQVLL
151 SDRYFNKTCG LCGNFNIFAE DDFMTQEGTL TSDPYDFANS WALSSGEQWC
201 ERASPPSSSC NISSGEMQKG LWEQCQLLKS TSVFARCHPL VDPEPFVALC
251 EKTLCECAGG LECACPALLE YARTCAQEGM VLYGWTDHSA CSPVCPAGME
301 YRQCVSPCAR TCQSLHINEM CQERCVDGCS CPEGQLLDEG LCVESTECPC
351 VHSGKRYPPG TSLSRDCNTC ICRNSQWICS NEECPGECLV TGQSHFKSFD
401 NRYFTFSGIC QYLLARDCQD HSFSIVIETV QCADDRDAVC TRSVTVRLPG
451 LHNSLVKLKH GAGVAMDGQD IQLPLLKGDL RIQHTVTASV RLSYGEDLQM
501 DWDGRGRLLV KLSPVYAGKT CGLCGNYNGN QGDDFLTPSG LAEPRVEDFG
551 NAWKLHGDCQ DLQKQHSDPC ALNPRMTRFS EEACAVLTSP TFEACHRAVS
601 PLPYLRNCRY DVCSCSDGRE CLCGALASYA AACAGRGVRV AWREPGRCEL
651 NCPKGQVYLQ CGTPCNLTCR SLSYPDEECN EACLEGCFCP PGLYMDERGD
701 CVPKAQCPCY YDGEIFQPED IFSDHHTMCY CEDGFMHCTM SGVPGSLLPD
751 AVLSSPLSHR SKRSLSCRPP MVKLVCPADN LRAEGLECTK TCQNYDLECM
801 SMGCVSGCLC PPGMVRHENR CVALERCPCF HQGKEYAPGE TVKIGCNTCV
851 CRDRKWNCTD HVCDATCSTI GMAHYLTFDG LKYLFPGECQ YVLVQDYCGS
901 NPGTFRILVG NKGCSHPSVK CKKRVTILVE GGEIELFDGE VNVKRPMKDE
951 THFEVVESGR YIILLLGKAL SVVWDRHLSI SVVLKQTYQE KVCGLCGNFD
1001 GIQNNDLTSS NLQVEEDPVD FGNSWKVSSQ CADTRKVPLD SSPATCHNNI
1051 MKQTMVDSSC RILTSDVFQD CNKLVDPEPY LDVCIYDTCS CESIGDCAAF
1101 CDTIAAYAHV CAQHGKVVTW RTATLCPQSC EERNLRENGY EAEWRYNSCA
1151 PACQVTCQHP EPLACPVQCV EGCHAHCPPG KILDELLQTC VDPEDCPVCE
1201 VAGRRFASGK KVTLNPSDPE HCQICHCDVV NLTCEACQEP GGLVPRSFLL
1251 RNPNDKYEPF WEDEESDKTH TCPPCPAPEL LGGPSVFLFP PKPKDTLMIS
1301 RTPEVTCVVV DVSHEDPEVK FNWYVDGVEV HNAKTKPREE QYNSTYRVVS
1351 VLTVLHQDWL NGKEYKCKVS NKALPAPIEK TISKAKGQPR EPQVYTLPPS
1401 RDELTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT PPVLDSDGSF
1451 FLYSKLTVDK SRWQQGNVFS CSVMHEALHN HYTQKSLSLS PGK
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[0225] **pSYN VWF052 nucleotide sequence (VWF D'D3-Fc with non-cleavable 48 amino acid long GS linker)** (SEQ ID NO: 42)

```
  1 ATGATTCCTG CCAGATTTGC CGGGGTGCTG CTTGCTCTGG CCCTCATTTT
 51 GCCAGGGACC CTTTGTGCAG AAGGAACTCG CGGCAGGTCA TCCACGGCCC
101 GATGCAGCCT TTTCGGAAGT GACTTCGTCA ACACCTTTGA TGGGAGCATG
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 151 TACAGCTTTG CGGGATACTG CAGTTACCTC CTGGCAGGGG GCTGCCAGAA
 201 ACGCTCCTTC TCGATTATTG GGGACTTCCA GAATGGCAAG AGAGTGAGCC
 251 TCTCCGTGTA TCTTGGGGAA TTTTTTGACA TCCATTTGTT TGTCAATGGT
 301 ACCGTGACAC AGGGGGACCA AAGAGTCTCC ATGCCCTATG CCTCCAAAGG
 351 GCTGTATCTA GAAACTGAGG CTGGGTACTA CAAGCTGTCC GGTGAGGCCT
 401 ATGGCTTTGT GGCCAGGATC GATGGCAGCG GCAACTTTCA AGTCCTGCTG
 451 TCAGACAGAT ACTTCAACAA GACCTGCGGG CTGTGTGGCA ACTTTAACAT
 501 CTTTGCTGAA GATGACTTTA TGACCCAAGA AGGGACCTTG ACCTCGGACC
 551 CTTATGACTT TGCCAACTCA TGGGCTCTGA GCAGTGGAGA ACAGTGGTGT
 601 GAACGGGCAT CTCCTCCCAG CAGCTCATGC AACATCTCCT CTGGGGAAAT
 651 GCAGAAGGGC CTGTGGGAGC AGTGCCAGCT TCTGAAGAGC ACCTCGGTGT
 701 TTGCCCGCTG CCACCCTCTG GTGGACCCCG AGCCTTTTGT GGCCCTGTGT
 751 GAGAAGACTT TGTGTGAGTG TGCTGGGGGG CTGGAGTGCG CCTGCCCTGC
 801 CCTCCTGGAG TACGCCCGGA CCTGTGCCCA GGAGGGAATG GTGCTGTACG
 851 GCTGGACCGA CCACAGCGCG TGCAGCCCAG TGTGCCCTGC TGGTATGGAG
 901 TATAGGCAGT GTGTGTCCCC TTGCGCCAGG ACCTGCCAGA GCCTGCACAT
 951 CAATGAAATG TGTCAGGAGC GATGCGTGGA TGGCTGCAGC TGCCCTGAGG
1001 GACAGCTCCT GGATGAAGGC CTCTGCGTGG AGAGCACCGA GTGTCCCTGC
1051 GTGCATTCCG GAAAGCGCTA CCCTCCCGGC ACCTCCCTCT CTCGAGACTG
1101 CAACACCTGC ATTTGCCGAA ACAGCCAGTG GATCTGCAGC AATGAAGAAT
1151 GTCCAGGGGA GTGCCTTGTC ACTGGTCAAT CCCACTTCAA GAGCTTTGAC
1201 AACAGATACT TCACCTTCAG TGGGATCTGC CAGTACCTGC TGGCCCGGGA
1251 TTGCCAGGAC CACTCCTTCT CCATTGTCAT TGAGACTGTC CAGTGTGCTG
1301 ATGACCGCGA CGCTGTGTGC ACCCGCTCCG TCACCGTCCG GCTGCCTGGC
1351 CTGCACAACA GCCTTGTGAA ACTGAAGCAT GGGGCAGGAG TTGCCATGGA
1401 TGGCCAGGAC ATCCAGCTCC CCCTCCTGAA AGGTGACCTC CGCATCCAGC
1451 ATACAGTGAC GGCCTCCGTG CGCCTCAGCT ACGGGGAGGA CCTGCAGATG
1501 GACTGGGATG GCCGCGGGAG GCTGCTGGTG AAGCTGTCCC CCGTCTATGC
1551 CGGGAAGACC TGCGGCCTGT GTGGGAATTA CAATGGCAAC CAGGGCGACG
1601 ACTTCCTTAC CCCCTCTGGG CTGGCGGAGC CCCGGGTGGA GGACTTCGGG
1651 AACGCCTGGA AGCTGCACGG GGACTGCCAG GACCTGCAGA AGCAGCACAG
1701 CGATCCCTGC GCCCTCAACC CGCGCATGAC CAGGTTCTCC GAGGAGGCGT
1751 GCGCGGTCCT GACGTCCCCC ACATTCGAGG CCTGCCATCG TGCCGTCAGC
1801 CCGCTGCCCT ACCTGCGGAA CTGCCGCTAC GACGTGTGCT CCTGCTCGGA
1851 CGGCCGCGAG TGCCTGTGCG GCGCCCTGGC CAGCTATGCC GCGGCCTGCG
1901 CGGGGAGAGG CGTGCGCGTC GCGTGGCGCG AGCCAGGCCG CTGTGAGCTG
1951 AACTGCCCGA AAGGCCAGGT GTACCTGCAG TGCGGGACCC CCTGCAACCT
2001 GACCTGCCGC TCTCTCTCTT ACCCGGATGA GGAATGCAAT GAGGCCTGCC
2051 TGGAGGGCTG CTTCTGCCCC CCAGGGCTCT ACATGGATGA GAGGGGGGAC
2101 TGCGTGCCCA AGGCCCAGTG CCCCTGTTAC TATGACGGTG AGATCTTCCA
2151 GCCAGAAGAC ATCTTCTCAG ACCATCACAC CATGTGCTAC TGTGAGGATG
2201 GCTTCATGCA CTGTACCATG AGTGGAGTCC CCGGAAGCTT GCTGCCTGAC
2251 GCTGTCCTCA GCAGTCCCCT GTCTCATCGC AGCAAAAGGA GCCTATCCTG
2301 TCGGCCCCCC ATGGTCAAGC TGGTGTGTCC CGCTGACAAC CTGCGGGCTG
2351 AAGGGCTCGA GTGTACCAAA ACGTGCCAGA ACTATGACCT GGAGTGCATG
2401 AGCATGGGCT GTGTCTCTGG CTGCCTCTGC CCCCCGGGCA TGGTCCGGCA
2451 TGAGAACAGA TGTGTGGCCC TGGAAAGGTG TCCCTGCTTC CATCAGGGCA
2501 AGGAGTATGC CCCTGGAGAA ACAGTGAAGA TTGGCTGCAA CACTTGTGTC
2551 TGTCGGGACC GGAAGTGGAA CTGCACAGAC CATGTGTGTG ATGCCACGTG
2601 CTCCACGATC GGCATGGCCC ACTACCTCAC CTTCGACGGG CTCAAATACC
2651 TGTTCCCCGG GGAGTGCCAG TACGTTCTGG TGCAGGATTA CTGCGGCAGT
2701 AACCCTGGGA CCTTTCGGAT CCTAGTGGGG AATAAGGGAT GCAGCCACCC
2751 CTCAGTGAAA TGCAAGAAAC GGGTCACCAT CCTGGTGGAG GGAGGAGAGA
2801 TTGAGCTGTT TGACGGGGAG GTGAATGTGA AGAGGCCCAT GAAGGATGAG
2851 ACTCACTTTG AGGTGGTGGA GTCTGGCCGG TACATCATTC TGCTGCTGGG
2901 CAAAGCCCTC TCCGTGGTCT GGGACCGCCA CCTGAGCATC TCCGTGGTCC
2951 TGAAGCAGAC ATACCAGGAG AAAGTGTGTG GCCTGTGTGG GAATTTTGAT
3001 GGCATCCAGA ACAATGACCT CACCAGCAGC AACCTCCAAG TGGAGGAAGA
3051 CCCTGTGGAC TTTGGGAACT CCTGGAAAGT GAGCTCGCAG TGTGCTGACA
3101 CCAGAAAAGT GCCTCTGGAC TCATCCCCTG CCACCTGCCA TAACAACATC
3151 ATGAAGCAGA CGATGGTGGA TTCCTCCTGT AGAATCCTTA CCAGTGACGT
3201 CTTCCAGGAC TGCAACAAGC TGGTGGACCC CGAGCCATAT CTGGATGTCT
3251 GCATTTACGA CACCTGCTCC TGTGAGTCCA TTGGGGACTG CGCCGCATTC
3301 TGCGACACCA TTGCTGCCTA TGCCCACGTG TGTGCCCAGC ATGGCAAGGT
3351 GGTGACCTGG AGGACGGCCA CATTGTGCCC CCAGAGCTGC GAGGAGAGGA
3401 ATCTCCGGGA GAACGGGTAT GAGGCTGAGT GGCGCTATAA CAGCTGTGCA
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3451 CCTGCCTGTC AAGTCACGTG TCAGCACCCT GAGCCACTGG CCTGCCCTGT
3501 GCAGTGTGTG GAGGGCTGCC ATGCCCACTG CCCTCCAGGG AAAATCCTGG
3551 ATGAGCTTTT GCAGACCTGC GTTGACCCTG AAGACTGTCC AGTGTGTGAG
3601 GTGGCTGGCC GGCGTTTTGC CTCAGGAAAG AAAGTCACCT TGAATCCCAG
3651 TGACCCTGAG CACTGCCAGA TTTGCCACTG TGATGTTGTC AACCTCACCT
3701 GTGAAGCCTG CCAGGAGCCG ATATCTGGCG GTGGAGGTTC CGGTGGCGGG
3751 GGATCCGGCG GTGGAGGTTC CGGCGGTGGA GGTTCCGGTG GCGGGGGATC
3801 CGGTGGCGGG GGATCCGGGG GCGGAGGGGG CAGCGGCGGT GGAGGTTCCG
3851 GTGGCGGGGG ATCCGACAAA ACTCACACAT GCCCACCGTG CCCAGCTCCA
3901 GAACTCCTGG GCGGACCGTC AGTCTTCCTC TTCCCCCCAA AACCCAAGGA
3951 CACCCTCATG ATCTCCCGGA CCCCTGAGGT CACATGCGTG GTGGTGGACG
4001 TGAGCCACGA AGACCCTGAG GTCAAGTTCA ACTGGTACGT GGACGGCGTG
4051 GAGGTGCATA ATGCCAAGAC AAAGCCGCGG GAGGAGCAGT ACAACAGCAC
4101 GTACCGTGTG GTCAGCGTCC TCACCGTCCT GCACCAGGAC TGGCTGAATG
4151 GCAAGGAGTA CAAGTGCAAG GTCTCCAACA AAGCCCTCCC AGCCCCCATC
4201 GAGAAAACCA TCTCCAAAGC CAAAGGGCAG CCCCGAGAAC CACAGGTGTA
4251 CACCCTGCCC CCATCCCGGG ATGAGCTGAC CAAGAACCAG GTCAGCCTGA
4301 CCTGCCTGGT CAAAGGCTTC TATCCCAGCG ACATCGCCGT GGAGTGGGAG
4351 AGCAATGGGC AGCCGGAGAA CAACTACAAG ACCACGCCTC CCGTGTTGGA
4401 CTCCGACGGC TCCTTCTTCC TCTACAGCAA GCTCACCGTG GACAAGAGCA
4451 GGTGGCAGCA GGGGAACGTC TTCTCATGCT CCGTGATGCA TGAGGCTCTG
4501 CACAACCACT ACACGCAGAA GAGCCTCTCC CTGTCTCCGG GTAAATGA
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[0226] **pSYN VWF052 protein sequence (VWF D'D3-Fc with non-cleavable 48 amino acid long GS linker)** (SEQ ID NO: 43)

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  1 MIPARFAGVL LALALILPGT LCAEGTRGRS STARCSLFGS DFVNTFDGSM
 51 YSFAGYCSYL LAGGCQKRSF SIIGDFQNGK RVSLSVYLGE FFDIHLFVNG
101 TVTQGDQRVS MPYASKGLYL ETEAGYYKLS GEAYGFVARI DGSGNFQVLL
151 SDRYFNKTCG LCGNFNIFAE DDFMTQEGTL TSDPYDFANS WALSSGEQWC
201 ERASPPSSSC NISSGEMQKG LWEQCQLLKS TSVFARCHPL VDPEPFVALC
251 EKTLCECAGG LECACPALLE YARTCAQEGM VLYGWTDHSA CSPVCPAGME
301 YRQCVSPCAR TCQSLHINEM CQERCVDGCS CPEGQLLDEG LCVESTECPC
351 VHSGKRYPPG TSLSRDCNTC ICRNSQWICS NEECPGECLV TGQSHFKSFD
401 NRYFTFSGIC QYLLARDCQD HSFSIVIETV QCADDRDAVC TRSVTVRLPG
451 LHNSLVKLKH GAGVAMDGQD IQLPLLKGDL RIQHTVTASV RLSYGEDLQM
501 DWDGRGRLLV KLSPVYAGKT CGLCGNYNGN QGDDFLTPSG LAEPRVEDFG
551 NAWKLHGDCQ DLQKQHSDPC ALNPRMTRFS EEACAVLTSP TFEACHRAVS
601 PLPYLRNCRY DVCSCSDGRE CLCGALASYA AACAGRGVRV AWREPGRCEL
651 NCPKGQVYLQ CGTPCNLTCR SLSYPDEECN EACLEGCFCP PGLYMDERGD
701 CVPKAQCPCY YDGEIFQPED IFSDHHTMCY CEDGFMHCTM SGVPGSLLPD
751 AVLSSPLSHR SKRSLSCRPP MVKLVCPADN LRAEGLECTK TCQNYDLECM
801 SMGCVSGCLC PPGMVRHENR CVALERCPCF HQGKEYAPGE TVKIGCNTCV
851 CRDRKWNCTD HVCDATCSTI GMAHYLTFDG LKYLFPGECQ YVLVQDYCGS
901 NPGTFRILVG NKGCSHPSVK CKKRVTILVE GGEIELFDGE VNVKRPMKDE
951 THFEVVESGR YIILLLGKAL SVVWDRHLSI SVVLKQTYQE KVCGLCGNFD
1001 GIQNNDLTSS NLQVEEDPVD FGNSWKVSSQ CADTRKVPLD SSPATCHNNI
1051 MKQTMVDSSC RILTSDVFQD CNKLVDPEPY LDVCIYDTCS CESIGDCAAF
1101 CDTIAAYAHV CAQHGKVVTW RTATLCPQSC EERNLRENGY EAEWRYNSCA
1151 PACQVTCQHP EPLACPVQCV EGCHAHCPPG KILDELLQTC VDPEDCPVCE
1201 VAGRRFASGK KVTLNPSDPE HCQICHCDVV NLTCEACQEP ISGGGGSGGG
1251 GSGGGGSGGG GSGGGGSGGG GSGGGGGSGG GGSGGGGSDK THTCPPCPAP
1301 ELLGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE VKFNWYVDGV
1351 EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK VSNKALPAPI
1401 EKTISKAKGQ PREPQVYTLP PSRDELTKNQ VSLTCLVKGF YPSDIAVEWE
1451 SNGQPENNYK TTPPVLDSDG SFFLYSKLTV DKSRWQQGNV FSCSVMHEAL
1501 HNHYTQKSLS LSPGK*
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[0227] **pSYN VWF054 nucleotide sequence (VWF D'D3-Fc with 40 amino acid long little a1 linker)** (SEQ ID NO: 44)

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  1 ATGATTCCTG CCAGATTTGC CGGGGTGCTG CTTGCTCTGG CCCTCATTTT
 51 GCCAGGGACC CTTTGTGCAG AAGGAACTCG CGGCAGGTCA TCCACGGCCC
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 101 GATGCAGCCT TTTCGGAAGT GACTTCGTCA ACACCTTTGA TGGGAGCATG
 151 TACAGCTTTG CGGGATACTG CAGTTACCTC CTGGCAGGGG GCTGCCAGAA
 201 ACGCTCCTTC TCGATTATTG GGGACTTCCA GAATGGCAAG AGAGTGAGCC
 251 TCTCCGTGTA TCTTGGGGAA TTTTTTGACA TCCATTTGTT TGTCAATGGT
 301 ACCGTGACAC AGGGGGACCA AAGAGTCTCC ATGCCCTATG CCTCCAAAGG
 351 GCTGTATCTA GAAACTGAGG CTGGGTACTA CAAGCTGTCC GGTGAGGCCT
 401 ATGGCTTTGT GGCCAGGATC GATGGCAGCG GCAACTTTCA AGTCCTGCTG
 451 TCAGACAGAT ACTTCAACAA GACCTGCGGG CTGTGTGGCA ACTTTAACAT
 501 CTTTGCTGAA GATGACTTTA TGACCCAAGA AGGGACCTTG ACCTCGGACC
 551 CTTATGACTT TGCCAACTCA TGGGCTCTGA GCAGTGGAGA ACAGTGGTGT
 601 GAACGGGCAT CTCCTCCCAG CAGCTCATGC AACATCTCCT CTGGGGAAAT
 651 GCAGAAGGGC CTGTGGGAGC AGTGCCAGCT TCTGAAGAGC ACCTCGGTGT
 701 TTGCCCGCTG CCACCCTCTG GTGGACCCCG AGCCTTTTGT GGCCCTGTGT
 751 GAGAAGACTT TGTGTGAGTG TGCTGGGGGG CTGGAGTGCG CCTGCCCTGC
 801 CCTCCTGGAG TACGCCCGGA CCTGTGCCCA GGAGGGAATG GTGCTGTACG
 851 GCTGGACCGA CCACAGCGCG TGCAGCCCAG TGTGCCCTGC TGGTATGGAG
 901 TATAGGCAGT GTGTGTCCCC TTGCGCCAGG ACCTGCCAGA GCCTGCACAT
 951 CAATGAAATG TGTCAGGAGC GATGCGTGGA TGGCTGCAGC TGCCCTGAGG
1001 GACAGCTCCT GGATGAAGGC CTCTGCGTGG AGAGCACCGA GTGTCCCTGC
1051 GTGCATTCCG GAAAGCGCTA CCCTCCCGGC ACCTCCCTCT CTCGAGACTG
1101 CAACACCTGC ATTTGCCGAA ACAGCCAGTG GATCTGCAGC AATGAAGAAT
1151 GTCCAGGGGA GTGCCTTGTC ACTGGTCAAT CCCACTTCAA GAGCTTTGAC
1201 AACAGATACT TCACCTTCAG TGGGATCTGC CAGTACCTGC TGGCCCGGGA
1251 TTGCCAGGAC CACTCCTTCT CCATTGTCAT TGAGACTGTC CAGTGTGCTG
1301 ATGACCGCGA CGCTGTGTGC ACCCGCTCCG TCACCGTCCG GCTGCCTGGC
1351 CTGCACAACA GCCTTGTGAA ACTGAAGCAT GGGGCAGGAG TTGCCATGGA
1401 TGGCCAGGAC ATCCAGCTCC CCCTCCTGAA AGGTGACCTC CGCATCCAGC
1451 ATACAGTGAC GGCCTCCGTG CGCCTCAGCT ACGGGGAGGA CCTGCAGATG
1501 GACTGGGATG GCCGCGGGAG GCTGCTGGTG AAGCTGTCCC CCGTCTATGC
1551 CGGGAAGACC TGCGGCCTGT GTGGGAATTA CAATGGCAAC CAGGGCGACG
1601 ACTTCCTTAC CCCCTCTGGG CTGGCGGAGC CCCGGGTGGA GGACTTCGGG
1651 AACGCCTGGA AGCTGCACGG GGACTGCCAG GACCTGCAGA AGCAGCACAG
1701 CGATCCCTGC GCCCTCAACC CGCGCATGAC CAGGTTCTCC GAGGAGGCGT
1751 GCGCGGTCCT GACGTCCCCC ACATTCGAGG CCTGCCATCG TGCCGTCAGC
1801 CCGCTGCCCT ACCTGCGGAA CTGCCGCTAC GACGTGTGCT CCTGCTCGGA
1851 CGGCCGCGAG TGCCTGTGCG GCGCCCTGGC CAGCTATGCC GCGGCCTGCG
1901 CGGGGAGAGG CGTGCGCGTC GCGTGGCGCG AGCCAGGCCG CTGTGAGCTG
1951 AACTGCCCGA AAGGCCAGGT GTACCTGCAG TGCGGGACCC CCTGCAACCT
2001 GACCTGCCGC TCTCTCTCTT ACCCGGATGA GGAATGCAAT GAGGCCTGCC
2051 TGGAGGGCTG CTTCTGCCCC CCAGGGCTCT ACATGGATGA GAGGGGGGAC
2101 TGCGTGCCCA AGGCCCAGTG CCCCTGTTAC TATGACGGTG AGATCTTCCA
2151 GCCAGAAGAC ATCTTCTCAG ACCATCACAC CATGTGCTAC TGTGAGGATG
2201 GCTTCATGCA CTGTACCATG AGTGGAGTCC CCGGAAGCTT GCTGCCTGAC
2251 GCTGTCCTCA GCAGTCCCCT GTCTCATCGC AGCAAAAGGA GCCTATCCTG
2301 TCGGCCCCCC ATGGTCAAGC TGGTGTGTCC CGCTGACAAC CTGCGGGCTG
2351 AAGGGCTCGA GTGTACCAAA ACGTGCCAGA ACTATGACCT GGAGTGCATG
2401 AGCATGGGCT GTGTCTCTGG CTGCCTCTGC CCCCCGGGCA TGGTCCGGCA
2451 TGAGAACAGA TGTGTGGCCC TGGAAAGGTG TCCCTGCTTC CATCAGGGCA
2501 AGGAGTATGC CCCTGGAGAA ACAGTGAAGA TTGGCTGCAA CACTTGTGTC
2551 TGTCGGGACC GGAAGTGGAA CTGCACAGAC CATGTGTGTG ATGCCACGTG
2601 CTCCACGATC GGCATGGCCC ACTACCTCAC CTTCGACGGG CTCAAATACC
2651 TGTTCCCCGG GGAGTGCCAG TACGTTCTGG TGCAGGATTA CTGCGGCAGT
2701 AACCCTGGGA CCTTTCGGAT CCTAGTGGGG AATAAGGGAT GCAGCCACCC
2751 CTCAGTGAAA TGCAAGAAAC GGGTCACCAT CCTGGTGGAG GGAGGAGAGA
2801 TTGAGCTGTT TGACGGGGAG GTGAATGTGA AGAGGCCCAT GAAGGATGAG
2851 ACTCACTTTG AGGTGGTGGA GTCTGGCCGG TACATCATTC TGCTGCTGGG
2901 CAAAGCCCTC TCCGTGGTCT GGGACCGCCA CCTGAGCATC TCCGTGGTCC
2951 TGAAGCAGAC ATACCAGGAG AAAGTGTGTG GCCTGTGTGG GAATTTTGAT
3001 GGCATCCAGA ACAATGACCT CACCAGCAGC AACCTCCAAG TGGAGGAAGA
3051 CCCTGTGGAC TTTGGGAACT CCTGGAAAGT GAGCTCGCAG TGTGCTGACA
3101 CCAGAAAAGT GCCTCTGGAC TCATCCCCTG CCACCTGCCA TAACAACATC
3151 ATGAAGCAGA CGATGGTGGA TTCCTCCTGT AGAATCCTTA CCAGTGACGT
3201 CTTCCAGGAC TGCAACAAGC TGGTGGACCC CGAGCCATAT CTGGATGTCT
3251 GCATTTACGA CACCTGCTCC TGTGAGTCCA TTGGGGACTG CGCCGCATTC
3301 TGCGACACCA TTGCTGCCTA TGCCCACGTG TGTGCCCAGC ATGGCAAGGT
3351 GGTGACCTGG AGGACGGCCA CATTGTGCCC CCAGAGCTGC GAGGAGAGGA
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3401 ATCTCCGGGA GAACGGGTAT GAGGCTGAGT GGCGCTATAA CAGCTGTGCA
3451 CCTGCCTGTC AAGTCACGTG TCAGCACCCT GAGCCACTGG CCTGCCCTGT
3501 GCAGTGTGTG GAGGGCTGCC ATGCCCACTG CCCTCCAGGG AAAATCCTGG
3551 ATGAGCTTTT GCAGACCTGC GTTGACCCTG AAGACTGTCC AGTGTGTGAG
3601 GTGGCTGGCC GGCGTTTTGC CTCAGGAAAG AAAGTCACCT TGAATCCCAG
3651 TGACCCTGAG CACTGCCAGA TTTGCCACTG TGATGTTGTC AACCTCACCT
3701 GTGAAGCCTG CCAGGAGCCG ATATCTATGA AAAATAATGA AGAAGCGGAA
3751 GACTATGATG ATGATCTTAC TGATTCTGAA ATGGATGTGG TCAGGTTTGA
3801 TGATGACAAC TCTCCTTCCT TTATCCAAAT TCGCTCAGTT GACAAAACTC
3851 ACACATGCCC ACCGTGCCCA GCTCCAGAAC TCCTGGGCGG ACCGTCAGTC
3901 TTCCTCTTCC CCCCAAAACC CAAGGACACC CTCATGATCT CCCGGACCCC
3951 TGAGGTCACA TGCGTGGTGG TGGACGTGAG CCACGAAGAC CCTGAGGTCA
4001 AGTTCAACTG GTACGTGGAC GGCGTGGAGG TGCATAATGC CAAGACAAAG
4051 CCGCGGGAGG AGCAGTACAA CAGCACGTAC CGTGTGGTCA GCGTCCTCAC
4101 CGTCCTGCAC CAGGACTGGC TGAATGGCAA GGAGTACAAG TGCAAGGTCT
4151 CCAACAAAGC CCTCCCAGCC CCCATCGAGA AAACCATCTC CAAAGCCAAA
4201 GGGCAGCCCC GAGAACCACA GGTGTACACC CTGCCCCCAT CCCGGGATGA
4251 GCTGACCAAG AACCAGGTCA GCCTGACCTG CCTGGTCAAA GGCTTCTATC
4301 CCAGCGACAT CGCCGTGGAG TGGGAGAGCA ATGGGCAGCC GGAGAACAAC
4351 TACAAGACCA CGCCTCCCGT GTTGGACTCC GACGGCTCCT TCTTCCTCTA
4401 CAGCAAGCTC ACCGTGGACA AGAGCAGGTG GCAGCAGGGG AACGTCTTCT
4451 CATGCTCCGT GATGCATGAG GCTCTGCACA ACCACTACAC GCAGAAGAGC
4501 CTCTCCCTGT CTCCGGGTAA ATGA
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[0228] **pSYN VWF054 protein sequence (VWF D'D3-Fc with 40 amino acid long little a1 linker)** (SEQ ID NO: 45)

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  1 MIPARFAGVL LALALILPGT LCAEGTRGRS STARCSLFGS DFVNTFDGSM
 51 YSFAGYCSYL LAGGCQKRSF SIIGDFQNGK RVSLSVYLGE FFDIHLFVNG
101 TVTQGDQRVS MPYASKGLYL ETEAGYYKLS GEAYGFVARI DGSGNFQVLL
151 SDRYFNKTCG LCGNFNIFAE DDFMTQEGTL TSDPYDFANS WALSSGEQWC
201 ERASPPSSSC NISSGEMQKG LWEQCQLLKS TSVFARCHPL VDPEPFVALC
251 EKTLCECAGG LECACPALLE YARTCAQEGM VLYGWTDHSA CSPVCPAGME
301 YRQCVSPCAR TCQSLHINEM CQERCVDGCS CPEGQLLDEG LCVESTECPC
351 VHSGKRYPPG TSLSRDCNTC ICRNSQWICS NEECPGECLV TGQSHFKSFD
401 NRYFTFSGIC QYLLARDCQD HSFSIVIETV QCADDRDAVC TRSVTVRLPG
451 LHNSLVKLKH GAGVAMDGQD IQLPLLKGDL RIQHTVTASV RLSYGEDLQM
501 DWDGRGRLLV KLSPVYAGKT CGLCGNYNGN QGDDFLTPSG LAEPRVEDFG
551 NAWKLHGDCQ DLQKQHSDPC ALNPRMTRFS EEACAVLTSP TFEACHRAVS
601 PLPYLRNCRY DVCSCSDGRE CLCGALASYA AACAGRGVRV AWREPGRCEL
651 NCPKGQVYLQ CGTPCNLTCR SLSYPDEECN EACLEGCFCP PGLYMDERGD
701 CVPKAQCPCY YDGEIFQPED IFSDHHTMCY CEDGFMHCTM SGVPGSLLPD
751 AVLSSPLSHR SKRSLSCRPP MVKLVCPADN LRAEGLECTK TCQNYDLECM
801 SMGCVSGCLC PPGMVRHENR CVALERCPCF HQGKEYAPGE TVKIGCNTCV
851 CRDRKWNCTD HVCDATCSTI GMAHYLTFDG LKYLFPGECQ YVLVQDYCGS
901 NPGTFRILVG NKGCSHPSVK CKKRVTILVE GGEIELFDGE VNVKRPMKDE
951 THFEVVESGR YIILLLGKAL SVVWDRHLSI SVVLKQTYQE KVCGLCGNFD
1001 GIQNNDLTSS NLQVEEDPVD FGNSWKVSSQ CADTRKVPLD SSPATCHNNI
1051 MKQTMVDSSC RILTSDVFQD CNKLVDPEPY LDVCIYDTCS CESIGDCAAF
1101 CDTIAAYAHV CAQHGKVVTW RTATLCPQSC EERNLRENGY EAEWRYNSCA
1151 PACQVTCQHP EPLACPVQCV EGCHAHCPPG KILDELLQTC VDPEDCPVCE
1201 VAGRRFASGK KVTLNPSDPE HCQICHCDVV NLTCEACQEP ESMKNNEEAE
1251 DYDDDLTDSE MDVVRFDDDN SPSFIQIRSV DKTHTCPPCP APELLGGPSV
1301 FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK
1351 PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK
1401 GQPREPQVYT LPPSRDELTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN
1451 YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS
1501 LSLSPGK*
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[0229] **pSYN VWF055 nucleotide sequence (VWF D'D3-Fc with 34 amino acid long little a2 linker)** (SEQ ID NO: 46)

```
1 ATGATTCCTG CCAGATTTGC CGGGGTGCTG CTTGCTCTGG CCCTCATTTT
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  51 GCCAGGGACC CTTTGTGCAG AAGGAACTCG CGGCAGGTCA TCCACGGCCC
 101 GATGCAGCCT TTTCGGAAGT GACTTCGTCA ACACCTTTGA TGGGAGCATG
 151 TACAGCTTTG CGGGATACTG CAGTTACCTC CTGGCAGGGG GCTGCCAGAA
 201 ACGCTCCTTC TCGATTATTG GGGACTTCCA GAATGGCAAG AGAGTGAGCC
 251 TCTCCGTGTA TCTTGGGGAA TTTTTTGACA TCCATTTGTT TGTCAATGGT
 301 ACCGTGACAC AGGGGGACCA AAGAGTCTCC ATGCCCTATG CCTCCAAAGG
 351 GCTGTATCTA GAAACTGAGG CTGGGTACTA CAAGCTGTCC GGTGAGGCCT
 401 ATGGCTTTGT GGCCAGGATC GATGGCAGCG GCAACTTTCA AGTCCTGCTG
 451 TCAGACAGAT ACTTCAACAA GACCTGCGGG CTGTGTGGCA ACTTTAACAT
 501 CTTTGCTGAA GATGACTTTA TGACCCAAGA AGGGACCTTG ACCTCGGACC
 551 CTTATGACTT TGCCAACTCA TGGGCTCTGA GCAGTGGAGA ACAGTGGTGT
 601 GAACGGGCAT CTCCTCCCAG CAGCTCATGC AACATCTCCT CTGGGGAAAT
 651 GCAGAAGGGC CTGTGGGAGC AGTGCCAGCT TCTGAAGAGC ACCTCGGTGT
 701 TTGCCCGCTG CCACCCTCTG GTGGACCCCG AGCCTTTTGT GGCCCTGTGT
 751 GAGAAGACTT TGTGTGAGTG TGCTGGGGGG CTGGAGTGCG CCTGCCCTGC
 801 CCTCCTGGAG TACGCCCGGA CCTGTGCCCA GGAGGGAATG GTGCTGTACG
 851 GCTGGACCGA CCACAGCGCG TGCAGCCCAG TGTGCCCTGC TGGTATGGAG
 901 TATAGGCAGT GTGTGTCCCC TTGCGCCAGG ACCTGCCAGA GCCTGCACAT
 951 CAATGAAATG TGTCAGGAGC GATGCGTGGA TGGCTGCAGC TGCCCTGAGG
1001 GACAGCTCCT GGATGAAGGC CTCTGCGTGG AGAGCACCGA GTGTCCCTGC
1051 GTGCATTCCG GAAAGCGCTA CCCTCCCGGC ACCTCCCTCT CTCGAGACTG
1101 CAACACCTGC ATTTGCCGAA ACAGCCAGTG GATCTGCAGC AATGAAGAAT
1151 GTCCAGGGGA GTGCCTTGTC ACTGGTCAAT CCCACTTCAA GAGCTTTGAC
1201 AACAGATACT TCACCTTCAG TGGGATCTGC CAGTACCTGC TGGCCCGGGA
1251 TTGCCAGGAC CACTCCTTCT CCATTGTCAT TGAGACTGTC CAGTGTGCTG
1301 ATGACCGCGA CGCTGTGTGC ACCCGCTCCG TCACCGTCCG GCTGCCTGGC
1351 CTGCACAACA GCCTTGTGAA ACTGAAGCAT GGGGCAGGAG TTGCCATGGA
1401 TGGCCAGGAC ATCCAGCTCC CCCTCCTGAA AGGTGACCTC CGCATCCAGC
1451 ATACAGTGAC GGCCTCCGTG CGCCTCAGCT ACGGGGAGGA CCTGCAGATG
1501 GACTGGGATG GCCGCGGGAG GCTGCTGGTG AAGCTGTCCC CCGTCTATGC
1551 CGGGAAGACC TGCGGCCTGT GTGGGAATTA CAATGGCAAC CAGGGCGACG
1601 ACTTCCTTAC CCCCTCTGGG CTGGCGGAGC CCCGGGTGGA GGACTTCGGG
1651 AACGCCTGGA AGCTGCACGG GGACTGCCAG GACCTGCAGA AGCAGCACAG
1701 CGATCCCTGC GCCCTCAACC CGCGCATGAC CAGGTTCTCC GAGGAGGCGT
1751 GCGCGGTCCT GACGTCCCCC ACATTCGAGG CCTGCCATCG TGCCGTCAGC
1801 CCGCTGCCCT ACCTGCGGAA CTGCCGCTAC GACGTGTGCT CCTGCTCGGA
1851 CGGCCGCGAG TGCCTGTGCG GCGCCCTGGC CAGCTATGCC GCGGCCTGCG
1901 CGGGGAGAGG CGTGCGCGTC GCGTGGCGCG AGCCAGGCCG CTGTGAGCTG
1951 AACTGCCCGA AAGGCCAGGT GTACCTGCAG TGCGGGACCC CCTGCAACCT
2001 GACCTGCCGC TCTCTCTCTT ACCCGGATGA GGAATGCAAT GAGGCCTGCC
2051 TGGAGGGCTG CTTCTGCCCC CCAGGGCTCT ACATGGATGA GAGGGGGGAC
2101 TGCGTGCCCA AGGCCCAGTG CCCCTGTTAC TATGACGGTG AGATCTTCCA
2151 GCCAGAAGAC ATCTTCTCAG ACCATCACAC CATGTGCTAC TGTGAGGATG
2201 GCTTCATGCA CTGTACCATG AGTGGAGTCC CCGGAAGCTT GCTGCCTGAC
2251 GCTGTCCTCA GCAGTCCCCT GTCTCATCGC AGCAAAAGGA GCCTATCCTG
2301 TCGGCCCCCC ATGGTCAAGC TGGTGTGTCC CGCTGACAAC CTGCGGGCTG
2351 AAGGGCTCGA GTGTACCAAA ACGTGCCAGA ACTATGACCT GGAGTGCATG
2401 AGCATGGGCT GTGTCTCTGG CTGCCTCTGC CCCCCGGGCA TGGTCCGGCA
2451 TGAGAACAGA TGTGTGGCCC TGGAAAGGTG TCCCTGCTTC CATCAGGGCA
2501 AGGAGTATGC CCCTGGAGAA ACAGTGAAGA TTGGCTGCAA CACTTGTGTC
2551 TGTCGGGACC GGAAGTGGAA CTGCACAGAC CATGTGTGTG ATGCCACGTG
2601 CTCCACGATC GGCATGGCCC ACTACCTCAC CTTCGACGGG CTCAAATACC
2651 TGTTCCCCGG GGAGTGCCAG TACGTTCTGG TGCAGGATTA CTGCGGCAGT
2701 AACCCTGGGA CCTTTCGGAT CCTAGTGGGG AATAAGGGAT GCAGCCACCC
2751 CTCAGTGAAA TGCAAGAAAC GGGTCACCAT CCTGGTGGAG GGAGGAGAGA
2801 TTGAGCTGTT TGACGGGGAG GTGAATGTGA AGAGGCCCAT GAAGGATGAG
2851 ACTCACTTTG AGGTGGTGGA GTCTGGCCGG TACATCATTC TGCTGCTGGG
2901 CAAAGCCCTC TCCGTGGTCT GGGACCGCCA CCTGAGCATC TCCGTGGTCC
2951 TGAAGCAGAC ATACCAGGAG AAAGTGTGTG GCCTGTGTGG GAATTTTGAT
3001 GGCATCCAGA ACAATGACCT CACCAGCAGC AACCTCCAAG TGGAGGAAGA
3051 CCCTGTGGAC TTTGGGAACT CCTGGAAAGT GAGCTCGCAG TGTGCTGACA
3101 CCAGAAAAGT GCCTCTGGAC TCATCCCCTG CCACCTGCCA TAACAACATC
3151 ATGAAGCAGA CGATGGTGGA TTCCTCCTGT AGAATCCTTA CCAGTGACGT
3201 CTTCCAGGAC TGCAACAAGC TGGTGGACCC CGAGCCATAT CTGGATGTCT
3251 GCATTTACGA CACCTGCTCC TGTGAGTCCA TTGGGGACTG CGCCGCATTC
3301 TGCGACACCA TTGCTGCCTA TGCCCACGTG TGTGCCCAGC ATGGCAAGGT
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3351 GGTGACCTGG AGGACGGCCA CATTGTGCCC CCAGAGCTGC GAGGAGAGGA
3401 ATCTCCGGGA GAACGGGTAT GAGGCTGAGT GGCGCTATAA CAGCTGTGCA
3451 CCTGCCTGTC AAGTCACGTG TCAGCACCCT GAGCCACTGG CCTGCCCTGT
3501 GCAGTGTGTG GAGGGCTGCC ATGCCCACTG CCCTCCAGGG AAAATCCTGG
3551 ATGAGCTTTT GCAGACCTGC GTTGACCCTG AAGACTGTCC AGTGTGTGAG
3601 GTGGCTGGCC GGCGTTTTGC CTCAGGAAAG AAAGTCACCT TGAATCCCAG
3651 TGACCCTGAG CACTGCCAGA TTTGCCACTG TGATGTTGTC AACCTCACCT
3701 GTGAAGCCTG CCAGGAGCCG ATATCTGACA AGAACACTGG TGATTATTAC
3751 GAGGACAGTT ATGAAGATAT TTCAGCATAC TTGCTGAGTA AAAACAATGC
3801 CATTGAACCA AGAAGCTTCT CTGACAAAAC TCACACATGC CCACCGTGCC
3851 CAGCTCCAGA ACTCCTGGGC GGACCGTCAG TCTTCCTCTT CCCCCCAAAA
3901 CCCAAGGACA CCCTCATGAT CTCCCGGACC CCTGAGGTCA CATGCGTGGT
3951 GGTGGACGTG AGCCACGAAG ACCCTGAGGT CAAGTTCAAC TGGTACGTGG
4001 ACGGCGTGGA GGTGCATAAT GCCAAGACAA AGCCGCGGGA GGAGCAGTAC
4051 AACAGCACGT ACCGTGTGGT CAGCGTCCTC ACCGTCCTGC ACCAGGACTG
4101 GCTGAATGGC AAGGAGTACA AGTGCAAGGT CTCCAACAAA GCCCTCCCAG
4151 CCCCCATCGA GAAAACCATC TCCAAAGCCA AAGGGCAGCC CCGAGAACCA
4201 CAGGTGTACA CCCTGCCCCC ATCCCGGGAT GAGCTGACCA AGAACCAGGT
4251 CAGCCTGACC TGCCTGGTCA AAGGCTTCTA TCCCAGCGAC ATCGCCGTGG
4301 AGTGGGAGAG CAATGGGCAG CCGGAGAACA ACTACAAGAC CACGCCTCCC
4351 GTGTTGGACT CCGACGGCTC CTTCTTCCTC TACAGCAAGC TCACCGTGGA
4401 CAAGAGCAGG TGGCAGCAGG GGAACGTCTT CTCATGCTCC GTGATGCATG
4451 AGGCTCTGCA CAACCACTAC ACGCAGAAGA GCCTCTCCCT GTCTCCGGGT
4501 AAATGA
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[0230] **pSYN VWF055 protein sequence (VWF D'D3-Fc with 34 amino acid long little a2 linker)** (SEQ ID NO: 47)

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  1 MIPARFAGVL LALALILPGT LCAEGTRGRS STARCSLFGS DFVNTFDGSM
 51 YSFAGYCSYL LAGGCQKRSF SIIGDFQNGK RVSLSVYLGE FFDIHLFVNG
101 TVTQGDQRVS MPYASKGLYL ETEAGYYKLS GEAYGFVARI DGSGNFQVLL
151 SDRYFNKTCG LCGNFNIFAE DDFMTQEGTL TSDPYDFANS WALSSGEQWC
201 ERASPPSSSC NISSGEMQKG LWEQCQLLKS TSVFARCHPL VDPEPFVALC
251 EKTLCECAGG LECACPALLE YARTCAQEGM VLYGWTDHSA CSPVCPAGME
301 YRQCVSPCAR TCQSLHINEM CQERCVDGCS CPEGQLLDEG LCVESTECPC
351 VHSGKRYPPG TSLSRDCNTC ICRNSQWICS NEECPGECLV TGQSHFKSFD
401 NRYFTFSGIC QYLLARDCQD HSFSIVIETV QCADDRDAVC TRSVTVRLPG
451 LHNSLVKLKH GAGVAMDGQD IQLPLLKGDL RIQHTVTASV RLSYGEDLQM
501 DWDGRGRLLV KLSPVYAGKT CGLCGNYNGN QGDDFLTPSG LAEPRVEDFG
551 NAWKLHGDCQ DLQKQHSDPC ALNPRMTRFS EEACAVLTSP TFEACHRAVS
601 PLPYLRNCRY DVCSCSDGRE CLCGALASYA AACAGRGVRV AWREPGRCEL
651 NCPKGQVYLQ CGTPCNLTCR SLSYPDEECN EACLEGCFCP PGLYMDERGD
701 CVPKAQCPCY YDGEIFQPED IFSDHHTMCY CEDGFMHCTM SGVPGSLLPD
751 AVLSSPLSHR SKRSLSCRPP MVKLVCPADN LRAEGLECTK TCQNYDLECM
801 SMGCVSGCLC PPGMVRHENR CVALERCPCF HQGKEYAPGE TVKIGCNTCV
851 CRDRKWNCTD HVCDATCSTI GMAHYLTFDG LKYLFPGECQ YVLVQDYCGS
901 NPGTFRILVG NKGCSHPSVK CKKRVTILVE GGEIELFDGE VNVKRPMKDE
951 THFEVVESGR YIILLLGKAL SVVWDRHLSI SVVLKQTYQE KVCGLCGNFD
1001 GIQNNDLTSS NLQVEEDPVD FGNSWKVSSQ CADTRKVPLD SSPATCHNNI
1051 MKQTMVDSSC RILTSDVFQD CNKLVDPEPY LDVCIYDTCS CESIGDCAAF
1101 CDTIAAYAHV CAQHGKVVTW RTATLCPQSC EERNLRENGY EAEWRYNSCA
1151 PACQVTCQHP EPLACPVQCV EGCHAHCPPG KILDELLQTC VDPEDCPVCE
1201 VAGRRFASGK KVTLNPSDPE HCQICHCDVV NLTCEACQEP ISDKNTGDYY
1251 EDSYEDISAY LLSKNNAIEP RSFSDKTHTC PPCPAPELLG GPSVFLFPPK
1301 PKDTLMISRT PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY
1351 NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI SKAKGQPREP
1401 QVYTLPPSRD ELTKNQVSLT CLVKGFYPSD IAVEWESNGQ PENNYKTTPP
1451 VLDSDGSFFL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG
1501 K*
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[0231] **pSYN VWF056 nucleotide sequence (VWF D'D3-Fc with 46 amino acid little a3 linker)** (SEQ ID NO: 31)

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   1 ATGATTCCTG CCAGATTTGC CGGGGTGCTG CTTGCTCTGG CCCTCATTTT
  51 GCCAGGGACC CTTTGTGCAG AAGGAACTCG CGGCAGGTCA TCCACGGCCC
 101 GATGCAGCCT TTTCGGAAGT GACTTCGTCA ACACCTTTGA TGGGAGCATG
 151 TACAGCTTTG CGGGATACTG CAGTTACCTC CTGGCAGGGG GCTGCCAGAA
 201 ACGCTCCTTC TCGATTATTG GGGACTTCCA GAATGGCAAG AGAGTGAGCC
 251 TCTCCGTGTA TCTTGGGGAA TTTTTTGACA TCCATTTGTT TGTCAATGGT
 301 ACCGTGACAC AGGGGGACCA AAGAGTCTCC ATGCCCTATG CCTCCAAAGG
 351 GCTGTATCTA GAAACTGAGG CTGGGTACTA CAAGCTGTCC GGTGAGGCCT
 401 ATGGCTTTGT GGCCAGGATC GATGGCAGCG GCAACTTTCA AGTCCTGCTG
 451 TCAGACAGAT ACTTCAACAA GACCTGCGGG CTGTGTGGCA ACTTTAACAT
 501 CTTTGCTGAA GATGACTTTA TGACCCAAGA AGGGACCTTG ACCTCGGACC
 551 CTTATGACTT TGCCAACTCA TGGGCTCTGA GCAGTGGAGA ACAGTGGTGT
 601 GAACGGGCAT CTCCTCCCAG CAGCTCATGC AACATCTCCT CTGGGGAAAT
 651 GCAGAAGGGC CTGTGGGAGC AGTGCCAGCT TCTGAAGAGC ACCTCGGTGT
 701 TTGCCCGCTG CCACCCTCTG GTGGACCCCG AGCCTTTTGT GGCCCTGTGT
 751 GAGAAGACTT TGTGTGAGTG TGCTGGGGGG CTGGAGTGCG CCTGCCCTGC
 801 CCTCCTGGAG TACGCCCGGA CCTGTGCCCA GGAGGGAATG GTGCTGTACG
 851 GCTGGACCGA CCACAGCGCG TGCAGCCCAG TGTGCCCTGC TGGTATGGAG
 901 TATAGGCAGT GTGTGTCCCC TTGCGCCAGG ACCTGCCAGA GCCTGCACAT
 951 CAATGAAATG TGTCAGGAGC GATGCGTGGA TGGCTGCAGC TGCCCTGAGG
1001 GACAGCTCCT GGATGAAGGC CTCTGCGTGG AGAGCACCGA GTGTCCCTGC
1051 GTGCATTCCG GAAAGCGCTA CCCTCCCGGC ACCTCCCTCT CTCGAGACTG
1101 CAACACCTGC ATTTGCCGAA ACAGCCAGTG GATCTGCAGC AATGAAGAAT
1151 GTCCAGGGGA GTGCCTTGTC ACTGGTCAAT CCCACTTCAA GAGCTTTGAC
1201 AACAGATACT TCACCTTCAG TGGGATCTGC CAGTACCTGC TGGCCCGGGA
1251 TTGCCAGGAC CACTCCTTCT CCATTGTCAT TGAGACTGTC CAGTGTGCTG
1301 ATGACCGCGA CGCTGTGTGC ACCCGCTCCG TCACCGTCCG GCTGCCTGGC
1351 CTGCACAACA GCCTTGTGAA ACTGAAGCAT GGGGCAGGAG TTGCCATGGA
1401 TGGCCAGGAC ATCCAGCTCC CCCTCCTGAA AGGTGACCTC CGCATCCAGC
1451 ATACAGTGAC GGCCTCCGTG CGCCTCAGCT ACGGGGAGGA CCTGCAGATG
1501 GACTGGGATG GCCGCGGGAG GCTGCTGGTG AAGCTGTCCC CCGTCTATGC
1551 CGGGAAGACC TGCGGCCTGT GTGGGAATTA CAATGGCAAC CAGGGCGACG
1601 ACTTCCTTAC CCCCTCTGGG CTGGCGGAGC CCCGGGTGGA GGACTTCGGG
1651 AACGCCTGGA AGCTGCACGG GGACTGCCAG GACCTGCAGA AGCAGCACAG
1701 CGATCCCTGC GCCCTCAACC CGCGCATGAC CAGGTTCTCC GAGGAGGCGT
1751 GCGCGGTCCT GACGTCCCCC ACATTCGAGG CCTGCCATCG TGCCGTCAGC
1801 CCGCTGCCCT ACCTGCGGAA CTGCCGCTAC GACGTGTGCT CCTGCTCGGA
1851 CGGCCGCGAG TGCCTGTGCG GCGCCCTGGC CAGCTATGCC GCGGCCTGCG
1901 CGGGGAGAGG CGTGCGCGTC GCGTGGCGCG AGCCAGGCCG CTGTGAGCTG
1951 AACTGCCCGA AAGGCCAGGT GTACCTGCAG TGCGGGACCC CCTGCAACCT
2001 GACCTGCCGC TCTCTCTCTT ACCCGGATGA GGAATGCAAT GAGGCCTGCC
2051 TGGAGGGCTG CTTCTGCCCC CCAGGGCTCT ACATGGATGA GAGGGGGGAC
2101 TGCGTGCCCA AGGCCCAGTG CCCCTGTTAC TATGACGGTG AGATCTTCCA
2151 GCCAGAAGAC ATCTTCTCAG ACCATCACAC CATGTGCTAC TGTGAGGATG
2201 GCTTCATGCA CTGTACCATG AGTGGAGTCC CCGGAAGCTT GCTGCCTGAC
2251 GCTGTCCTCA GCAGTCCCCT GTCTCATCGC AGCAAAAGGA GCCTATCCTG
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2501 AGGAGTATGC CCCTGGAGAA ACAGTGAAGA TTGGCTGCAA CACTTGTGTC
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2601 CTCCACGATC GGCATGGCCC ACTACCTCAC CTTCGACGGG CTCAAATACC
2651 TGTTCCCCGG GGAGTGCCAG TACGTTCTGG TGCAGGATTA CTGCGGCAGT
2701 AACCCTGGGA CCTTTCGGAT CCTAGTGGGG AATAAGGGAT GCAGCCACCC
2751 CTCAGTGAAA TGCAAGAAAC GGGTCACCAT CCTGGTGGAG GGAGGAGAGA
2801 TTGAGCTGTT TGACGGGGAG GTGAATGTGA AGAGGCCCAT GAAGGATGAG
2851 ACTCACTTTG AGGTGGTGGA GTCTGGCCGG TACATCATTC TGCTGCTGGG
2901 CAAAGCCCTC TCCGTGGTCT GGGACCGCCA CCTGAGCATC TCCGTGGTCC
2951 TGAAGCAGAC ATACCAGGAG AAAGTGTGTG GCCTGTGTGG GAATTTTGAT
3001 GGCATCCAGA ACAATGACCT CACCAGCAGC AACCTCCAAG TGGAGGAAGA
3051 CCCTGTGGAC TTTGGGAACT CCTGGAAAGT GAGCTCGCAG TGTGCTGACA
3101 CCAGAAAAGT GCCTCTGGAC TCATCCCCTG CCACCTGCCA TAACAACATC
3151 ATGAAGCAGA CGATGGTGGA TTCCTCCTGT AGAATCCTTA CCAGTGACGT
3201 CTTCCAGGAC TGCAACAAGC TGGTGGACCC CGAGCCATAT CTGGATGTCT
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3351 GGTGACCTGG AGGACGGCCA CATTGTGCCC CCAGAGCTGC GAGGAGAGGA
3401 ATCTCCGGGA GAACGGGTAT GAGGCTGAGT GGCGCTATAA CAGCTGTGCA
3451 CCTGCCTGTC AAGTCACGTG TCAGCACCCT GAGCCACTGG CCTGCCCTGT
3501 GCAGTGTGTG GAGGGCTGCC ATGCCCACTG CCCTCCAGGG AAAATCCTGG
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3601 GTGGCTGGCC GGCGTTTTGC CTCAGGAAAG AAAGTCACCT TGAATCCCAG
3651 TGACCCTGAG CACTGCCAGA TTTGCCACTG TGATGTTGTC AACCTCACCT
3701 GTGAAGCCTG CCAGGAGCCG ATATCTGAAA TAACTCGTAC TACTCTTCAG
3751 TCAGATCAAG AGGAAATTGA CTATGATGAT ACCATATCAG TTGAAATGAA
3801 GAAGGAAGAT TTTGACATTT ATGATGAGGA TGAAAATCAG AGCCCCCGCA
3851 GCTTTCAAGA CAAAACTCAC ACATGCCCAC CGTGCCCAGC TCCAGAACTC
3901 CTGGGCGGAC CGTCAGTCTT CCTCTTCCCC CCAAAACCCA AGGACACCCT
3951 CATGATCTCC CGGACCCCTG AGGTCACATG CGTGGTGGTG GACGTGAGCC
4001 ACGAAGACCC TGAGGTCAAG TTCAACTGGT ACGTGGACGG CGTGGAGGTG
4051 CATAATGCCA AGACAAAGCC GCGGGAGGAG CAGTACAACA GCACGTACCG
4101 TGTGGTCAGC GTCCTCACCG TCCTGCACCA GGACTGGCTG AATGGCAAGG
4151 AGTACAAGTG CAAGGTCTCC AACAAAGCCC TCCCAGCCCC CATCGAGAAA
4201 ACCATCTCCA AAGCCAAAGG GCAGCCCCGA GAACCACAGG TGTACACCCT
4251 GCCCCCATCC CGGGATGAGC TGACCAAGAA CCAGGTCAGC CTGACCTGCC
4301 TGGTCAAAGG CTTCTATCCC AGCGACATCG CCGTGGAGTG GGAGAGCAAT
4351 GGGCAGCCGG AGAACAACTA CAAGACCACG CCTCCCGTGT TGGACTCCGA
4401 CGGCTCCTTC TTCCTCTACA GCAAGCTCAC CGTGGACAAG AGCAGGTGGC
4451 AGCAGGGGAA CGTCTTCTCA TGCTCCGTGA TGCATGAGGC TCTGCACAAC
4501 CACTACACGC AGAAGAGCCT CTCCCTGTCT CCGGGTAAAT GA
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[0232] **pSYN VWF056 protein sequence (VWF D'D3-Fc with 46 amino acid long little a3 linker)** (SEQ ID NO: 39)

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   1 MIPARFAGVL LALALILPGT LCAEGTRGRS STARCSLFGS DFVNTFDGSM
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 101 TVTQGDQRVS MPYASKGLYL ETEAGYYKLS GEAYGFVARI DGSGNFQVLL
 151 SDRYFNKTCG LCGNFNIFAE DDFMTQEGTL TSDPYDFANS WALSSGEQWC
 201 ERASPPSSSC NISSGEMQKG LWEQCQLLKS TSVFARCHPL VDPEPFVALC
 251 EKTLCECAGG LECACPALLE YARTCAQEGM VLYGWTDHSA CSPVCPAGME
 301 YRQCVSPCAR TCQSLHINEM CQERCVDGCS CPEGQLLDEG LCVESTECPC
 351 VHSGKRYPPG TSLSRDCNTC ICRNSQWICS NEECPGECLV TGQSHFKSFD
 401 NRYFTFSGIC QYLLARDCQD HSFSIVIETV QCADDRDAVC TRSVTVRLPG
 451 LHNSLVKLKH GAGVAMDGQD IQLPLLKGDL RIQHTVTASV RLSYGEDLQM
 501 DWDGRGRLLV KLSPVYAGKT CGLCGNYNGN QGDDFLTPSG LAEPRVEDFG
 551 NAWKLHGDCQ DLQKQHSDPC ALNPRMTRFS EEACAVLTSP TFEACHRAVS
 601 PLPYLRNCRY DVCSCSDGRE CLCGALASYA AACAGRGVRV AWREPGRCEL
 651 NCPKGQVYLQ CGTPCNLTCR SLSYPDEECN EACLEGCFCP PGLYMDERGD
 701 CVPKAQCPCY YDGEIFQPED IFSDHHTMCY CEDGFMHCTM SGVPGSLLPD
 751 AVLSSPLSHR SKRSLSCRPP MVKLVCPADN LRAEGLECTK TCQNYDLECM
 801 SMGCVSGCLC PPGMVRHENR CVALERCPCF HQGKEYAPGE TVKIGCNTCV
 851 CRDRKWNCTD HVCDATCSTI GMAHYLTFDG LKYLFPGECQ YVLVQDYCGS
 901 NPGTFRILVG NKGCSHPSVK CKKRVTILVE GGEIELFDGE VNVKRPMKDE
 951 THFEVVESGR YIILLLGKAL SVVWDRHLSI SVVLKQTYQE KVCGLCGNFD
1001 GIQNNDLTSS NLQVEEDPVD FGNSWKVSSQ CADTRKVPLD SSPATCHNNI
1051 MKQTMVDSSC RILTSDVFQD CNKLVDPEPY LDVCIYDTCS CESIGDCAAF
1101 CDTIAAYAHV CAQHGKVVTW RTATLCPQSC EERNLRENGY EAEWRYNSCA
1151 PACQVTCQHP EPLACPVQCV EGCHAHCPPG KILDELLQTC VDPEDCPVCE
1201 VAGRRFASGK KVTLNPSDPE HCQICHCDVV NLTCEACQEP ISEITRTTLQ
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1351 HNAKTKPREE QYNSTYRVVS VLTVLHQDWL NGKEYKCKVS NKALPAPIEK
1401 TISKAKGQPR EPQVYTLPPS RDELTKNQVS LTCLVKGFYP SDIAVEWESN
1451 GQPENNYKTT PPVLDSDGSF FLYSKLTVDK SRWQQGNVFS CSVMHEALHN
1501 HYTQKSLSLS PGK*
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pSYN-FVIII-155 mature Protein sequencing (SEQ ID NO: 50):

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DSYEDISAYLLSKNNAIEPRSFSQNPPVLKAHQAEITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQ
SPRSFQKKTRHYFIAAVERLWDYGMSSSPHVLRNRAQSGSVPQFKKVVFQEFTDGSFTQPLYRGELNEHLGL
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DCKAWAYFSDVDLEKDVHSGLIGPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAP
CNIQMEDPTFKENYRFHAINGYIMDTLPGLVMAQDQRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMA
LYNLYPGVFETVEMLPSKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQW
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CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ
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[0233] pSYN-FVIII-155 DNA sequencing (SEQ ID NO:51):

ATGCAAATAGAGCTCTCCACCTGCTTCTTTCTGTGCCTTTTGCGATTCTGCTTTAGTGCCACCAGAAGATAC
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GCTGAGGTTTATGATACAGTGGTCATTACACTTAAGAACATGGCTTCCCATCCTGTCAGTCTTCATGCTGTT
GGTGTATCCTACTGGAAAGCTTCTGAGGGAGCTGAATATGATGATCAGACCAGTCAAAGGGAGAAAGAAGAT
GATAAAGTCTTCCCTGGTGGAAGCCATACATATGTCTGGCAGGTCCTGAAAGAGAATGGTCCAATGGCCTCT
GACCCACTGTGCCTTACCTACTCATATCTTTCTCATGTGGACCTGGTAAAAGACTTGAATTCAGGCCTCATT
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AACCGAAGCTGGTACCTCACAGAGAATATACAACGCTTTCTCCCCAATCCAGCTGGAGTGCAGCTTGAGGAT
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TGTTTGCATGAGGTGGCATACTGGTACATTCTAAGCATTGGAGCACAGACTGACTTCCTTTCTGTCTTCTTC
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CAAAAGAAAACACGACACTATTTTATTGCTGCAGTGGAGAGGCTCTGGGATTATGGGATGAGTAGCTCCCCA
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AGAGCAGAAGTTGAAGATAATATCATGGTAACTTTCAGAAATCAGGCCTCTCGTCCCTATTCCTTCTATTCT
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ACCAAAACTTACTTTTGGAAAGTGCAACATCATATGGCACCCACTAAAGATGAGTTTGACTGCAAAGCCTGG
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GAAGATCCCACTTTTAAAGAGAATTATCGCTTCCATGCAATCAATGGCTACATAATGGATACACTACCTGGC
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GCTTCTGGACACATTAGAGATTTTCAGATTACAGCTTCAGGACAATATGGACAGTGGGCCCCAAAGCTGGCC
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CAGTTTATCATCATGTATAGTCTTGATGGGAAGAAGTGGCAGACTTATCGAGGAAATTCCACTGGAACCTTA
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TACATCCGTTTGCACCCAACTCATTATAGCATTCGCAGCACTCTTCGCATGGAGTTGATGGGCTGTGATTTA
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TTTACCAATATGTTTGCCACCTGGTCTCCTTCAAAAGCTCGACTTCACCTCCAAGGGAGGAGTAATGCCTGG
AGACCTCAGGTGAATAATCCAAAAGAGTGGCTGCAAGTGGACTTCCAGAAGACAATGAAAGTCACAGGAGTA
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GGCCATCAGTGGACTCTCTTTTTTCAGAATGGCAAAGTAAAGGTTTTTCAGGGAAATCAAGACTCCTTCACA
CCTGTGGTGAACTCTCTAGACCCACCGTTACTGACTCGCTACCTTCGAATTCACCCCCAGAGTTGGGTGCAC
CAGATTGCCCTGAGGATGGAGGTTCTGGGCTGCGAGGCACAGGACCTCTACGACAAAACTCACACATGCCCA
CCGTGCCCAGCTCCAGAACTCCTGGGCGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTC
ATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTC
AACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACG
TACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTC
TCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAG
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TTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCT
CCCGTGTTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAG
GGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTG
TCTCCGGGTAAA

[0234] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art, readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or

phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance.

[0235] Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

[0236] All patents and publications cited herein are incorporated by reference herein in their entirety.

[0237] This application claims the benefit of priority of U.S. Provisional Patent Application No. 61/840,864 filed on June 28, 2013. The contents of the above application are incorporated herein by reference in their entirety.

WHAT IS CLAIMED IS:

1. A chimeric molecule comprising a Von Willebrand Factor (VWF) protein, a heterologous moiety (H1), and a VWF linker connecting the VWF protein with the heterologous moiety, wherein the VWF linker comprises a polypeptide selected from:

i. an a2 region from Factor VIII (FVIII);

ii. an a1 region from FVIII;

iii. an a3 region from FVIII;

iv. a thrombin cleavage site which comprises X-V-P-R (SEQ ID NO: 3) and a PAR1 exosite interaction motif, wherein X is an aliphatic amino acid; or

v. any combination thereof.

2. The chimeric molecule of claim 1, wherein the VWF linker comprises the a2 region which comprises an amino acid sequence at least about 80%, about 85%, about 90%, about 95%, or 100% identical to Glu720 to Arg740 corresponding to full-length FVIII, wherein the a2 region is capable of being cleaved by thrombin.

3. The chimeric molecule of claim 1 or 2, wherein the a2 region comprises ISDKNTGDYYEDSYEDISAYLLSKNNAIEPRSFS (SEQ ID NO: 29).

4. The chimeric molecule of claim 1, wherein the VWF linker comprises the a1 region which comprises an amino acid sequence at least about 80%, about 85%, about 90%, about 95%, or 100% identical to Met337 to Arg372 corresponding to full-length FVIII, wherein the a1 region is capable of being cleaved by thrombin.

5. The chimeric molecule of claim 1 or 4, wherein the a1 region comprises ISMKNNEEAEDYDDDLTDSEMDVVRFDDDNSPSFIQIRSV (SEQ ID NO: 28).

6. The chimeric molecule of claim 1, wherein the VWF linker comprises the a3 region which comprises an amino acid sequence at least about 80%, about 85%, about 90%, about 95%, or 100% identical to Glu1649 to Arg1689 corresponding to full-length FVIII, wherein the a3 region is capable of being cleaved by thrombin.

7. The chimeric molecule of claim 1 or 6, wherein the a3 region comprises ISEITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQSPRSFQ (SEQ ID NO: 30).

8. The chimeric molecule of claim 1, wherein the VWF linker comprises the thrombin cleavage site which comprises X-V-P-R (SEQ ID NO: 3) and the PAR1 exosite interaction motif and wherein the PAR1 exosite interaction motif comprises S-F-L-L-R-N (SEQ ID NO: 4).

9. The chimeric molecule of claim 1 or 8, wherein the PAR1 exosite interaction motif further comprises an amino acid sequence selected from P, P-N, P-N-D, P-N-D-K (SEQ ID NO: 5), P-N-D-K-Y (SEQ ID NO: 6), P-N-D-K-Y-E (SEQ ID NO: 7), P-N-D-K-Y-E-P (SEQ ID NO: 8), P-N-D-K-Y-E-P-F (SEQ ID NO: 9), P-N-D-K-Y-E-P-F-W (SEQ ID NO: 10), P-N-D-K-Y-E-P-F-W-E (SEQ ID NO: 11), P-N-D-K-Y-E-P-F-W-E-D (SEQ ID NO: 12), P-N-D-K-Y-E-P-F-W-E-D-E (SEQ ID NO: 13), P-N-D-K-Y-E-P-F-W-E-D-E-E (SEQ ID NO: 14), P-N-D-K-Y-E-P-F-W-E-D-E-E-S (SEQ ID NO: 20), or any combination thereof.

10. The chimeric molecule of any one of claims 1, 8, or 9, wherein the aliphatic amino acid is selected from Glycine, Alanine, Valine, Leucine, or Isoleucine.

11. The chimeric molecule of any one of claims 1 or 8 to 10, wherein the VWF linker comprises L-V-P-R-S-F-L-L-R-N (SEQ ID NOs: 4 and 21).

12. The chimeric molecule of any one of claims 1 to 11, wherein thrombin cleaves the VWF linker faster than thrombin would cleave the thrombin cleavage site if the thrombin cleavage site were substituted for the VWF linker in the chimeric molecule.

13. The chimeric molecule of claim 12, wherein thrombin cleaves the VWF linker at least about 10 times, at least about 20 times, at least about 30 times, at least about 40 times, at least about 50 times, at least about 60 times, at least about 70 times, at least about 80 times, at least about 90 times or at least about 100 times faster than thrombin would cleave the thrombin cleavage site if the thrombin cleavage site were substituted for the VWF linker in the chimeric molecule.

14. The chimeric molecule of any one of claims 1 to 13, wherein the VWF linker further comprises one or more amino acids having a length of at least about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1200, 1400, 1600, 1800, or 2000 amino acids.

15. The chimeric molecule of claim 14, wherein the one or more amino acids comprise a gly peptide.

16. The chimeric molecule of claim 14 or 15, wherein the one or more amino acids comprise GlyGly.

17. The chimeric molecule of claim 14, wherein the one or more amino acids comprise a gly/ser peptide.

18. The chimeric molecule of claim 17, wherein the gly/ser peptide has a formula of $(Gly_4Ser)n$ or $S(Gly_4Ser)n$, wherein n is a positive integer selected from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, or 100.

19. The chimeric molecule of claim 18, wherein the $(Gly_4Ser)n$ linker is $(Gly_4Ser)_3$ (SEQ ID NO: 48) or $(Gly_4Ser)_4$ (SEQ ID NO: 49).

20. The chimeric molecule of any one of claims 1 to 19, wherein the VWF protein comprises a D' domain and a D3 domain of VWF, wherein the D' domain and D3 domain are capable of binding to FVIII.

21. The chimeric molecule of claim 20, wherein the D' domain of the VWF protein comprises an amino acid sequence at least about 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 764 to 866 of SEQ ID NO: 2.

22. The chimeric molecule of claim 20 or 21, wherein the D3 domain of the VWF protein comprises an amino acid sequence at least about 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 867 to 1240 of SEQ ID NO: 2.

23. The chimeric molecule of any one of claims 20 to 22, wherein the VWF protein contains at least one amino acid substitution at a residue corresponding to residue 1099, residue 1142, or both residues 1099 and 1142 of SEQ ID NO: 2.

24. The chimeric molecule of any one of claims 1 to 23, wherein in the sequence of the VWF protein, an amino acid other than cysteine is substituted for a residue corresponding to residue 1099, residue 1142, or both residues 1099 and 1142 of SEQ ID NO: 2.

25. The chimeric molecule of any one of claims 1 to 23, wherein the sequence of the VWF protein comprises amino acids 764 to 1240 of SEQ ID NO: 2.

26. The chimeric molecule of any one of claims 20 to 25, wherein the VWF protein further comprises the D1 domain, the D2 domain, or the D1 and D2 domains of VWF.

27. The chimeric molecule of any one of claims 20 to 26, wherein the VWF protein further comprises a VWF domain selected from the group consisting of the A1 domain, the A2 domain, the A3 domain, the D4 domain, the B1 domain, the B2 domain, the B3 domain, the C1 domain, the C2 domain, the CK domain, one or more fragments thereof, and any combination thereof.

28. The chimeric molecule of any one of claims 20 to 27, wherein the VWF protein consists essentially of or consists of: (1) the D' and D3 domains of VWF or fragments thereof; (2) the D1, D', and D3 domains of VWF or fragments thereof; (3) the D2, D', and D3 domains of

VWF or fragments thereof; (4) the D1, D2, D', and D3 domains of VWF or fragments thereof; or (5) the D1, D2, D', D3, and A1 domains of VWF or fragments thereof.

29. The chimeric molecule of any one of claims 1 to 28, further comprising a signal peptide of VWF, which is operably linked to the VWF protein.

30. The chimeric molecule of any one of claims 1 to 29, wherein the VWF protein is pegylated, glycosylated, hesylated, or polysialylated.

31. The chimeric molecule of any one of claims 1 to 30, wherein the heterologous moiety (H1) is capable of extending the half-life of the chimeric molecule.

32. The chimeric molecule of claim 31, wherein the heterologous moiety (H1) comprises an immunoglobulin constant region or a portion thereof, albumin, albumin-binding moiety, PAS, HAP, the C-terminal peptide (CTP) of the β subunit of human chorionic gonadotropin, PSA, polyethylene glycol (PEG), hydroxyethyl starch (HES), albumin-binding small molecules, or any combination thereof.

33. The chimeric molecule of claim 32, wherein the immunoglobulin constant region or a portion thereof comprises an FcRn binding partner.

34. The chimeric molecule of claim 32, wherein the immunoglobulin constant region or a portion thereof comprises an Fc region.

35. The chimeric molecule of any one of claims 1 to 31, wherein the heterologous moiety (H1) comprises a clearance receptor, or fragment thereof, wherein the clearance receptor blocks binding of the recombinant FVIII protein to FVIII clearance receptors.

36. The chimeric molecule of claim 35, wherein the clearance receptor is a low-density lipoprotein receptor-related protein 1 (LRP1) or FVIII-binding fragment thereof.

37. The chimeric molecule of any one of claims 1 to 36, which further comprises a second polypeptide chain comprising a FVIII protein and a second heterologous moiety (H2), wherein the FVIII protein is associated with the VWF protein.

38. The chimeric molecule of any one of claims 1 to 36, which further comprises a FVIII protein and a second heterologous moiety (H2), which are linked directly or via a linker to the VWF protein or the heterologous moiety.

39. The chimeric molecule of claim 37 or 38, wherein the second heterologous moiety is selected from an immunoglobulin constant region or a portion thereof, albumin, albumin-binding polypeptide, PAS, the C-terminal peptide (CTP) of the β subunit of human chorionic

gonadotropin, polyethylene glycol (PEG), hydroxyethyl starch (HES), albumin-binding small molecules, or any combination thereof.

40. The chimeric molecule of any one of 37 to 39, wherein the second heterologous moiety (H2) is capable of extending the half-life of the chimeric molecule.

41. The chimeric molecule of claim 40, wherein the second heterologous moiety (H2) comprises a polypeptide, a non-polypeptide moiety, or both.

42. The chimeric molecule of claim 39 or 40, wherein the second heterologous moiety (H2) comprises an immunoglobulin constant region or a portion thereof.

43. The chimeric molecule of claim 42, wherein the immunoglobulin constant region or a portion thereof comprises an FcRn binding partner.

44. The chimeric molecule of claim 42, wherein the immunoglobulin constant region or a portion thereof comprises a second Fc region.

45. The chimeric molecule of any one of claims 37 to 44, wherein the first heterologous moiety and the second heterologous moiety are identical or different.

46. The chimeric molecule of any one of claims 37 to 45, wherein the first heterologous moiety and the second heterologous moiety are associated with each other.

47. The chimeric molecule of claim 46, wherein the association between the first polypeptide chain and the second polypeptide is a covalent bond.

48. The chimeric molecule of claim 46, wherein the association between the first heterologous moiety and the second heterologous moiety is a disulfide bond.

49. The chimeric molecule of any one of claims 37 to 48, wherein the first heterologous moiety is a first FcRn binding partner and the second heterologous moiety is a second FcRn binding partner.

50. The chimeric molecule of any one of claims 37 to 48, wherein the first heterologous moiety is a first Fc region and the second heterologous moiety is a second Fc region.

51. The chimeric molecule of any one of claims 37 to 48, wherein the FVIII protein is linked to the second heterologous moiety by a FVIII linker.

52. The chimeric molecule of claim 51, wherein the second linker is a cleavable linker.

53. The chimeric molecule of claim 51 or 52, wherein the FVIII linker is identical to the VWF linker.

54. The chimeric molecule of claim 51 or 52, wherein the FVIII linker is different from the VWF linker.

55. The chimeric molecule of any one of claims 37 and 39 to 50, comprising a formula selected from:

(a) V-L1-H1: H2-L2-C, or

(b) C-L2-H2:H1-L1-V;

wherein V is the VWF protein;

L1 is the VWF linker;

L2 is an optional FVIII linker;

H1 is the first heterologous moiety;

H2 is the second heterologous moiety;

C is a FVIII protein;

(-) is a peptide bond or one or more amino acids; and

(:) is a covalent bond between the H1 and the H2.

56. The chimeric molecule of any one of claims 38 to 50, comprising a formula selected from:

(a) V-L1-H1-L3-C-L2-H2,

(b) H2-L2-C-L3-H1-L1-V,

(c) C-L2-H2-L3-V-L1-H1,

(d) H1-L1-V-L3-H2-L2-C,

(e) H1-L1-V-L3-C-L2-H2,

(f) H2-L2-C-L3-V-L1-H1,

(g) V-L1-H1-L3-H2-L2-C, or

(h) C-L2-H2-L3-H1-L1-V,

wherein V comprises the VWF protein;

L1 is the VWF linker;

L2 is an optional FVIII linker;

L3 is a processable linker that is processed by a protease,

H1 is the first heterologous moiety;

H2 is the second heterologous moiety;

C comprises the FVIII protein; and

(-) is a peptide bond or one or more amino acids.

57. The chimeric molecule of claim 56, wherein the protease is a proprotein convertase.

58. The chimeric molecule of claim 57, wherein the proprotein convertease is selected from PC5, PC7, PACE, furin, or any combination thereof.

59. The chimeric molecule of any one of claims 37 to 58, wherein the VWF protein inhibits or prevents binding of endogenous VWF to the FVIII protein.

60. The chimeric molecule of any one of claims 37 to 59, wherein the FVIII protein comprises a third heterologous moiety (H3).

61. The chimeric molecule of any one of claims 37 to 60, wherein the FVIII protein comprises a fourth heterologous moiety (H4).

62. The chimeric molecule of any one of claims 37 to 61, wherein the FVIII protein comprises a fifth heterologous moiety (H5).

63. The chimeric molecule of any one of claims 37 to 62, wherein the FVIII protein comprises the sixth heterologous moiety (H6).

64. The chimeric molecule of claim 63, wherein one or more of the third heterologous moiety (H3), the fourth heterologous moiety (H4), the fifth heterologous moiety (H5), the sixth heterologous moiety (H6) are capable of extending the half-life of the chimeric molecule.

65. The chimeric molecule of 63 or 64, wherein the third heterologous moiety (H3), the fourth heterologous moiety (H4), the fifth heterologous moiety (H5), and the sixth heterologous moiety (H6) are linked to the C terminus or N terminus of the FVIII protein or inserted between two amino acids of the FVIII protein.

66. The chimeric molecule of any one of claims 63 to 65, wherein one or more of the third heterologous moiety (H3), the fourth heterologous moiety (H4), the fifth heterologous moiety (H5), and the sixth heterologous moiety (H6) comprise an amino acid sequence of at least about 50 amino acids, at least about 100 amino acids, at least about 150 amino acids, at least about 200 amino acids, at least about 250 amino acids, at least about 300 amino acids, at least about 350 amino acids, at least about 400 amino acids, at least about 450 amino acids, at least about 500 amino acids, at least about 550 amino acids, at least about 600 amino acids, at least about 650 amino acids, at least about 700 amino acids, at least about 750 amino acids, at least about 800 amino acids, at least about 850 amino acids, at least about 900 amino acids, at least about 950 amino acids, or at least about 1000 amino acids.

67. The chimeric molecule of any one of claims 37 to 66, which the half-life of the FVIII is extended at least about 1.5 times, at least about 2 times, at least about 2.5 times, at least about 3 times, at least about 4 times, at least about 5 times, at least about 6 times, at least about 7

times, at least about 8 times, at least about 9 times, at least about 10 times, at least about 11 times, or at least about 12 times longer than wild-type FVIII.

68. A polynucleotide or a set of polynucleotides encoding the chimeric molecule of any one of claims 1 to 67 or any complementary sequence thereof.

69. The polynucleotide or the set of polynucleotides of claim 68, further comprising a polynucleotide chain, which encodes PC5 or PC7.

70. A vector or a set of vectors comprising the polynucleotide or the set of polynucleotides of claim 68 or 69 and one or more promoter operably linked to the polynucleotide or the set of polynucleotides.

71. The vector or the set of vectors of claim 70, further comprising an additional vector, which comprises a polynucleotide chain encoding PC5 or PC7.

72. A host cell comprising the polynucleotide or the set of polynucleotides of any one of claims 68 or 69 or the vector or the set of vectors of claim 70 or 71.

73. The host cell of claim 72, which is a mammalian cell.

74. The host cell of claim 73, wherein the mammalian cell is selected from a HEK293 cell, CHO cell, or BHK cell.

75. A pharmaceutical composition comprising the chimeric molecule of any one of claims 1 to 67, the polynucleotide or the set of polynucleotides of claim 68 or 69, the vector or the set of vectors of claim 70 or 71, or the host cell of any one of claims 72 or 73, and a pharmaceutically acceptable carrier.

76. The composition of claim 75, wherein the chimeric molecule comprising the FVIII protein has extended half-life compared to wild type FVIII protein.

77. The composition of claim 76, wherein the half-life of the chimeric molecule is extended at least about 1.5 times, at least about 2 times, at least about 2.5 times, at least about 3 times, at least about 4 times, at least about 5 times, at least about 6 times, at least about 7 times, at least about 8 times, at least about 9 times, at least about 10 times, at least about 11 times, or at least about 12 times longer than wild type FVIII.

78. A method of reducing a frequency or degree of a bleeding episode in a subject in need thereof comprising administering an effective amount of the chimeric molecule of any one of claims 67, the polynucleotide or the set of polynucleotides of claim 68 or 69, the vector or the set of vectors of claim 70 or 71, the host cell of claim 72 or 73, or the composition of claim 76 or 77.

79. A method of preventing an occurrence of a bleeding episode in a subject in need thereof comprising administering an effective amount of the chimeric molecule of any one of claims 67, the polynucleotide or the set of polynucleotides of claim 68 or 69, the vector or the set of vectors of claim 70 or 71, the host cell of claim 72 or 73, or the composition of claim 76 or 77.

80. The method of claim 78 or 79, wherein the bleeding episode is derived from a bleeding coagulation disorder, hemarthrosis, muscle bleed, oral bleed, hemorrhage, hemorrhage into muscles, oral hemorrhage, trauma, trauma capitis, gastrointestinal bleeding, intracranial hemorrhage, intra-abdominal hemorrhage, intrathoracic hemorrhage, bone fracture, central nervous system bleeding, bleeding in the retropharyngeal space, bleeding in the retroperitoneal space, bleeding in the illiopsoas sheath, or any combination thereof.

81. The method of any one of claims 78 to 80, wherein the chimeric molecule of any one of claims 67, the polynucleotide or the set of polynucleotides of claim 68 or 69, the vector or the set of vectors of claim 70 or 71, the host cell of claim 72 or 73, or the composition of claim 76 or 77 is administered by a route selected from the group consisting of topical administration, intraocular administration, parenteral administration, intrathecal administration, subdural administration and oral administration.

82. A method of making a chimeric molecule, comprising transfecting one or more host cell with the polynucleotide or the set of polynucleotides of claim 68 or 69 or the vector or the set of vectors of claim 70 or 71 and expressing the chimeric molecule in the host cell.

83. The method of claim 82, further comprising isolating the chimeric molecule.

84. A method of improving FVIII activity of a chimeric FVIII protein comprising a VWF protein fused to a first heterologous moiety and a FVIII protein fused to a second heterologous moiety, the method comprising inserting a VWF linker between the VWF protein and the first heterologous moiety, wherein the VWF linker comprises a polypeptide selected from:

i. an a2 region from Factor VIII (FVIII);

ii. an a1 region from FVIII;

iii. an a3 region from FVIII;

iv. a thrombin cleavage site which comprises X-V-P-R (SEQ ID NO: 3) and a PAR1 exosite interaction motif, wherein X is an aliphatic amino acid; or

v. any combination thereof.

85. The method of claim 84, wherein the FVIII activity is measured by aPTT assay or ROTEM assay.

86. A chimeric molecule comprising a first polypeptide which comprises an amino acid sequence at least about 80%, at least about 90%, at least about 95%, or 100% identical to FVIII155 (SEQ ID NO: 50) and a second polypeptide which comprises an amino acid sequence at least about 80%, at least about 90%, at least about 95%, or 100% identical to a sequence selected from VWF039 (SEQ ID NO: 41), VWF054 (SEQ ID NO: 45), VWF055 (SEQ ID NO: 47), or VWF056 (SEQ ID NO: 39).

87. A polynucleotide comprising a nucleic acid sequence encoding the chimeric protein of claim 86.

88. A vector comprising the polynucleotide of claim 87.

89. A host cell comprising the vector of claim 88.

90. A pharmaceutical composition comprising the chimeric protein of claim 86, the polynucleotide of claim 87, the vector of claim 88, the host cell of claim 89, or the composition of claim 90.

91. A method of preventing an occurrence of a bleeding episode in a subject in need thereof comprising administering an effective amount of the chimeric molecule of claim 86, the polynucleotide of claim 87, the vector of claim 88, the host cell of claim 89, or the composition of claim 90.

FIG. 1.

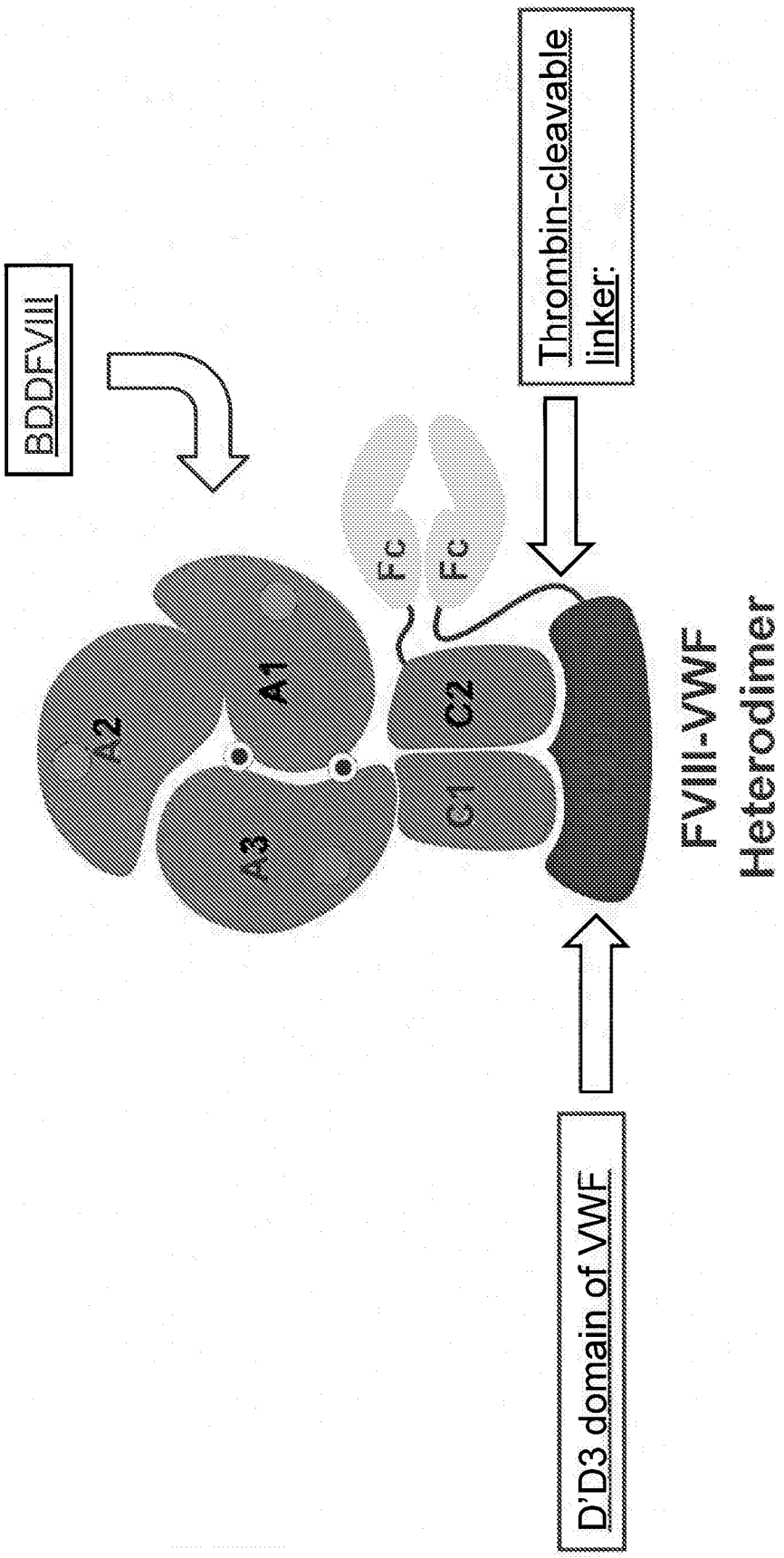

BDDFVIII
Thrombin-cleavable linker:
Fc
Fc
A2
A1
A3
C2
C1
FVIII-VWF Heterodimer
D'D3 domain of VWF

FIG. 2

Construct	Type & length of linker between VWF-D'D3 and Fc
VWF-031	48aa (LVPR site)
VWF-035	73aa (LVPR site)
VWF-036	98aa (LVPR site)
VWF-039	26aa (LVPR+PAR1site)
VWF-051	54aa (ALRPRVV site)
VWF-052	48aa (No thrombin site)
VWF-054	40aa (little a1 from FVIII)
VWF-055	34aa (little a2 from FVIII)
VWF-056	46aa (little a3 from FVIII)

FIG. 3

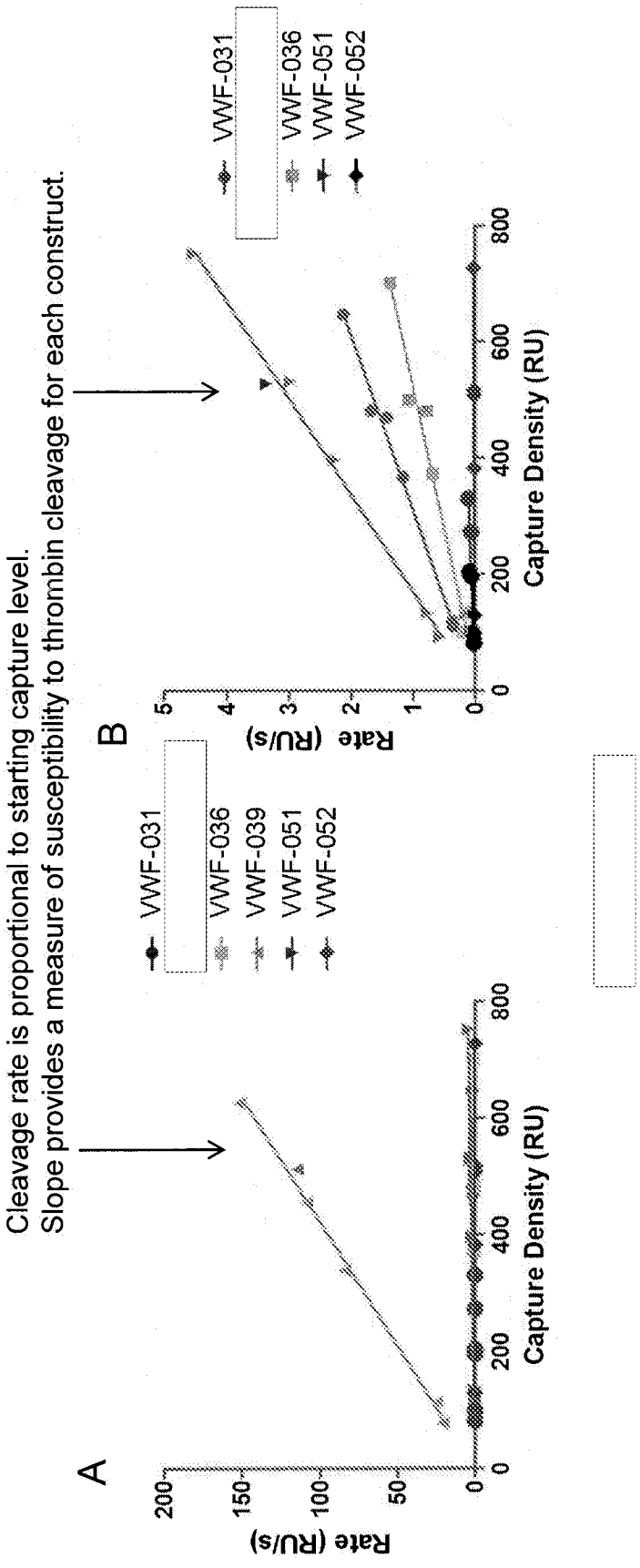

Cleavage rate is proportional to starting capture level.
Slope provides a measure of susceptibility to thrombin cleavage for each construct.
A
B
Rate (RU/s)
Capture Density (RU)
VWF-031
VWF-036
VWF-039
VWF-051
VWF-052

FIG. 4

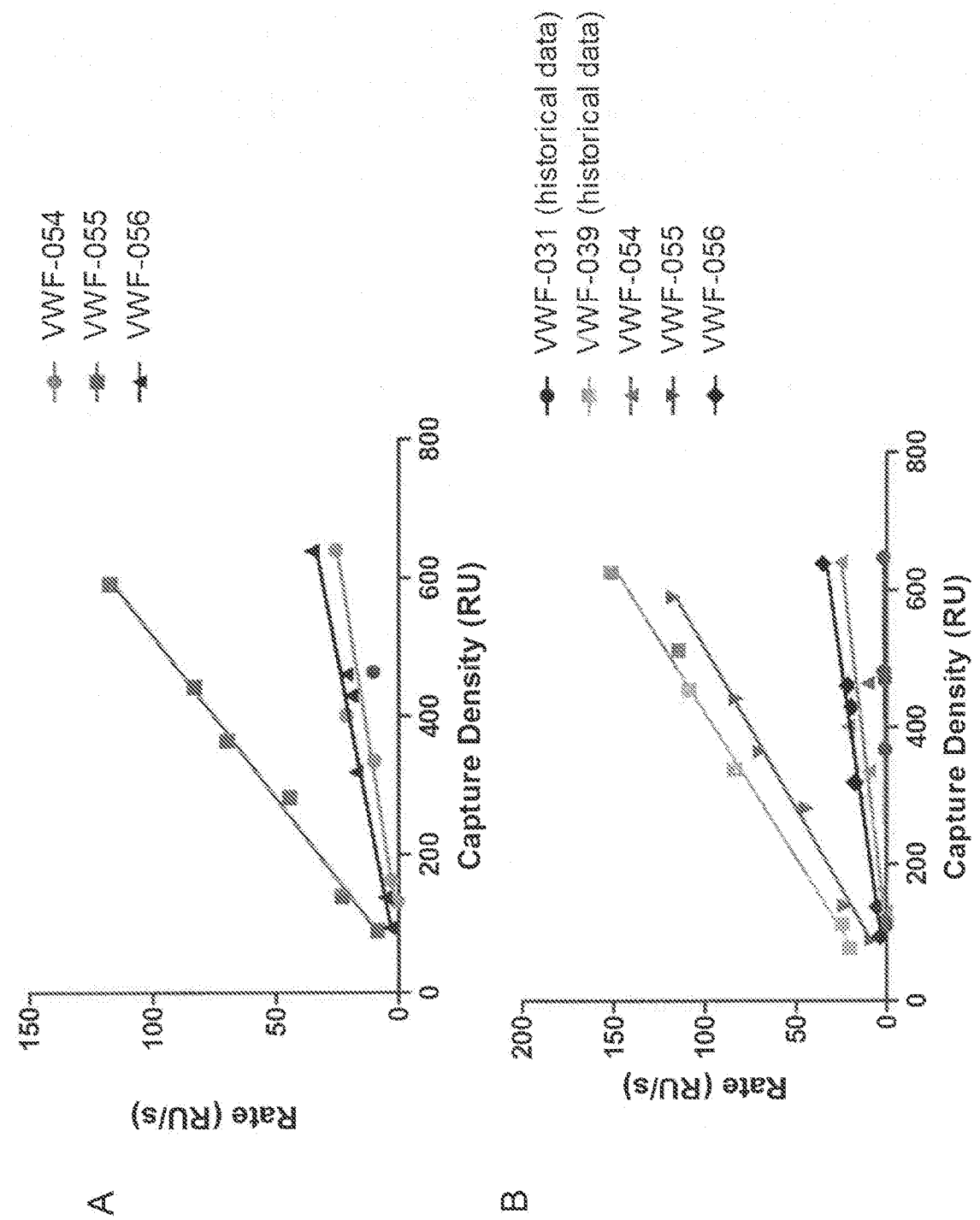
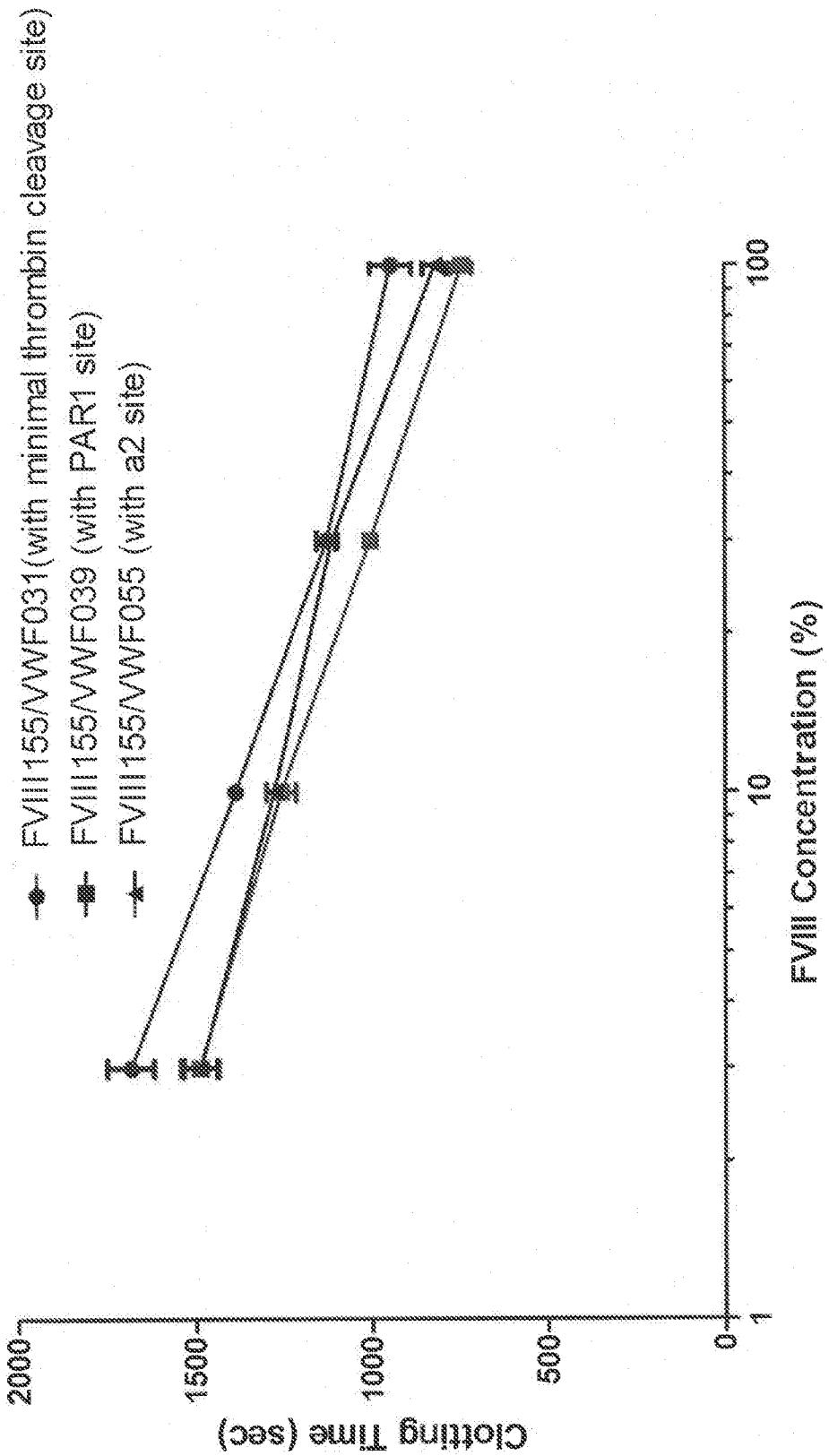

A
Rate (RU/s)
Capture Density (RU)
VWF-054
VWF-055
VWF-056
B
Rate (RU/s)
Capture Density (RU)
VWF-031 (historical data)
VWF-039 (historical data)
VWF-054
VWF-055
VWF-056

FIG. 5

Construct	Slope (1/s)
VWF-031	$3.32 \times 10^{-3} \pm 0.16 \times 10^{-3}$
VWF-036	$2.06 \times 10^{-3} \pm 0.18 \times 10^{-3}$
VWF-039	$235.4 \times 10^{-3} \pm 9.1 \times 10^{-3}$
VWF-051	$6.00 \times 10^{-3} \pm 0.27 \times 10^{-3}$
VWF-052	$0.02 \times 10^{-3} \pm 0.006 \times 10^{-3}$
VWF-054	$46.35 \times 10^{-3} \pm 11.53 \times 10^{-3}$
VWF-055	$213.5 \times 10^{-3} \pm 8.5 \times 10^{-3}$
VWF-056	$55.6 \times 10^{-3} \pm 4.5 \times 10^{-3}$

$slope_{VWF-039}/slope_{VWF-031} = 71$
$slope_{VWF-055}/slope_{VWF-031} = 65$
$slope_{VWF-051}/slope_{VWF-031} = 1.8$

FIG. 6.


HemA Donor Whole Blood ROTEM
FVIII155/VWF031(with minimal thrombin cleavage site)
FVIII155/VWF039 (with PAR1 site)
FVIII155/VWF055 (with a2 site)
Clotting Time (sec)
2000
1500
1000
500
0
1
10
100
FVIII Concentration (%)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/044718

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 38/37 (2014.01)
CPC - A61K 38/37 (2014.09)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 38/00; 38/37; C07K 14/755 (2014.01)
CPC - A61K 38/00; 38/37; C07K 14/755

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
US Class - 514/1.1, 14.1; 530/300, 350, 383
CPC - A61K 38/00; 38/37; C07K 14/755 (2014.09) (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatBase, Google Patents, Google, PubMed
Search terms used: FVIII, vonWillebrand factor, VWF, coagulation, XTEN, chimeric protein, fusion, half-life, heterologous

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2011/0183907 A1 (WEIMER et al) 28 July 2011 (28.07.2011) entire document	1, 2, 4, 6, 84 ---------- 8, 9, 85
Y	US 2011/0287517 A1 (STEWARD et al) 24 November 2011 (24.11.2011) entire document	8
Y	US 2012/0121706 A1 (KULIOPULOS et al) 17 May 2012 (17.05.2012) entire document	9
Y	WO 2010/060081 A1 (TANG et al) 27 May 2010 (27.05.2010) entire document	85
Y	US 2013/0108629 A1 (DUMONT et al) 02 May 2013 (02.05.2013) entire document	86-90
Y	US 2012/0289468 A1 (BARNETT) 15 November 2012 (15.11.2012) entire document	86-90
P, X	WO 2013/122617 A1 (SCHELLENBERGER et al) 22 August 2013 (22.08.2013) entire document	1-4, 6, 8, 9, 84-90
X	WO 2013/106787 A1 (CHABRA et al) 18 July 2013 (18.07.2013) entire document	1-4, 6, 8, 9, 84-90
A	PETERS et al. "Biochemical and functional characterization of a recombinant monomeric factor VIII–Fc fusion protein," Journal of Thrombosis and Haemostasis, 27 January 2013 (27.01.2013), Vol. 11, Pgs. 132-141. entire document	1-4, 6, 8, 9, 84-90

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 October 2014	04 NOV 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/044718

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 3-20, 28-30, 39, 41, 45, 47, and 50 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/044718

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5, 7, 10-83, 91
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.