RECOMBINANT VWF FORMULATIONS

This application claims priority of U.S. Provisional Application No. 61/017,418, filed December 28, 2007, and U.S. Provisional Application No. 61/017,881 filed December 31, 2007, each of which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

[0001] Generally, the invention relates to formulations of recombinant VWF and methods for making a composition comprising recombinant VWF.

BACKGROUND OF THE INVENTION

[0002] Von Willebrand factor (VWF) is a glycoprotein circulating in plasma as a series of multimers ranging in size from about 500 to 20,000 kD. Multimeric forms of VWF are composed of 250 kD polypeptide subunits linked together by disulfide bonds. VWF mediates initial platelet adhesion to the sub-endothelium of the damaged vessel wall. Only the larger multimers exhibit hemostatic activity. It is assumed that endothelial cells secrete large polymeric forms of VWF and those forms of VWF which have a low molecular weight (low molecular weight VWF) arise from proteolytic cleavage. The multimers having large molecular masses are stored in the Weibel-Pallade bodies of endothelial cells and liberated upon stimulation.

[0003] VWF is synthesized by endothelial cells and megakaryocytes as prepro-VWF that consists to a large extent of repeated domains. Upon cleavage of the signal peptide, pro-VWF dimerizes through disulfide linkages at its C-terminal region. The dimers serve as protomers for multimerization, which is governed by disulfide linkages between the free end termini. The assembly to multimers is followed by the proteolytic removal of the propeptide sequence (Leyte et al., Biochem. J. 274 (1991), 257-261).

[0004] The primary translation product predicted from the cloned cDNA of VWF is a 2813-residue precursor polypeptide (prepro-VWF). The prepro-VWF consists of a 22 amino acid signal peptide and a 741 amino acid propeptide, with the mature VWF comprising 2050 amino acids (Ruggeri Z.A., and Ware, J., FASEB J., 308-316 (1993)).

[0005] Defects in VWF are causal to Von Willebrand disease (VWD), which is characterized by a more or less pronounced bleeding phenotype. VWD type 3 is the most severe form in which VWF is completely missing, and VWD type 1 relates to a quantitative loss of VWF and its phenotype can be very mild. VWD type 2 relates to qualitative defects of VWF and can be as severe as VWD type 3. VWD type 2 has many sub forms, some being associated with the loss or the decrease of high molecular weight multimers. Von Willebrand syndrome type 2a (VWS-2A) is characterized by a loss of both intermediate and large multimers. VWS-2B is characterized by a loss of highest-molecular-weight multimers. Other diseases and disorders related to VWF are known in the art.

[0006] US. Patent Nos. 6,531,577, 7,166,709, and European Patent Application No. 04380188.5, describe plasma-derived VWF formulations. However, in addition to quantity and purity issues with plasma-derived VWF, there is also a risk of blood-born pathogens (e.g., viruses and Variant Creutzfeldt-Jakob disease (vCJD).

[0007] Thus there exists a need in the art to develop a stable pharmaceutical formulation comprising recombinant VWF.

SUMMARY OF THE INVENTION

[0008] The present invention provides formulations useful for compositions comprising recombinant VWF, resulting in a highly stable pharmaceutical composition. The stable pharmaceutical composition is useful as a therapeutic agent in the treatment of individuals suffering from disorders or conditions that can benefit from the administration of recombinant VWF.

[0009] In one embodiment, the invention provides a stable liquid pharmaceutical formulation of a recombinant von Willebrand Factor (rVWF) comprising: (a) a rVWF; (b) a buffering agent; (c) one or more salts; (d) optionally a stabilizing agent; and (e) optionally a surfactant; wherein the rVWF comprises a polypeptide selected from the group consisting of: a) the amino acid sequence set out in SEQ ID NO: 3; b) a biologically active analog, fragment or variant of a); c) a polypeptide encoded by the polynucleotide set out in SEQ ID NO: 1; d) a biologically active analog, fragment or variant of c); and e) a polypeptide encoded by a polynucleotide that hybridizes to the polynucleotide set out in SEQ ID NO: 1 under moderately stringent hybridization conditions; wherein the buffer is comprised of a pH buffering agent in a range of about 0.1 mM to about 500 mM and wherein the pH is in a range of about 2.0 to about 12.0; wherein the salt is at a concentration of about 1 to 500 mM;

wherein the stabilizing agent is at a concentration of about 0.1 to 1000 mM; and wherein the surfactant is at a concentration of about 0.01 g/L to 0.5 g/L.

[0010] In another embodiment, the aforementioned formulation is provided wherein the rVWF comprises the amino acid sequence set out in SEQ ID NO: 3. In another embodiment, an aforementioned formulation is provided wherein the buffering agent is selected from the group consisting of sodium citrate, glycine, histidine, Tris and combinations of these agents. In yet another embodiment, an aforementioned formulation is provided wherein the buffering agent is citrate. In still another embodiment of the invention, the aforementioned formulation is provided wherein pH is in the range of 6.0-8.0, or 6.5-7.3. In a related embodiment, the aforementioned formulation is provided wherein the pH is 7.0. In another embodiment, an aforementioned formulation is provided wherein the buffering agent is citrate and the pH is 7.0.

[0011] In still another embodiment, an aforementioned formulation is provided wherein the salt is selected from the group consisting of calcium chloride, sodium chloride and magnesium chloride. In another embodiment, the aforementioned formulation is provided wherein the salt is at a concentration range of 0.5 to 300 mM. In another embodiment, the aforementioned formulation is provided wherein the salt is calcium chloride at a concentration of 10 mM.

[0012] In another embodiment, an aforementioned formulation is provided wherein the rVWF comprises the amino acid sequence set out in SEQ ID NO: 3; wherein the buffering agent is citrate and the pH is 7.0; and wherein the salt is calcium chloride at a concentration of 10 mM. In still another embodiment, an aforementioned formulation is provided wherein the rVWF comprises the amino acid sequence set out in SEQ ID NO: 3; wherein the buffering agent is sodium citrate and the pH is 7.0; and wherein the salt is calcium chloride at a concentration of 10 mM and NaCl at a concentration of 100 mM.

[0013] Other formulations are also contemplated by the instant invention. For example, in one embodiment, an aforementioned formulation is provided wherein the one or more buffering agents is histidine and Tris at a concentration of 3.3 mM each. In another embodiment, the aforementioned formulation is provided wherein the pH is 7.0. In yet another embodiment, an aforementioned formulation is provided wherein the first salt is sodium chloride at a concentration of 30 mM and the second salt is calcium chloride at a concentration of 0.56 mM.

[0014] In still another embodiment of the invention, an aforementioned formulation is provided wherein the stabilizing agent is selected from the group consisting of mannitol, lactose, sorbitol, xylitol, sucrose, trehalose, mannose, maltose, lactose, glucose, raffinose, cellobiose,

gentiobiose, isomaltose, arabinose, glucosamine, fructose and combinations of these stabilizing agents. In another embodiment, the aforementioned formulation is provided wherein the stabilizing agents are trehalose at a concentration of 7.8 mM and mannitol at a concentration of 58.6 mM.

[0015] In another embodiment, an aforementioned formulation is provided wherein the surfactant is selected from the group consisting of digitonin, Triton X-100, Triton X-114, TWEEN-20, TWEEN-80 and combinations of these surfactants. In another embodiment, the aforementioned formulation is provided wherein the surfactant is TWEEN-80 at 0.03 g/L.

[0016] In one embodiment of the invention, an aforementioned formulation is provided wherein the rVWF comprises amino acid sequence set out in SEQ ID NO: 3; wherein the buffering agents are histidine at a concentration of 3.3 mM and Tris at a concentration of 3.3 mM at pH 7.0; wherein the first salt is sodium chloride at a concentration of 30 mM and the second salt is calcium chloride at a concentration of 0.56 mM; wherein the stabilizing agents are trehalose at a concentration of 7.8 mM mannitol at a concentration of 58.6 mM; and wherein the surfactant is TWEEN-80 at 0.03 g/L.

BRIEF DESCRIPTION OF THE FIGURES

[0017] **Figure 1** shows that rVWF is not stable in Advate buffer after 26 weeks, due to the presence of glutathione.

[0018] **Figure 2** shows that rVWF is stable in Advate 1:3 buffer for up to 12 weeks at 4 °C.

[0019] **Figure 3** shows that the stability of a citrate-based formulation is better than Advate 1:3 buffer formulation containing 0.1M glutathione.

[0020] **Figure 4** shows shows that rVWF concentration is stable over 26 weeks in Advate buffer.

[0021] **Figure 5** shows that rVWF concentration is stable over time in Advate 1:3 buffer.

[0022] **Figure 6** shows that rVWF concentration is stable over time in citrate-based buffer.

[0023] **Figure 7** shows that most excipients increase the unfolding temperature of rVWF by about 1 or 2 °C.

[0024] **Figure 8** shows that 10 mM $CaCl_2$ increases unfolding temperature of rVWF by about 8 °C to about 67 °C.

[0025] **Figure 9** shows that the effect of $CaCl_2$ is similar at pH 7.3 and pH 6.5.

DETAILED DESCRIPTION OF THE INVENTION

Definition of terms

[0026] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton, et al., DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY (2d ed. 1994); THE CAMBRIDGE DICTIONARY OF SCIENCE AND TECHNOLOGY (Walker ed., 1988); THE GLOSSARY OF GENETICS, 5TH ED., R. Rieger, et al. (eds.), Springer Verlag (1991); and Hale and Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY (1991).

[0027] Each publication, patent application, patent, and other reference cited herein is incorporated by reference in its entirety to the extent that it is not inconsistent with the present disclosure.

[0028] It is noted here that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

[0029] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0030] The term "comprising," with respect to a peptide compound, means that a compound may include additional amino acids at either or both amino and carboxy termini of the given sequence. Of course, these additional amino acids should not significantly interfere with the activity of the compound. With respect to a composition of the instant invention, the term "comprising" means that a composition may include additional components. These additional components should not significantly interfere with the activity of the composition.

[0031] The term "pharmacologically active" means that a substance so described is determined to have activity that affects a medical parameter (e.g., but not limited to blood pressure, blood cell count, cholesterol level) or disease state (e.g., but not limited to cancer, autoimmune disorders).

[0032] As used herein the terms "express," "expressing" and "expression" mean allowing or causing the information in a gene or DNA sequence to become manifest, for example, producing a

protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form an "expression product" such as a protein. The expression product itself, e.g. the resulting protein, may also be said to be "expressed." An expression product can be characterized as intracellular, extracellular or secreted. The term "intracellular" means inside a cell. The term "extracellular" means outside a cell, such as a transmembrane protein. A substance is "secreted" by a cell if it appears in significant measure outside the cell, from somewhere on or inside the cell.

[0033] As used herein a "polypeptide" refers to a polymer composed of amino acid residues, structural variants, related naturally-occurring structural variants, and synthetic non-naturally occurring analogs thereof linked via peptide bonds. Synthetic polypeptides can be prepared, for example, using an automated polypeptide synthesizer. The term "protein" typically refers to large polypeptides. The term "peptide" typically refers to short polypeptides.

[0034] As used herein a "fragment" of a polypeptide is meant to refer to any portion of a polypeptide or protein smaller than the full-length polypeptide or protein expression product.

[0035] As used herein an "analog" refers to any of two or more polypeptides substantially similar in structure and having the same biological activity, but can have varying degrees of activity, to either the entire molecule, or to a fragment thereof. Analogs differ in the composition of their amino acid sequences based on one or more mutations involving substitution of one or more amino acids for other amino acids. Substitutions can be conservative or non-conservative based on the physico-chemical or functional relatedness of the amino acid that is being replaced and the amino acid replacing it.

[0036] As used herein a "variant" refers to a polypeptide, protein or analog thereof that is modified to comprise additional chemical moieties not normally a part of the molecule. Such moieties may modulate the molecule's solubility, absorption, biological half-life, etc. The moieties may alternatively decrease the toxicity of the molecule and eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences (1980). Procedure for coupling such moieties to a molecule are well known in the art. For example, the variant may be a blood clotting factor having a chemical modification which confers a longer half-life in vivo to the protein. In various aspects, polypeptides are modified by glycosylation, pegylation, and/or polysialylation.

[0037] **Recombinant VWF**

[0038] The polynucleotide and amino acid sequences of prepro-VWF are set out in SEQ ID NO:1 and SEQ ID NO:2, respectively, and are available at GenBank Accession Nos. NM_000552 and NP_000543, respectively. The amino acid sequence corresponding to the mature VWF protein is set out in SEQ ID NO: 3 (corresponding to amino acids 764-2813 of the full length prepro-VWF amino acid sequence).

[0039] One form of useful rVWF has at least the property of in vivo-stabilizing, e.g. binding, of at least one Factor VIII (FVIII) molecule and having optionally a glycosylation pattern which is pharmacologically acceptable. Specific examples thereof include VWF without A2 domain thus resistant to proteolysis (Lankhof et al., Thromb. Haemost. 77: 1008-1013, 1997), and the VWF fragment from Val 449 to Asn 730 including the glycoprotein Ib-binding domain and binding sites for collagen and heparin (Pietu et al., Biochem. Biophys. Res. Commun. 164: 1339-1347, 1989). The determination of the ability of a VWF to stabilize at least one FVIII molecule can be carried out in VWF-deficient mammals according to methods known in the state in the art.

[0040] The rVWF of the present invention may be produced by any method known in the art. One specific example is disclosed in WO86/06096 published on Oct. 23, 1986 and U.S. Patent Application No. 07/559,509, filed on Jul. 23, 1990, which is incorporated herein by reference with respect to the methods of producing recombinant VWF. Thus, methods are known in the art for (i) the production of recombinant DNA by genetic engineering, e.g. via reverse transcription of RNA and/or amplification of DNA, (ii) introducing recombinant DNA into procaryotic or eucaryotic cells by transfection, e.g. via electroporation or microinjection, (iii) cultivating said transformed cells, e.g. in a continuous or batchwise manner, (iv) expressing VWF, e.g. constitutively or upon induction, and (v) isolating said VWF, e.g. from the culture medium or by harvesting the transformed cells, in order to (vi) obtain purified rVWF, e.g. via anion exchange chromatography or affinity chromatography. A recombinant VWF may be made in transformed host cells using recombinant DNA techniques well known in the art. For instance, sequences coding for the polypeptide could be excised from DNA using suitable restriction enzymes.

[0041] Alternatively, the DNA molecule could be synthesized using chemical synthesis techniques, such as the phosphoramidate method. Also, a combination of these techniques could be used.

[0042] The invention also provides vectors encoding polypeptides of the invention in an appropriate host. The vector comprises the polynucleotide that encodes the polypeptide operatively linked to appropriate expression control sequences. Methods of effecting this operative linking,

either before or after the polynucleotide is inserted into the vector, are well known. Expression control sequences include promoters, activators, enhancers, operators, ribosomal binding sites, start signals, stop signals, cap signals, polyadenylation signals, and other signals involved with the control of transcription or translation. The resulting vector having the polynucleotide therein is used to transform an appropriate host. This transformation may be performed using methods well known in the art.

[0043] Any of a large number of available and well-known host cells may be used in the practice of this invention. The selection of a particular host is dependent upon a number of factors recognized by the art, including, for example, compatibility with the chosen expression vector, toxicity of the peptides encoded by the DNA molecule, rate of transformation, ease of recovery of the peptides, expression characteristics, bio-safety and costs. A balance of these factors must be struck with the understanding that not all host cells are equally effective for the expression of a particular DNA sequence. Within these general guidelines, useful microbial host cells include bacteria, yeast and other fungi, insects, plants, mammalian (including human) cells in culture, or other hosts known in the art.

[0044] Next, the transformed host is cultured and purified. Host cells may be cultured under conventional fermentation conditions so that the desired compounds are expressed. Such fermentation conditions are well known in the art. Finally, the polypeptides are purified from culture by methods well known in the art.

[0045] Depending on the host cell utilized to express a compound of the invention, carbohydrate (oligosaccharide) groups may conveniently be attached to sites that are known to be glycosylation sites in proteins. Generally, O-linked oligosaccharides are attached to serine (Ser) or threonine (Thr) residues while N-linked oligosaccharides are attached to asparagine (Asn) residues when they are part of the sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. X is preferably one of the 19 naturally occurring amino acids not counting proline. The structures of N-linked and O-linked oligosaccharides and the sugar residues found in each type are different. One type of sugar that is commonly found on both is N-acetylneuraminic acid (referred to as sialic acid). Sialic acid is usually the terminal residue of both N-linked and O-linked oligosaccharides and, by virtue of its negative charge, may confer acidic properties to the glycosylated compound. Such site(s) may be incorporated in the linker of the compounds of this invention and are preferably glycosylated by a cell during recombinant production of the polypeptide compounds (e.g., in mammalian cells such as CHO, BHK, COS). However, such sites may further be glycosylated by synthetic or semi-synthetic procedures known in the art.

[0046] Alternatively, the compounds may be made by synthetic methods. For example, solid phase synthesis techniques may be used. Suitable techniques are well known in the art, and include those described in Merrifield (1973), Chem. Polypeptides, pp. 335-61 (Katsoyannis and Panayotis eds.); Merrifield (1963), J. Am. Chem. Soc. 85: 2149; Davis et al. (1985), Biochem. Intl. 10: 394-414; Stewart and Young (1969), Solid Phase Peptide Synthesis; U.S. Pat. No. 3,941,763; Finn et al. (1976), The Proteins (3rd ed.) 2: 105-253; and Erickson et al. (1976), The Proteins (3rd ed.) 2: 257-527. Solid phase synthesis is the preferred technique of making individual peptides since it is the most cost-effective method of making small peptides.

[0047] **Fragments, variants and analogs of VWF**

[0048] Methods for preparing polypeptide fragments, variants or analogs are well-known in the art.

[0049] Fragments of a polypeptide are prepared using, without limitation, enzymatic cleavage (e.g., trypsin, chymotrypsin) and also using recombinant means to generate a polypeptide fragments having a specific amino acid sequence. Polypeptide fragments may be generated comprising a region of the protein having a particular activity, such as a multimerization domain or any other identifiable VWF domain known in the art.

[0050] Methods of making polypeptide analogs are also well-known. Amino acid sequence analogs of a polypeptide can be substitutional, insertional, addition or deletion analogs. Deletion analogs, including fragments of a polypeptide, lack one or more residues of the native protein which are not essential for function or immunogenic activity. Insertional analogs involve the addition of, e.g., amino acid(s) at a non-terminal point in the polypeptide. This analog may include insertion of an immunoreactive epitope or simply a single residue. Addition analogs, including fragments of a polypeptide, include the addition of one or more amino acids at either of both termini of a protein and include, for example, fusion proteins.

[0051] Substitutional analogs typically exchange one amino acid of the wild-type for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide without the loss of other functions or properties. In one aspect, substitutions are conservative substitutions. By "conservative amino acid substitution" is meant substitution of an amino acid with an amino acid having a side chain of a similar chemical character. Similar amino acids for making conservative substitutions include those having an acidic side chain (glutamic acid, aspartic acid); a basic side chain (arginine, lysine, histidine); a polar amide side chain (glutamine, asparagine); a hydrophobic, aliphatic side chain (leucine, isoleucine, valine, alanine, glycine); an

aromatic side chain (phenylalanine, tryptophan, tyrosine); a small side chain (glycine, alanine, serine, threonine, methionine); or an aliphatic hydroxyl side chain (serine, threonine).

[0052] Analogs may be substantially homologous or substantially identical to the recombinant VWF from which they are derived. Preferred analogs are those which retain at least some of the biological activity of the wild-type polypeptide, *e.g.* blood clotting activity.

[0053] Polypeptide variants contemplated include polypeptides chemically modified by such techniques as ubiquitination, glycosylation, including polysialation, conjugation to therapeutic or diagnostic agents, labeling, covalent polymer attachment such as pegylation (derivatization with polyethylene glycol), introduction of non-hydrolyzable bonds, and insertion or substitution by chemical synthesis of amino acids such as ornithine, which do not normally occur in human proteins. Variants retain the same or essentially the same binding properties of non-modified molecules of the invention. Such chemical modification may include direct or indirect (e.g., via a linker) attachment of an agent to the VWF polypeptide. In the case of indirect attachment, it is contemplated that the linker may be hydrolyzable or non-hydrolyzable.

[0054] Preparing pegylated polypeptide analogs will generally comprise the steps of (a) reacting the polypeptide with polyethylene glycol (such as a reactive ester or aldehyde derivative of PEG) under conditions whereby the binding construct polypeptide becomes attached to one or more PEG groups, and (b) obtaining the reaction product(s). In general, the optimal reaction conditions for the acylation reactions will be determined based on known parameters and the desired result. For example, the larger the ratio of PEG: protein, the greater the percentage of poly-pegylated product. In some embodiments, the binding construct will have a single PEG moiety at the N-terminus. Polyethylene glycol (PEG) may be attached to the blood clotting factor to provide a longer half-life *in vivo*. The PEG group may be of any convenient molecular weight and may be linear or branched. The average molecular weight of the PEG ranges from about 2 kiloDalton ("kD") to about 100 kDa, from about 5 kDa to about 50 kDa, or from about 5 kDa to about 10 kDa. The PEG groups are attached to the blood clotting factor via acylation or reductive alkylation through a natural or engineered reactive group on the PEG moiety (e.g., an aldehyde, amino, thiol, or ester group) to a reactive group on the blood clotting factor (e.g., an aldehyde, amino, or ester group) or by any other technique known in the art.

[0055] Methods for preparing polysialylated polypeptide are described in United States Patent Publication 20060160948, Fernandes et Gregoriadis; Biochim. Biophys. Acta 1341: 26-34, 1997, and Saenko et al., Haemophilia 12:42-51, 2006. Briefly, a solution of colominic acid

containing 0.1 M $NaIO_4$ is stirred in the dark at room temperature to oxidize the CA. The activated CA solution is dialyzed against, e.g., 0.05 M sodium phosphate buffer, pH 7.2 in the dark and this solution was added to a rVWF solution and incubated for 18 h at room temperature in the dark under gentle shaking. Free reagents can then be separated from the rVWF-polysialic acid conjugate by ultrafiltration/diafiltration. Conjugation of rVWF with polysialic acid may also be achieved using glutaraldehyde as cross-linking reagent (Migneault et al., Biotechniques 37: 790-796, 2004).

[0056] It is further contemplated that a polypeptide of the invention may be a fusion protein with a second agent which is a polypeptide. In one embodiment, the second agent which is a polypeptide, without limitation, is an enzyme, a growth factor, an antibody, a cytokine, a chemokine, a cell-surface receptor, the extracellular domain of a cell surface receptor, a cell adhesion molecule, or fragment or active domain of a protein described above. In a related embodiment, the second agent is a blood clotting factor such as Factor VIII, Factor VII, Factor IX. The fusion protein contemplated is made by chemical or recombinant techniques well-known in the art.

[0057] It is also contemplated that prepro-VWF and pro-VWF polypeptides may provide a therapeutic benefit in the formulations of the present invention. For example, US Patent No. 7,005,502 describes a pharmaceutical preparation comprising substantial amounts of pro-VWF that induces thrombin gerneation *in vitro*. In addition to recombinant, biologically active fragments, variants, or analogs of the naturally-occuring mature VWF, the present invention contemplates the use of recombinant biologically active fragments, variants, or analogs of the prepro-VWF (set out in SEQ ID NO:2) or pro-VWF polypeptides (amino acid residues 23 to 764 of SEQ ID NO: 2) in the formulations described herein.

[0058] Polynucleotides encoding fragments, variants and analogs may be readily generated by a worker of skill to encode biologically active fragments, variants, or analogs of the naturally-occurring molecule that possess the same or similar biological activity to the naturally-occurring molecule. These polynucleotides can be prepared using PCR techniques, digestion/ligation of DNA encoding molecule, and the like. Thus, one of skill in the art will be able to generate single base changes in the DNA strand to result in an altered codon and a missense mutation, using any method known in the art, including, but not limited to site-specific mutagenesis. As used herein, the phrase "moderately stringent hybridization conditions" means, for example, hybridization at 42°C in 50% formamide and washing at 60°C in 0.1 x SSC, 0.1% SDS. It is understood by those of skill in the art that variation in these conditions occurs based on the length and GC nucleotide base content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining exact

hybridization conditions. See Sambrook et al., 9.47-9.51 in Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

[0059] Formulations and excipients in general

[0060] Excipients are additives that are included in a formulation because they either impart or enhance the stability and delivery of a drug product. Regardless of the reason for their inclusion, excipients are an integral component of a drug product and therefore need to be safe and well tolerated by patients. For protein drugs, the choice of excipients is particularly important because they can affect both efficacy and immunogenicity of the drug. Hence, protein formulations need to be developed with appropriate selection of excipients that afford suitable stability, safety, and marketability.

[0061] The principal challenge in developing formulations for therapeutic proteins is stabilizing the product against the stresses of manufacturing, shipping and storage. The role of formulation excipients is to provide stabilization against these stresses. Excipients may also be employed to reduce viscosity of high concentration protein formulations in order to enable their delivery and enhance patient convenience. In general, excipients can be classified on the basis of the mechanisms by which they stabilize proteins against various chemical and physical stresses. Some excipients are used to alleviate the effects of a specific stress or to regulate a particular susceptibility of a specific protein. Other excipients have more general effects on the physical and covalent stabilities of proteins. The excipients described herein are organized either by their chemical type or their functional role in formulations. Brief descriptions of the modes of stabilization are provided when discussing each excipient type.

[0062] Given the teachings and guidance provided herein, those skilled in the art will know what amount or range of excipient can be included in any particular formulation to achieve a biopharmaceutical formulation of the invention that promotes retention in stability of the biopharmaceutical (e.g., a polypeptide). For example, the amount and type of a salt to be included in a biopharmaceutical formulation of the invention can be selected based on the desired osmolality (i.e., isotonic, hypotonic or hypertonic) of the final solution as well as the amounts and osmolality of other components to be included in the formulation. Similarly, by exemplification with reference to the type of polyol or sugar included in a formulation, the amount of such an excipient will depend on its osmolality.

[0063] By way of example, inclusion of about 5% sorbitol can achieve isotonicity while about 9% of a sucrose excipient is needed to achieve isotonicity. Selection of the amount or range of

concentrations of one or more excipients that can be included within a biopharmaceutical formulation of the invention has been exemplified above by reference to salts, polyols and sugars. However, those skilled in the art will understand that the considerations described herein and further exemplified by reference to specific excipients are equally applicable to all types and combinations of excipients including, for example, salts, amino acids, other tonicity agents, surfactants, stabilizers, bulking agents, cryoprotectants, lyoprotectants, anti-oxidants, metal ions, chelating agents and/or preservatives.

[0064] Further, where a particular excipient is reported in molar concentration, those skilled in the art will recognize that the equivalent percent (%) w/v (e.g., (grams of substance in a solution sample/mL of solution) X 100%) of solution is also contemplated.

[0065] Of course, a person having ordinary skill in the art would recognize that the concentrations of the excipients described herein share an interdependency within a particular formulation. By way of example, the concentration of a bulking agent may be lowered where, e.g., there is a high polypeptide concentration or where, e.g., there is a high stabilizing agent concentration. In addition, a person having ordinary skill in the art would recognize that, in order to maintain the isotonicity of a particular formulation in which there is no bulking agent, the concentration of a stabilizing agent would be adjusted accordingly (i.e., a "tonicifying" amount of stabilizer would be used). Common excipients are known in the art and can be found in Powell et al., Compendium of Excipients fir Parenteral Formulations (1998), PDA J. Pharm. Sci. Technology, 52:238-311.

[0066] Buffers and buffering agents

[0067] The stability of a pharmacologically active polypeptide formulation is usually observed to be maximal in a narrow pH range. This pH range of optimal stability needs to be identified early during pre-formulation studies. Several approaches, such as accelerated stability studies and calorimetric screening studies, have been demonstrated to be useful in this endeavor (Remmele R.L. Jr., et al., *Biochemistry*, 38(16): 5241-7 (1999)). Once a formulation is finalized, the drug product must be manufactured and maintained throughout its shelf-life. Hence, buffering agents are almost always employed to control pH in the formulation.

[0068] Organic acids, phosphates and Tris have been employed routinely as buffers in protein formulations. The buffer capacity of the buffering species is maximal at a pH equal to the pKa and decreases as pH increases or decreases away from this value. Ninety percent of the

buffering capacity exists within one pH unit of its pKa. Buffer capacity also increases proportionally with increasing buffer concentration.

[0069] Several factors need to be considered when choosing a buffer. First and foremost, the buffer species and its concentration need to be defined based on its pKa and the desired formulation pH. Equally important is to ensure that the buffer is compatible with the polypeptide and other formulation excipients, and does not catalyze any degradation reactions. A third important aspect to be considered is the sensation of stinging and irritation the buffer may induce upon administration. For example, citrate is known to cause stinging upon injection (Laursen T, et al., *Basic Clin Pharmacol Toxicol.*, 98(2): 218-21 (2006)). The potential for stinging and irritation is greater for drugs that are administered via the subcutaneous (SC) or intramuscular (IM) routes, where the drug solution remains at the site for a relatively longer period of time than when administered by the IV route where the formulation gets diluted rapidly into the blood upon administration. For formulations that are administered by direct IV infusion, the total amount of buffer (and any other formulation component) needs to be monitored. One has to be particularly careful about potassium ions administered in the form of the potassium phosphate buffer, which can induce cardiovascular effects in a patient (Hollander-Rodriguez JC, et al., *Am. Fam. Physician.*, 73(2): 283-90 (2006)).

[0070] The buffer system present in the compositions is selected to be physiologically compatible and to maintain a desired pH of the pharmaceutical formulation. In one embodiment, the pH of the solution is between pH 2.0 and pH 12.0. For example, the pH of the solution may be 2.0, 2.3, 2.5, 2.7, 3.0, 3.3, 3.5, 3.7, 4.0, 4.3, 4.5, 4.7, 5.0, 5.3, 5.5, 5.7, 6.0, 6.3, 6.5, 6.7, 7.0, 7.3, 7.5, 7.7, 8.0, 8.3, 8.5, 8.7, 9.0, 9.3, 9.5, 9.7, 10.0, 10.3, 10.5, 10.7, 11.0, 11.3, 11.5, 11.7, or 12.0.

[0071] The pH buffering compound may be present in any amount suitable to maintain the pH of the formulation at a predetermined level. In one embodiment, the pH buffering concentration is between 0.1 mM and 500 mM (1 M). For example, it is contemplated that the pH buffering agent is at least 0.1, 0.5, 0.7, 0.8 0.9, 1.0, 1.2, 1.5, 1.7, 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, 90, 100, 200, 500 mM.

[0072] Exemplary pH buffering agents used to buffer the formulation as set out herein include, but are not limited to glycine, histidine, glutamate, succinate, phosphate, acetate, citrate, Tris and amino acids or mixtures of amino acids, including, but not limited to aspartate, histidine, and glycine..

[0073] **Salts**

[0074] Salts are often added to increase the ionic strength of the formulation, which can be important for protein solubility, physical stability, and isotonicity. Salts can affect the physical stability of proteins in a variety of ways. Ions can stabilize the native state of proteins by binding to charged residues on the protein's surface. Alternatively, salts can stabilize the denatured state by binding to peptide groups along the protein backbone (-CONH-). Salts can also stabilize the protein native conformation by shielding repulsive electrostatic interactions between residues within a protein molecule. Salts in protein formulations can also shield attractive electrostatic interactions between protein molecules that can lead to protein aggregation and insolubility. In formulations provided, the salt concentration is between 0.1, 1, 10, 20, 30, 40, 50, 80, 100, 120, 150, 200, 300, and 500 mM.

[0075] **Stabilizers and bulking agents**

[0076] In the present pharmaceutical formulations, a stabilizer (or a combination of stabilizers) may be added to prevent or reduce storage-induced aggregation and chemical degradation. A hazy or turbid solution upon reconstitution indicates that the protein has precipitated or at least aggregated. The term "stabilizer" means an excipient capable of preventing aggregation or other physical degradation, as well as chemical degradation (for example, autolysis, deamidation, oxidation, etc.) in an aqueous state. Stabilizers that are conventionally employed in pharmaceutical compositions include, but are not limited to, sucrose, trehalose, mannose, maltose, lactose, glucose, raffinose, cellobiose, gentiobiose, isomaltose, arabinose, glucosamine, fructose, mannitol, sorbitol, glycine, arginine HCL, poly-hydroxy compounds, including polysaccharides such as dextran, starch, hydroxyethyl starch, cyclodextrins, N-methyl pyrollidene, cellulose and hyaluronic acid, sodium chloride, [Carpenter et al., Develop. Biol. Standard 74:225, (1991)]. In the present formulations, the stabilizer is incorporated in a concentration of about 0.1, 0.5, 0.7, 0.8 0.9, 1.0, 1.2, 1.5, 1.7, 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, 90, 100, 200, 500, 700, 900, or 1000 mM.

[0077] If desired, the formulations also include appropriate amounts of bulking and osmolarity regulating agents. Bulking agents include, for example, mannitol, glycine, sucrose, polymers such as dextran, polyvinylpyrolidone, carboxymethylcellulose, lactose, sorbitol, trehalose, or xylitol. In one embodiment, the bulking agent is mannitol. The bulking agent is incorporated in a concentration of about 0.1, 0.5, 0.7, 0.8 0.9, 1.0, 1.2, 1.5, 1.7, 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, 90, 100, 200, 500, 700, 900, or 1000 mM.

[0078] **Surfactants**

[0079] Protein molecules have a high propensity to interact with surfaces making them susceptible to adsorption and denaturation at air-liquid, vial-liquid, and liquid-liquid (silicone oil) interfaces. This degradation pathway has been observed to be inversely dependent on protein concentration and results in either the formation of soluble and insoluble protein aggregates or the loss of protein from solution via adsorption to surfaces. In addition to container surface adsorption, surface-induced degradation is exacerbated with physical agitation, as would be experienced during shipping and handling of the product.

[0080] Surfactants are commonly used in protein formulations to prevent surface-induced degradation. Surfactants are amphipathic molecules with the capability of out-competing proteins for interfacial positions. Hydrophobic portions of the surfactant molecules occupy interfacial positions (e.g., air/liquid), while hydrophilic portions of the molecules remain oriented towards the bulk solvent. At sufficient concentrations (typically around the detergent's critical micellar concentration), a surface layer of surfactant molecules serve to prevent protein molecules from adsorbing at the interface. Thereby, surface-induced degradation is minimized. The most commonly used surfactants are fatty acid esters of sorbitan polyethoxylates, i.e. polysorbate 20 and polysorbate 80. The two differ only in the length of the aliphatic chain that imparts hydrophobic character to the molecules, C-12 and C-18, respectively. Accordingly, polysorbate-80 is more surface-active and has a lower critical micellar concentration than polysorbate-20.

[0081] Detergents can also affect the thermodynamic conformational stability of proteins. Here again, the effects of a given detergent excipient will be protein specific. For example, polysorbates have been shown to reduce the stability of some proteins and increase the stability of others. Detergent destabilization of proteins can be rationalized in terms of the hydrophobic tails of the detergent molecules that can engage in specific binding with partially or wholly unfolded protein states. These types of interactions could cause a shift in the conformational equilibrium towards the more expanded protein states (i.e. increasing the exposure of hydrophobic portions of the protein molecule in complement to binding polysorbate). Alternatively, if the protein native state exhibits some hydrophobic surfaces, detergent binding to the native state may stabilize that conformation.

[0082] Another aspect of polysorbates is that they are inherently susceptible to oxidative degradation. Often, as raw materials, they contain sufficient quantities of peroxides to cause oxidation of protein residue side-chains, especially methionine. The potential for oxidative damage arising from the addition of stabilizer emphasizes the point that the lowest effective concentrations of excipients should be used in formulations. For surfactants, the effective concentration for a given protein will depend on the mechanism of stabilization. It has been postulated that if the mechanism

of surfactant stabilization is related to preventing surface-denaturation the effective concentration will be around the detergent's critical micellar concentration. Conversely, if the mechanism of stabilization is associated with specific protein-detergent interactions, the effective surfactant concentration will be related to the protein concentration and the stoichiometry of the interaction (Randolph T.W., et al., *Pharm Biotechnol.*, 13:159-75 (2002)).

[0083] Surfactants may also be added in appropriate amounts to prevent surface related aggregation phenomenon during freezing and drying [Chang, B, J. Pharm. Sci. 85:1325, (1996)]. Exemplary surfactants include anionic, cationic, nonionic, zwitterionic, and amphoteric surfactants including surfactants derived from naturally-occurring amino acids. Anionic surfactants include, but are not limited to, sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate, chenodeoxycholic acid, N-lauroylsarcosine sodium salt, lithium dodecyl sulfate, 1-octanesulfonic acid sodium salt, sodium cholate hydrate, sodium deoxycholate, and glycodeoxycholic acid sodium salt. Cationic surfactants include, but are not limited to, benzalkonium chloride or benzethonium chloride, cetylpyridinium chloride monohydrate, and hexadecyltrimethylammonium bromide. Zwitterionic surfactants include, but are not limited to, CHAPS, CHAPSO, SB3-10, and SB3-12. Non-ionic surfactants include, but are not limited to, digitonin, Triton X-100, Triton X-114, TWEEN-20, and TWEEN-80. Surfactants also include, but are not limited to lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 40, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, soy lecithin and other phospholipids such as dioleyl phosphatidyl choline (DOPC), dimyristoylphosphatidyl glycerol (DMPG), dimyristoylphosphatidyl choline (DMPC), and (dioleyl phosphatidyl glycerol) DOPG; sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. Compositions comprising these surfactants, either individually or as a mixture in different ratios, are therefore further provided. In the present formulations, the surfactant is incorporated in a concentration of about 0.01 to about 0.5 g/L.

[0084] **Other common excipient components**

[0085] Amino acids

[0086] Amino acids have found versatile use in protein formulations as buffers, bulking agents, stabilizers and antioxidants. Histidine and glutamic acid are employed to buffer protein formulations in the pH range of 5.5 – 6.5 and 4.0 – 5.5 respectively. The imidazole group of histidine has a pKa = 6.0 and the carboxyl group of glutamic acid side chain has a pKa of 4.3 which makes these amino acids suitable for buffering in their respective pH ranges. Glutamic acid is particularly useful in such cases (e.g., Stemgen®). Histidine is commonly found in marketed protein

formulations (e.g., Xolair®, Herceptin®, Recombinate®),and this amino acid provides an alternative to citrate, a buffer known to sting upon injection. Interestingly, histidine has also been reported to have a stabilizing effect, as observed in formulations with ABX-IL8 (an IgG2 antibody), with respect to aggregation when used at high concentrations in both liquid and lyophilized presentations (Chen B, et al., *Pharm Res.*, 20(12): 1952-60 (2003)). Histidine (up to 60 mM) was also observed to reduce the viscosity of a high concentration formulation of this antibody. However, in the same study, the authors observed increased aggregation and discoloration in histidine containing formulations during freeze-thaw studies of the antibody in stainless steel containers. The authors attributed this to an effect of iron ions leached from corrosion of steel containers. Another note of caution with histidine is that it undergoes photo-oxidation in the presence of metal ions (Tomita M, et al., *Biochemistry*, 8(12): 5149-60 (1969)). The use of methionine as an antioxidant in formulations appears promising; it has been observed to be effective against a number of oxidative stresses (Lam XM, et al., *J Pharm Sci.*, 86(11): 1250-5 (1997)).

[0087] The amino acids glycine, proline, serine and alanine have been shown to stabilize proteins by the mechanism of preferential exclusion. Glycine is also a commonly used bulking agent in lyophilized formulations (e.g., Neumega ®, Genotropin®, Humatrope®). Arginine has been shown to be an effective agent in inhibiting aggregation and has been used in both liquid and lyophilized formulations (e.g., Activase®, Avonex®, Enbrel® liquid). Furthermore, the enhanced efficiency of refolding of certain proteins in the presence of arginine has been attributed to its suppression of the competing aggregation reaction during refolding.

[0088] Antioxidants

[0089] Oxidation of protein residues arises from a number of different sources. Beyond the addition of specific antioxidants, the prevention of oxidative protein damage involves the careful control of a number of factors throughout the manufacturing process and storage of the product such as atmospheric oxygen, temperature, light exposure, and chemical contamination. The most commonly used pharmaceutical antioxidants are reducing agents, oxygen/free-radical scavengers, or chelating agents. Antioxidants in therapeutic protein formulations are water-soluble and remain active throughout the product shelf-life. Reducing agents and oxygen/free-radical scavengers work by ablating active oxygen species in solution. Chelating agents such as EDTA are effective by binding trace metal contaminants that promote free-radical formation. For example, EDTA was utilized in the liquid formulation of acidic fibroblast growth factor to inhibit the metal ion catalyzed oxidation of cysteine residues. EDTA has been used in marketed products like Kineret® and Ontak®.

[0090] In addition to the effectiveness of various excipients to prevent protein oxidation, the potential for the antioxidants themselves to induce other covalent or physical changes to the protein is of concern. For example, reducing agents can cause disruption of intramolecular disulfide linkages, which can lead to disulfide shuffling. In the presence of transition metal ions, ascorbic acid and EDTA have been shown to promote methionine oxidation in a number of proteins and peptides (Akers MJ, and Defelippis MR. Peptides and Proteins as Parenteral Solutions. In: Pharmaceutical Formulation Development of Peptides and Proteins. Sven Frokjaer, Lars Hovgaard, editors. Pharmaceutical Science. Taylor and Francis, UK (1999)); Fransson J.R., *J. Pharm. Sci.* 86(9): 4046-1050 (1997); Yin J, et al., *Pharm Res.*, 21(12): 2377-83 (2004)). Sodium thiosulfate has been reported to reduce the levels of light and temperature induced methionine-oxidation in rhuMab HER2; however, the formation of a thiosulfate-protein adduct was also reported in this study (Lam XM, Yang JY, et al., *J Pharm Sci.* 86(11): 1250-5 (1997)). Selection of an appropriate antioxidant is made according to the specific stresses and sensitivities of the protein.

[0091] Metal ions

[0092] In general, transition metal ions are undesired in protein formulations because they can catalyze physical and chemical degradation reactions in proteins. However, specific metal ions are included in formulations when they are co-factors to proteins and in suspension formulations of proteins where they form coordination complexes (e.g., zinc suspension of insulin). Recently, the use of magnesium ions (10 –120 mM) has been proposed to inhibit the isomerization of aspartic acid to isoaspartic acid (WO 2004039337).

[0093] Two examples where metal ions confer stability or increased activity in proteins are human deoxyribonuclease (rhDNase, Pulmozyme®), and Factor VIII. In the case of rhDNase, Ca^{+2} ions (up to 100 mM) increased the stability of the enzyme through a specific binding site (Chen B, et al., *J Pharm Sci.*, 88(4): 477-82 (1999)). In fact, removal of calcium ions from the solution with EGTA caused an increase in deamidation and aggregation. However, this effect was observed only with Ca^{+2} ions; other divalent cations Mg^{+2}, Mn^{+2} and Zn^{+2} were observed to destabilize rhDNase. Similar effects were observed in Factor VIII. Ca^{+2} and Sr^{+2} ions stabilized the protein while others like Mg^{+2}, Mn^{+2} and Zn^{+2}, Cu^{+2} and Fe^{+2} destabilized the enzyme (Fatouros, A., et al., *Int. J. Pharm.*, 155, 121–131 (1997). In a separate study with Factor VIII, a significant increase in aggregation rate was observed in the presence of Al^{+3} ions (Derrick TS, et al., *J. Pharm. Sci.*, 93(10): 2549-57 (2004)). The authors note that other excipients like buffer salts are often contaminated with Al^{+3} ions and illustrate the need to use excipients of appropriate quality in formulated products.

[0094] Preservatives

[0095] Preservatives are necessary when developing multi-use parenteral formulations that involve more than one extraction from the same container. Their primary function is to inhibit microbial growth and ensure product sterility throughout the shelf-life or term of use of the drug product. Commonly used preservatives include benzyl alcohol, phenol and m-cresol. Although preservatives have a long history of use, the development of protein formulations that includes preservatives can be challenging. Preservatives almost always have a destabilizing effect (aggregation) on proteins, and this has become a major factor in limiting their use in multi-dose protein formulations (Roy S, et al., *J Pharm Sci.*, 94(2): 382-96 (2005)).

[0096] To date, most protein drugs have been formulated for single-use only. However, when multi-dose formulations are possible, they have the added advantage of enabling patient convenience, and increased marketability. A good example is that of human growth hormone (hGH) where the development of preserved formulations has led to commercialization of more convenient, multi-use injection pen presentations. At least four such pen devices containing preserved formulations of hGH are currently available on the market. Norditropin® (liquid, Novo Nordisk), Nutropin AQ® (liquid, Genentech) & Genotropin (lyophilized – dual chamber cartridge, Pharmacia & Upjohn) contain phenol while Somatrope® (Eli Lilly) is formulated with m-cresol.

[0097] Several aspects need to be considered during the formulation development of preserved dosage forms. The effective preservative concentration in the drug product must be optimized. This requires testing a given preservative in the dosage form with concentration ranges that confer anti-microbial effectiveness without compromising protein stability. For example, three preservatives were successfully screened in the development of a liquid formulation for interleukin-1 receptor (Type I), using differential scanning calorimetry (DSC). The preservatives were rank ordered based on their impact on stability at concentrations commonly used in marketed products (Remmele RL Jr., et al., *Pharm Res.*, 15(2): 200-8 (1998)).

[0098] Some preservatives can cause injection site reactions, which is another factor that needs consideration when choosing a preservative. In clinical trials that focused on the evaluation of preservatives and buffers in Norditropin, pain perception was observed to be lower in formulations containing phenol and benzyl alcohol as compared to a formulation containing m-cresol (Kappelgaard A.M., *Horm Res.* 62 Suppl 3:98-103 (2004)). Interestingly, among the commonly used preservative, benzyl alcohol possesses anesthetic properties (Minogue SC, and Sun DA., *Anesth Analg.*, 100(3): 683-6 (2005)).

[0099] Lyophilization

[00100] It is also contemplated that the formulations comprising a VWF polypeptide of the invention may be lyophilized prior to administration. Lyophilization is carried out using techniques common in the art and should be optimized for the composition being developed [Tang et al., Pharm Res. 21:191-200, (2004) and Chang et al., Pharm Res. 13:243-9 (1996)].

[00101] A lyophilization cycle is, in one aspect, composed of three steps: freezing, primary drying, and secondary drying [A.P. Mackenzie, Phil Trans R Soc London, Ser B, Biol 278:167 (1977)]. In the freezing step, the solution is cooled to initiate ice formation. Furthermore, this step induces the crystallization of the bulking agent. The ice sublimes in the primary drying stage, which is conducted by reducing chamber pressure below the vapor pressure of the ice, using a vacuum and introducing heat to promote sublimation. Finally, adsorbed or bound water is removed at the secondary drying stage under reduced chamber pressure and at an elevated shelf temperature. The process produces a material known as a lyophilized cake. Thereafter the cake can be reconstituted with either sterile water or suitable diluent for injection.

[00102] The lyophilization cycle not only determines the final physical state of the excipients but also affects other parameters such as reconstitution time, appearance, stability and final moisture content. The composition structure in the frozen state proceeds through several transitions (e.g., glass transitions, wettings, and crystallizations) that occur at specific temperatures and can be used to understand and optimize the lyophilization process. The glass transition temperature (Tg and/or Tg') can provide information about the physical state of a solute and can be determined by differential scanning calorimetry (DSC). Tg and Tg' are an important parameter that must be taken into account when designing the lyophilization cycle. For example, Tg' is important for primary drying. Furthermore, in the dried state, the glass transition temperature provides information on the storage temperature of the final product.

[00103] Methods of Preparation

[00104] The present invention further contemplates methods for the preparation of pharmaceutical formulations. A variety of aqueous carriers, e.g., sterile water for injection, water with preservatives for multi dose use, or water with appropriate amounts of surfactants (for example, polysorbate-20), 0.4% saline, 0.3% glycine, or aqueous suspensions may contain the active compound in admixture with excipients suitable for the manufacture of aqueous suspensions. In various aspects, such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum

tragacanth and gum acacia; dispersing or wetting agents may be a naturally-occurring phosphatide, for example lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyl-eneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives, for example ethyl, or n-propyl, p-hydroxybenzoate.

[00105] **Administration**

[00106] To administer compositions to human or test animals, in one aspect, the compositions comprises one or more pharmaceutically acceptable carriers. The phrases "pharmaceutically" or "pharmacologically" acceptable refer to molecular entities and compositions that are stable, inhibit protein degradation such as aggregation and cleavage products, and in addition do not produce allergic, or other adverse reactions when administered using routes well-known in the art, as described below. "Pharmaceutically acceptable carriers" include any and all clinically useful solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like, including those agents disclosed above.

[00107] The pharmaceutical formulations may be administered orally, topically, transdermally, parenterally, by inhalation spray, vaginally, rectally, or by intracranial injection. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intracisternal injection, or infusion techniques. Administration by intravenous, intradermal, intramusclar, intramammary, intraperitoneal, intrathecal, retrobulbar, intrapulmonary injection and or surgical implantation at a particular site is contemplated as well. Generally, compositions are essentially free of pyrogens, as well as other impurities that could be harmful to the recipient.

[00108] Single or multiple administrations of the compositions can be carried out with the dose levels and pattern being selected by the treating physician. For the prevention or treatment of disease, the appropriate dosage will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether drug is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the drug, and the discretion of the attending physician.

[00109] **Kits**

[00110] As an additional aspect, the invention includes kits which comprise one or more pharmaceutical formulations packaged in a manner which facilitates their use for administration to subjects. In one embodiment, such a kit includes pharmaceutical formulation described herein (e.g., a composition comprising a therapeutic protein or peptide), packaged in a container such as a sealed bottle or vessel, with a label affixed to the container or included in the package that describes use of the compound or composition in practicing the method. In one embodiment, the pharmaceutical formulation is packaged in the container such that the amount of headspace in the container (e.g., the amount of air between the liquid formulation and the top of the container) is very small. Preferably, the amount of headspace is negligible (i.e., almost none). In one embodiment, the kit contains a first container having a therapeutic protein or peptide composition and a second container having a physiologically acceptable reconstitution solution for the composition. In one aspect, the pharmaceutical formulation is packaged in a unit dosage form. The kit may further include a device suitable for administering the pharmaceutical formulation according to a specific route of administration. Preferably, the kit contains a label that describes use of the pharmaceutical formulations.

[00111] **Dosages**

[00112] The dosage regimen involved in a method for treating a condition described herein will be determined by the attending physician, considering various factors which modify the action of drugs, e.g. the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. By way of example, a typical dose of a recombinant VWF of the present invention is approximately 50 U/kg, equal to 500 μg/kg.

[00113] Formulations of the invention may be administered by an initial bolus followed by a continuous infusion to maintain therapeutic circulating levels of drug product. As another example, the inventive compound may be administered as a one-time dose. Those of ordinary skill in the art will readily optimize effective dosages and administration regimens as determined by good medical practice and the clinical condition of the individual patient. The frequency of dosing will depend on the pharmacokinetic parameters of the agents and the route of administration. The optimal pharmaceutical formulation will be determined by one skilled in the art depending upon the route of administration and desired dosage. See for example, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712, the disclosure of which is hereby incorporated by reference. Such formulations may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the administered agents. Depending on the route of administration, a suitable dose may be calculated according to body weight, body surface area or

organ size. Appropriate dosages may be ascertained through use of established assays for determining blood level dosages in conjunction with appropriate dose-response data. The final dosage regimen will be determined by the attending physician, considering various factors which modify the action of drugs, e.g. the drug's specific activity, the severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding the appropriate dosage levels and duration of treatment for various diseases and conditions.

[00114] The following examples are not intended to be limiting but only exemplary of specific embodiments of the invention.

Example 1

Shaking experiments

[00115] In order to determine the amount of precipitation of rVWF in various formulations, the percent recovery of rVWF following turbulent shaking was tested under a variety of conditions.

[00116] rVWF in Advate buffer (90 mM NaCl, 1.68 mM $CaCl_2$, 10 mM L-histidine, 10 mM tris, 0.26 mM glutathione, 23.4 mM trehalose, 175.7 mM mannitol, and 0.1g/L TWEEN-80, pH 7.0)or Advate 1:3 buffer (Advate buffer diluted 3-fold in water) was subjected to turbulent shaking on a shaker at room temperature (RT) for 0 min, 1 min, 2.5 hrs, or 4 days, and percent recovery of the rVWF was measured relative to the starting material prior to shaking. As shown in Table 1, losses of about 40-80% were observed in the Advate buffer while losses of about 20-30% were observed in the Advate 1:3 buffer. VWF antigen VWF:Ag corresponds to the amount of VWF which can be detected in an VWF-specific ELISA using polyclonal anti-VWF antibody, while VWF:RCo corresponds to the amount of VWF which causes agglutination of stabilized platelets in the presence of ristocetin.. In both cases human reference plasma calibrated against the actual WHO standard was used as standard (1 ml of reference plasma usually contains 1U VWF).

[00117] **Table 1. Influence of turbulent shaking time on rVWF recovery**

rVWF	Turbulent shaking at RT	VWF:Ag [U/ml]	Recovery [%]	VWF:RCo [U/ml]	Recovery [%]	RCo/VWF:Ag [U/U]
Advate	0 min	213	100%	104	100%	0.49
	1 min	120	56%			
	2.5 hr	139	65%			
	4 d	37	17%	7	7%	0.19
Advate 1:3	0 min	206	100%	134	100%	0.65
	1 min	152	74%			
	2.5 hr	170	82%			
	4 d	138	67%	131	98%	0.95

[00118] The effect of freeze/thawing and lyophilization was also tested in the shaking experiments. Freezing was performed at –20°C in an –20°C cold room or on dry ice, thawing in both cases at RT and both started from the liquid formulations. As for lyophilization, the formulated VWF samples described herein were frozen within a pilot scale lyophilizer at <=-40°C and were lyophilized using a standard lyo program. Shaking was performed directly with the liquid formulations (2ml in 5 ml vials). As shown in Table 2, percent recovery of rVWF was higher in Advate 1:3 buffer compared to Advate buffer.

[00119] Table 2.

RVWF		VWF:Ag [U/ml]	VWF:Ag recovery [%]	VWF:RCo [U/ml]	VWF:RCo recovery [%]	RCo:Ag [U/U]
Advate	**Frozen**	213	100%	104	100%	0.49
	Frozen – 3x at -20°C	229	107%	84	81%	0.37
	Frozen – 3x with dry ice	231	108%	72	69%	0.31
	Lyo	242	113%	61	59%	0.25
	Starting material	213	100%	104	100%	0.49
	Heavily shaken for 4 days at RT	37.0	17%	7.2	6.9%	0.19
Advate 1:3	**Frozen**	206	100%	134	100%	0.65
	Frozen – 3x at -20°C	184	89%	132	99%	0.72
	Frozen – 3x with dry ice	195	94%	128	96%	0.66
	Lyo	195	94%	107	80%	0.55
	Starting material	206	100%	134	100%	0.65
	Heavily shaken for 4 days at RT	138	67%	131	98%	0.95

[00120] Percent recovery was also measured in the shaking experiments with rVWF being stored in syringes with headspace and without headspace. Interestingly, when rVWF is stored in syringes without headspace and shaken as described above, no rVWF precipitation was observed. In contrast, when rVWF is stored in syringes with headspace, some precipitation was observed.

[00121] In summary, turbulent shaking resulted in at least 30% loss of rVWF in Advate buffer or Advate 1:3 buffer, with Advate buffer showing higher loss of recovery compared to Advate 1:3 buffer. Interestingly, the same precipitates observed in the turbulent shaking experiments were not observed when rVWF was stored and transported ~5000 km in an automobile (representing

the expected shaking during transport). Precipitation of rVWF could be eliminated by storage in syringes without headspace.

Example 2

Stability of recombinant VWF

[00122] The stability of rVWF was tested by assessing the activity level of rVWF present in a various formulations.

[00123] As shown in Figure 1, rVWF is not stable in Advate buffer after 26 weeks due to the presence of 0.3 mM glutathione. As shown in Figure 2, however, rVWF is more stable in Advate 1:3 buffer (e.g., for up 12 weeks at 4°C.)

[00124] As shown in Figure 3, the stability of a citrate-based formulation (15 mM sodium citrate, 10 mM $CaCl_2$, 100 mM NaCl, pH 7.0) is better than Advate 1:3 buffer formulation containing 0.1M glutathione.

[00125] Likewise, the concentration of rVWF was measured over time in various buffers. As shown in Figure 4, Figure 5 and Figure 6, rVWF concentration is stable over time in Advate buffer, Advate 1:3 buffer, and citrate-based buffer, respectively.

Example 4

Characterization of the liquid formulations

[00126] Differential scanning calorimetry (DSC) was used to assess the extent of protein (rVWF) unfolding in various buffers. As shown in Table 3, Advate buffer pH 7.0 is the optimum for stabilization.

[00127] DSC is a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a sample and references are measured as a function of temperature. The result of a DSC experiment is a curve of heat flux versus temperature or versus time.

[00128] The Differential Scanning Calorimeter can scan through a range of temperatures while heating and cooling and it determines a phase transition, i.e. melting, crystallization, or glass transition, by measuring the amount of heat needed to reach a set temperature. The calorimeter was calibrated with a set of pure metals (zinc, indium, and tin) that have a known heat capacity, Cp and melting point, Tm. The respective reference buffer was placed into the reference capillary and the rVWF sample was placed into the sample capillary of the

instrument.

[00129] **Table 3. Unfolding temperature in various buffers**

Lot	Buffer	pH	T unfold [°C]
rVWF161A	Advate	7.0	66.0
rVWF161B	Immunate	6.8	64.5
rVWF161C	Citrate	6.8	61.2
rVWF161D	NovoSeven	6.8	64.9
rVWF158	Hepes	7.4	61.3

Buffer components and concentrations:

A) Advate:	5.26g/lNaCl	
	0.248g/l CaCl2	
	32g/l D-Mannitol	
	8g/l Trehalose	
	1.56g/l L-Histidine	
	1.2g/l Tris	
	0.08g/l Glutadione red.	pH=7.0
B) Immunate:	5.25g/l Glycin	
	2.2g/l NaCl	
	5.25g/l NaCit3	
	5.25g/l Lysin-HCl	
	0.62g/l CaCl2	pH=6.8
C) Citrat:	3g/l Glycin	
	2.92g/l NaCl	
	2.5g/l NaCit3	
	30g/l D-Mannitol	
	10g/l Trehalose	pH=6.8
D) NovoSeven:	0.75g/l Glycin	
	2.92g/l NaCl	
	1.47g/l CaCl2	
	30g/l D-Mannitol	pH=6,8

rVWF158: 20 mM Hepes, 150 mM NaCl, 5g/L sucrose; pH 7.4

[00130] Further, as shown in Figure 7, most formulation excipients increase the unfolding temperature by about 1-2 °C. Figure 8 shows that 10 mM $CaCl_2$ increases the unfolding

temperature by ~8 °C to ~67 °C, an unfolding temperature which can also be reached by Advate buffer. This effect of $CaCl_2$ is similar at pH 7.3 and 6.5, as shown in Figure 9. Finally, the effect of trehalose and sucrose were analyzed on the unfolding temperature. Compared to citrate alone, neither trehalose nor sucrose increased the unfolding temperature of rVWF. A summary of the unfolding temperature (Tmax) data for rVWF in the presence of various excipients is set out in Table 4.

Table 4.

15 mM Sodium Citrate buffer	-	15 mM Tris	15 mM Glycine	50 mM NaCl
ΔH [kJ/mol]	128494.3	656259.7	157352.2	124985.8
Unfolding T [°C] - Peak 1	58.6		59.1	61
Peak 2	65.2	68.5	65.5	
Peak 3	80.4		80.1	81
Peak 4				
15mM Sodium Citrate buffer	**15 mM Histidine**	**20.52 g/L Mannitol**	**10.26 g/L Trehalose**	
ΔH [kJ/mol]	134044.5	1588590.1	612235.9	
Unfolding T [°C] Peak 1	59.2	58.5	58.5	
Peak 2	65.2	65.5	71.3	
Peak 3	79.3	78.2	81.5	
Peak 4	88.5		92.7	
15mM Sodium Citrate buffer	**1 mM $CaCl_2$**	**10 mM $CaCl_2$**	**32 g/L Saccharose**	**0.25 mM Saccharose**
ΔH [kJ/mol]	266008.2	308171.3	115082.4	246904.6
Unfolding T [°C] - Peak 1	64.5	67.2	59.2	60
Peak 2			66	67
Peak 3	81	83.1	81.1	81.7
Peak 4	91.8	93		
15mM Sodium Citrate buffer	**0.1 g/L TWEEN-80**	**32 g/L Raffinose**	**Na_2HPO_4/NaH PO_4**	**7.8 mM Trehalose**
ΔH [kJ/mol]	338792.7	127329.2	197967.5	135573.3
Unfolding T [°C] - Peak 1	58.7	60.1	61.4	58.4
Peak 2	64.4	65.8		65.4
Peak 3	81.6	80.3	80.4	80.4
Peak 4			89.2	

[00131] In addition to the various buffers, DSC was used to assess unfolding temperature of rVWF at various pH values in Advate buffer. The results are shown in Table 5, below. Advate buffer pH 7.0 is the optimum for stabilization (i.e., highest unfolding temperature; Peak 1) of rVWF.

[00132] **Table 5.**

pH	Peak 1	Peak 2
5.0	59.5	62.0
6.0	65.2	75.4
7.0	67.2	82.8
8.0	66.6	85.6
9.0	65.0	84.9

[00133] The fluorescence spectrum of rVWF in Advate buffer and Advate 1:3 buffer was assessed after storage at various temperatures for various lengths of time. No (or only slight) change in fluorescence spectrum was observed after storage at 40 °C from 0 to 28 days in either Advate or Advate 1:3 buffers. No difference was observed at other temperatures.

[00134] Likewise, degradation of rVWF was assessed using gelfiltration (Superose 6). While some degradation was observed after 26 weeks at 4 °C in Advate buffer, almost no degradation of rVWF in Advate 1:3 buffer was observed after 26 weeks at 4 °C. At 40 °C, glutathione increased the amount of degradation over time (albeit to a slower extent in Advate 1:3 buffer).

[00135] Based on the above Examples, Advate 1:3 buffer offers an advantage with respect to freeze/thawing and recovery after lyophilization as compared to the undiluted Advate buffer. Moreover, Advate 1:3 buffer can stabilize (e.g., maintain biological activity) rVWF activity during incubation at 40 °C better that Advate buffer. rVWF in Advate 1:3 buffer is stable for 4 weeks of incubation at 4 °C. Finally, DSC has demonstrated that pH 7.0 is optimum for preventing degradation of rVWF (i.e., showed the highest unfolding temperature).

[00136] Thus, in view of the data presented herein, a formulation was proposed for rVWF including 15 mM citrate (or glycine or hitidine), 10 mM $CaCl_2$, pH 6.5-7.3, adjusted to the

desired osmolarity by NaCl. For example, in one embodiment, the citrate-based formula is 15 mM sodium citrate, 10 mM $CaCl_2$, 100 mM NaCl, pH 7.0.

[00137] Alternatively, an Advate or Advate 1:3 buffer, without glutathione, is also contemplated: Advate: 90mM NaCl, 1.68mM $CaCl_2$, 10mM L-histidine, 10mM Tris, 0.26mM glutathione, 23.4mM trehalose, 175.7mM mannitol, and 0.1g/L TWEEN-80, pH 7.0; Advate 1:3: 30 mM NaCl, 0.56 mM $CaCl_2$, 3.3 mM L-histidine, 3.3 mM tris, 7.8 mM trehalose, 58.6 mM mannitol, and 0.03g/L TWEEN-80, ph 7.0.

What is claimed is:

1. A stable liquid pharmaceutical formulation of a recombinant von Willebrand Factor (rVWF) comprising: (a) a rVWF; (b) a buffering agent; (c) one or more salts; (d) optionally a stabilizing agent; and (e) optionally a surfactant;

wherein said rVWF comprises a polypeptide selected from the group consisting of:

a) the amino acid sequence set out in SEQ ID NO: 3;

b) a biologically active analog, fragment or variant of a);

c) a polypeptide encoded by the polynucleotide set out in SEQ ID NO: 1;

d) a biologically active analog, fragment or variant of c); and

e) a polypeptide encoded by a polynucleotide that hybridizes to the polynucleotide set out in SEQ ID NO: 1 under moderately stringent hybridization conditions;

wherein said buffer is comprised of a pH buffering agent in a range of about 0.1 mM to about 500 mM and wherein the pH is in a range of about 2.0 to about 12.0;

wherein said salt is at a concentration of about 1 to 500 mM;

wherein said stabilizing agent is at a concentration of about 0.1 to 1000 mM; and

wherein said surfactant is at a concentration of about 0.01 g/L to 0.5 g/L.

2. The formulation of claim 1 wherein the rVWF comprises the amino acid sequence set out in SEQ ID NO: 3.

3. The formulation of claim 1 wherein the buffering agent is selected from the group consisting of sodium citrate, glycine, histidine, Tris and combinations of these agents.

4. The formulation of claim 3 wherein the buffering agent is sodium citrate at a concentration of 15 mM..

5. The formulation of claim 1 wherein pH is in the range of 6.0-8.0.

6. The formulation of claim 5 wherein pH is in the range of 6.5-7.3.

7. The formulation of claim 4 wherein the pH is 7.0.

8. The formulation of claim 1 wherein the buffering agent is citrate and the pH is 7.0.

9. The formulation of claim 1 wherein the salt is selected from the group consisting of calcium chloride, sodium chloride and magnesium chloride.

10. The formulation of claim 9 wherein the salt is at a concentration range of 0.5 to 300 mM.

11. The formulation of claim 10 wherein the salt is calcium chloride at a concentration of 10 mM.

12. The formulation of claim 1 wherein the rVWF comprises the amino acid sequence set out in SEQ ID NO: 3; wherein the buffering agent is citrate and the pH is 7.0; and wherein the salt is calcium chloride at a concentration of 10 mM.

13. The formulation of claim 1 wherein the rVWF comprises the amino acid sequence set out in SEQ ID NO: 3; wherein the buffering agent is sodium citrate at a concentration of 15 mM and the pH is 7.0; and wherein the salt is calcium chloride at a concentration of 10 mM and NaCl at a concentration of 100 mM.

14. The formulation of claim 3 wherein the one or more buffering agents is histidine and Tris at a concentration of 3.3 mM each.

15. The formulation of claim 3 wherein the pH is 7.0.

16. The formulation of claim 9 wherein the one or more salts is sodium chloride at a concentration of 30 mM and calcium chloride at a concentration of 0.56 mM.

17. The formulation of claim 1 wherein the stabilizing agent is selected from the group consisting of mannitol, lactose, sorbitol, xylitol, sucrose, trehalose, mannose, maltose, lactose, glucose, raffinose, cellobiose, gentiobiose, isomaltose, arabinose, glucosamine, fructose and combinations of these stabilizing agents.

18. The formulation of claim 17 wherein the stabilizing agents are trehalose at a concentration of 7.8 mM and mannitol at a concentration of 58.6 mM.

19. The formulation of claim 1 wherein the surfactant is selected from the group consisting of digitonin, Triton X-100, Triton X-114, TWEEN-20, TWEEN-80 and combinations of these surfactants.

20. The formulation of claim 1 wherein the surfactant is TWEEN-80 at 0.03 g/L.

21. The formulation of claim 1 wherein the rVWF comprises amino acid sequence set out in SEQ ID NO: 3; wherein the buffering agents are histidine at a concentration of 3.3 mM and Tris at a concentration of 3.3 mM at pH 7.0; wherein the salts are sodium chloride at a concentration of 30 mM and calcium chloride at a concentration of 0.56 mM; wherein the stabilizing agents are trehalose at a concentration of 7.8 mM and mannitol at a concentration of 58.6 mM.; and wherein the surfactant is TWEEN-80 at 0.03 g/L.

SEQUENCE LISTING

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<120> Recombinant VWF Formulations

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tcggtcgggc ttcacttacg ttctgcatga aggcgagtgc tgtggaaggt gcctgccatc 7740

tgcctgtgag gtggtgactg gctcaccgcg gggggactcc cagtcttcct ggaagagtgt 7800

cggctcccag tgggcctccc cggagaaccc ctgcctcatc aatgagtgtg tccgagtgaa 7860

ggaggaggtc tttatacaac aaaggaacgt ctcctgcccc cagctggagg tccctgtctg 7920

cccctcgggc tttcagctga gctgtaagac ctcagcgtgc tgcccaagct gtcgctgtga 7980

gcgcatggag gcctgcatgc tcaatggcac tgtcattggg cccgggaaga ctgtgatgat 8040

cgatgtgtgc acgacctgcc gctgcatggt gcaggtgggg gtcatctctg gattcaagct 8100

ggagtgcagg aagaccacct gcaacccctg ccccctgggt tacaaggaag aaaataacac 8160

aggtgaatgt tgtgggagat gtttgcctac ggcttgcacc attcagctaa gaggaggaca 8220

gatcatgaca ctgaagcgtg atgagacgct ccaggatggc tgtgatactc acttctgcaa 8280

ggtcaatgag agaggagagt acttctggga gaagagggtc acaggctgcc cacccttga 8340

tgaacacaag tgtctggctg agggaggtaa aattatgaaa attccaggca cctgctgtga 8400

cacatgtgag gagcctgagt gcaacgacat cactgccagg ctgcagtatg tcaaggtggg 8460

aagctgtaag tctgaagtag aggtggatat ccactactgc cagggcaaat gtgccagcaa 8520

agccatgtac tccattgaca tcaacgatgt gcaggaccag tgctcctgct gctctccgac 8580

acggacggag cccatgcagg tggccctgca ctgcaccaat ggctctgttg tgtaccatga 8640

ggttctcaat gccatggagt gcaaatgctc ccccaggaag tgcagcaagt gaggctgctg 8700

cagctgcatg ggtgcctgct gctgcctgcc ttggcctgat ggccaggcca gagtgctgcc 8760

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tcttgcaaaa ggc 8833

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<212> PRT
<213> Artificial Sequence

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<223> Synthetic Polypeptide

<220>
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<223> prepro-vWF

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Thr Leu Cys Ala Glu Gly Thr Arg Gly Arg Ser Ser Thr Ala Arg Cys
20 25 30

Ser Leu Phe Gly Ser Asp Phe Val Asn Thr Phe Asp Gly Ser Met Tyr
35 40 45

Ser Phe Ala Gly Tyr Cys Ser Tyr Leu Leu Ala Gly Gly Cys Gln Lys
50 55 60

Arg Ser Phe Ser Ile Ile Gly Asp Phe Gln Asn Gly Lys Arg Val Ser
65 70 75 80

Leu Ser Val Tyr Leu Gly Glu Phe Phe Asp Ile His Leu Phe Val Asn
85 90 95

Gly Thr Val Thr Gln Gly Asp Gln Arg Val Ser Met Pro Tyr Ala Ser
100 105 110

Lys Leu Glu Thr Glu Ala Gly Tyr Tyr Lys Leu Ser Gly Glu Ala Tyr
115 120 125

Gly Phe Val Ala Arg Ile Asp Gly Ser Gly Asn Phe Gln Val Leu Leu
130 135 140

Ser Asp Arg Tyr Phe Asn Lys Thr Cys Gly Leu Cys Gly Asn Phe Asn
145 150 155 160

Ile Phe Ala Glu Asp Asp Phe Met Thr Gln Glu Gly Thr Leu Thr Ser
165 170 175

```
Asp Pro Tyr Asp Phe Ala Asn Ser Trp Ala Leu Ser Ser Gly Glu Gln
            180                 185                 190

Trp Cys Glu Arg Pro Ser Ser Ser Cys Asn Ile Ser Ser Gly Glu Met
        195                 200                 205

Gln Lys Gly Leu Trp Glu Gln Cys Gln Leu Leu Lys Ser Thr Ser Val
    210                 215                 220

Phe Ala Arg Cys His Pro Leu Val Asp Pro Glu Pro Phe Cys Glu Lys
225                 230                 235                 240

Thr Leu Cys Glu Cys Ala Gly Gly Leu Glu Cys Ala Cys Pro Ala Leu
                245                 250                 255

Leu Glu Tyr Ala Arg Thr Cys Ala Gln Glu Gly Met Val Leu Tyr Gly
            260                 265                 270

Trp Thr Asp His Ser Ala Cys Ser Pro Val Cys Pro Ala Gly Met Glu
        275                 280                 285

Tyr Arg Gln Cys Val Ser Pro Cys Ala Arg Thr Cys Gln Ser Leu His
    290                 295                 300

Ile Asn Glu Met Cys Gln Glu Arg Cys Val Asp Gly Cys Ser Cys Pro
305                 310                 315                 320

Glu Gly Gln Leu Leu Asp Glu Gly Leu Cys Val Glu Ser Thr Glu Cys
                325                 330                 335

Pro Cys Val His Ser Gly Lys Arg Tyr Pro Pro Gly Thr Ser Leu Ser
            340                 345                 350

Arg Asp Cys Asn Thr Cys Ile Cys Arg Asn Ser Gln Trp Ile Cys Ser
        355                 360                 365

Asn Glu Glu Cys Pro Gly Glu Cys Leu Val Thr Gly Gln Ser His Phe
    370                 375                 380

Lys Ser Phe Asp Asn Arg Tyr Phe Thr Phe Ser Gly Ile Cys Gln Tyr
385                 390                 395                 400

Leu Leu Ala Arg Asp Cys Gln Asp His Ser Phe Ser Ile Val Ile Glu
                405                 410                 415

Thr Val Gln Cys Ala Asp Asp Arg Asp Ala Val Cys Thr Arg Ser Val
            420                 425                 430
```

```
Thr Val Arg Leu Pro Gly Leu His Asn Ser Leu Val Lys Leu Lys His
        435                 440                 445

Gly Ala Gly Val Ala Met Asp Gly Gln Asp Val Gln Leu Pro Leu Leu
    450                 455                 460

Lys Gly Asp Leu Arg Ile Gln His Thr Val Thr Ala Ser Val Arg Leu
465                 470                 475                 480

Ser Tyr Gly Glu Asp Leu Gln Met Asp Trp Asp Gly Arg Gly Arg Leu
                485                 490                 495

Leu Val Lys Leu Ser Pro Val Tyr Ala Gly Lys Thr Cys Gly Leu Cys
            500                 505                 510

Gly Asn Tyr Asn Gly Asn Gln Gly Asp Asp Phe Leu Thr Pro Ser Gly
        515                 520                 525

Leu Ala Glu Pro Arg Val Glu Asp Phe Gly Asn Ala Trp Lys Leu His
    530                 535                 540

Gly Asp Cys Gln Asp Leu Gln Lys Gln His Ser Asp Pro Cys Ala Leu
545                 550                 555                 560

Asn Pro Arg Met Thr Arg Phe Ser Glu Glu Ala Cys Ala Val Leu Thr
                565                 570                 575

Ser Pro Thr Phe Glu Ala Cys His Arg Ala Val Ser Pro Leu Pro Tyr
            580                 585                 590

Leu Arg Asn Cys Arg Tyr Asp Val Cys Ser Cys Ser Asp Gly Arg Glu
        595                 600                 605

Cys Leu Cys Gly Ser Tyr Ala Ala Ala Cys Ala Gly Arg Gly Val Arg
    610                 615                 620

Val Ala Trp Arg Glu Pro Gly Arg Cys Glu Leu Asn Cys Pro Lys Gly
625                 630                 635                 640

Gln Val Tyr Leu Gln Cys Gly Thr Pro Cys Asn Leu Thr Cys Arg Ser
                645                 650                 655

Leu Ser Tyr Pro Asp Glu Glu Cys Asn Glu Ala Cys Leu Glu Gly Cys
            660                 665                 670

Phe Cys Pro Pro Met Asp Glu Arg Gly Asp Cys Val Pro Lys Ala Gln
        675                 680                 685
```

Cys Pro Cys Tyr Tyr Asp Gly Glu Ile Phe Gln Pro Glu Asp Ile Phe
690 695 700

Ser Asp His His Thr Met Cys Tyr Cys Glu Asp Gly Phe Met His Cys
705 710 715 720

Thr Met Ser Gly Val Pro Gly Ser Leu Leu Pro Asp Ala Val Leu Ser
725 730 735

Ser Pro Leu Ser His Arg Ser Lys Arg Ser Leu Ser Cys Arg Pro Pro
740 745 750

Met Val Lys Leu Val Cys Pro Ala Asp Asn Leu Arg Ala Glu Gly Leu
755 760 765

Glu Cys Thr Lys Thr Cys Gln Asn Tyr Asp Leu Glu Cys Met Ser Met
770 775 780

Gly Cys Val Ser Gly Cys Leu Cys Pro Pro Gly Met Val Arg His Glu
785 790 795 800

Asn Arg Cys Glu Arg Cys Pro Cys Phe His Gln Gly Lys Glu Tyr Ala
805 810 815

Pro Gly Glu Thr Val Lys Ile Gly Cys Asn Thr Cys Val Cys Arg Asp
820 825 830

Arg Lys Trp Asn Cys Thr Asp His Val Cys Asp Ala Thr Cys Ser Thr
835 840 845

Ile Gly Met Ala His Tyr Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe
850 855 860

Pro Gly Glu Cys Gln Tyr Val Leu Val Gln Asp Tyr Cys Gly Ser Asn
865 870 875 880

Pro Gly Thr Phe Arg Ile Leu Val Gly Asn Lys Gly Cys Ser His Pro
885 890 895

Ser Val Lys Cys Lys Lys Arg Val Thr Ile Leu Val Glu Gly Gly Glu
900 905 910

Ile Glu Leu Phe Asp Gly Glu Val Asn Val Lys Arg Pro Met Lys Asp
915 920 925

Glu Thr His Phe Glu Val Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu
930 935 940

```
Leu Gly Lys Ala Leu Ser Val Val Trp Asp Arg His Leu Ser Ile Ser
945                 950                 955                 960

Val Val Leu Lys Gln Thr Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly
                965                 970                 975

Asn Phe Asp Gly Ile Gln Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln
            980                 985                 990

Val Glu Glu Asp Pro Val Asp Phe  Gly Asn Ser Trp Lys  Val Ser Ser
        995                 1000                1005

Gln Cys  Ala Asp Thr Arg Lys  Val Pro Leu Asp Ser  Ser Pro Ala
    1010                1015                1020

Thr Cys  His Asn Asn Ile Met  Lys Gln Thr Met Val  Asp Ser Ser
    1025                1030                1035

Cys Arg  Ile Leu Thr Ser Asp  Val Phe Gln Asp Cys  Asn Lys Leu
    1040                1045                1050

Val Asp  Pro Glu Pro Tyr Leu  Asp Val Cys Ile Tyr  Asp Thr Cys
    1055                1060                1065

Ser Cys  Glu Ser Ile Gly Asp  Cys Ala Cys Phe Cys  Asp Thr Ile
    1070                1075                1080

Ala Ala  Tyr Ala His Val Cys  Ala Gln His Gly Lys  Val Val Thr
    1085                1090                1095

Trp Arg  Thr Ala Thr Leu Cys  Pro Gln Ser Cys Glu  Glu Arg Asn
    1100                1105                1110

Leu Arg  Glu Asn Gly Tyr Glu  Cys Glu Trp Arg Tyr  Asn Ser Cys
    1115                1120                1125

Ala Pro  Ala Cys Gln Val Thr  Cys Gln His Pro Glu  Pro Leu Ala
    1130                1135                1140

Cys Pro  Val Gln Cys Val Glu  Gly Cys His Ala His  Cys Pro Pro
    1145                1150                1155

Gly Lys  Ile Leu Asp Glu Leu  Leu Gln Thr Cys Val  Asp Pro Glu
    1160                1165                1170

Asp Cys  Pro Val Cys Glu Val  Ala Gly Arg Arg Phe  Ala Ser Gly
    1175                1180                1185
```

```
Lys Lys  Val Thr Leu Asn Pro  Ser Asp Pro Glu His  Cys Gln Ile
    1190                 1195                 1200

Cys His  Cys Asp Val Val Asn  Leu Thr Cys Glu Ala  Cys Gln Glu
    1205                 1210                 1215

Pro Gly  Gly Leu Val Val Pro  Pro Thr Asp Ala Pro  Val Ser Pro
    1220                 1225                 1230

Thr Thr  Leu Tyr Val Glu Asp  Ile Ser Glu Pro Pro  Leu His Asp
    1235                 1240                 1245

Phe Tyr  Cys Ser Arg Leu Leu  Asp Leu Val Phe Leu  Leu Asp Gly
    1250                 1255                 1260

Ser Ser  Arg Leu Ser Glu Ala  Glu Phe Glu Val Leu  Lys Ala Phe
    1265                 1270                 1275

Val Val  Asp Met Met Glu Arg  Leu Arg Ile Ser Gln  Lys Trp Val
    1280                 1285                 1290

Arg Val  Ala Val Val Glu Tyr  His Asp Gly Ser His  Ala Tyr Ile
    1295                 1300                 1305

Gly Leu  Lys Asp Arg Lys Arg  Pro Ser Glu Leu Arg  Arg Ile Ala
    1310                 1315                 1320

Ser Gln  Val Lys Tyr Ala Gly  Ser Gln Val Ala Ser  Thr Ser Glu
    1325                 1330                 1335

Val Leu  Lys Tyr Thr Leu Phe  Gln Ile Phe Ser Lys  Ile Asp Arg
    1340                 1345                 1350

Pro Glu  Ala Ser Arg Ile Thr  Leu Leu Leu Met Ala  Ser Gln Glu
    1355                 1360                 1365

Pro Gln  Arg Met Ser Arg Asn  Phe Val Arg Tyr Val  Gln Gly Leu
    1370                 1375                 1380

Lys Lys  Lys Lys Val Ile Val  Ile Pro Val Gly Ile  Gly Pro His
    1385                 1390                 1395

Ala Asn  Leu Lys Gln Ile Arg  Leu Ile Glu Lys Gln  Ala Pro Glu
    1400                 1405                 1410

Asn Lys  Ala Phe Val Leu Ser  Ser Val Asp Glu Leu  Glu Gln Gln
    1415                 1420                 1425
```

```
Arg Asp Glu Ile Val Ser Tyr Leu Cys Asp Leu Ala Pro Glu Ala
    1430                1435                1440

Pro Pro Pro Thr Leu Pro Pro Asp Met Ala Gln Val Thr Val Gly
    1445                1450                1455

Pro Gly Leu Leu Gly Val Ser Thr Leu Gly Pro Lys Arg Asn Ser
    1460                1465                1470

Met Val Leu Asp Val Ala Phe Val Leu Glu Gly Ser Asp Lys Ile
    1475                1480                1485

Gly Glu Ala Asp Phe Asn Arg Ser Lys Glu Phe Met Glu Glu Val
    1490                1495                1500

Ile Gln Arg Met Asp Val Gly Gln Asp Ser Ile His Val Thr Val
    1505                1510                1515

Leu Gln Tyr Ser Tyr Met Val Thr Val Glu Tyr Pro Phe Ser Glu
    1520                1525                1530

Ala Gln Ser Lys Gly Asp Ile Leu Gln Arg Val Arg Glu Ile Arg
    1535                1540                1545

Tyr Gln Gly Gly Asn Arg Thr Asn Thr Gly Leu Ala Leu Arg Tyr
    1550                1555                1560

Leu Ser Asp His Ser Phe Leu Val Ser Gln Gly Asp Arg Glu Gln
    1565                1570                1575

Ala Pro Asn Leu Val Tyr Met Val Thr Gly Asn Pro Ala Ser Asp
    1580                1585                1590

Glu Ile Lys Arg Leu Pro Gly Asp Ile Gln Val Val Pro Ile Gly
    1595                1600                1605

Val Gly Pro Asn Ala Asn Val Gln Glu Leu Glu Arg Ile Gly Trp
    1610                1615                1620

Pro Asn Ala Pro Ile Leu Ile Gln Asp Phe Glu Thr Leu Pro Arg
    1625                1630                1635

Glu Ala Pro Asp Leu Val Leu Gln Arg Cys Cys Ser Gly Glu Gly
    1640                1645                1650

Leu Gln Ile Pro Thr Leu Ser Pro Ala Pro Asp Cys Ser Gln Pro
    1655                1660                1665
```

Leu Asp Val Ile Leu Leu Leu Asp Gly Ser Ser Ser Phe Pro Ala
1670 1675 1680

Ser Tyr Phe Asp Glu Met Lys Ser Phe Ala Lys Ala Phe Ile Ser
1685 1690 1695

Lys Ala Asn Ile Gly Pro Arg Leu Thr Gln Val Ser Val Leu Gln
1700 1705 1710

Tyr Gly Ser Ile Thr Thr Ile Asp Val Pro Trp Asn Val Val Pro
1715 1720 1725

Glu Lys Ala His Leu Leu Ser Leu Val Asp Val Met Gln Arg Glu
1730 1735 1740

Gly Gly Pro Ser Gln Ile Gly Asp Ala Leu Gly Phe Ala Val Arg
1745 1750 1755

Tyr Leu Thr Ser Glu Met His Gly Ala Arg Pro Gly Ala Ser Lys
1760 1765 1770

Ala Val Val Ile Leu Val Thr Asp Val Ser Val Asp Ser Val Asp
1775 1780 1785

Ala Ala Ala Asp Ala Ala Arg Ser Asn Arg Val Thr Val Phe Pro
1790 1795 1800

Ile Gly Ile Gly Asp Arg Tyr Asp Ala Ala Gln Leu Arg Ile Leu
1805 1810 1815

Ala Gly Pro Ala Gly Asp Ser Asn Val Val Lys Leu Gln Arg Ile
1820 1825 1830

Glu Asp Leu Pro Thr Met Val Thr Leu Gly Asn Ser Phe Leu His
1835 1840 1845

Lys Leu Cys Ser Gly Phe Val Arg Ile Cys Met Asp Glu Asp Gly
1850 1855 1860

Asn Glu Lys Arg Pro Gly Asp Val Trp Thr Leu Pro Asp Gln Cys
1865 1870 1875

His Thr Val Thr Cys Gln Pro Asp Gly Gln Thr Leu Leu Lys Ser
1880 1885 1890

His Arg Val Asn Cys Asp Arg Gly Leu Arg Pro Ser Cys Pro Asn
1895 1900 1905

```
Ser Gln  Ser Pro Val Lys Val  Glu Glu Thr Cys Gly  Cys Arg Trp
    1910                1915                1920

Thr Cys  Pro Cys Val Cys Thr  Gly Ser Ser Thr Arg  His Ile Val
    1925                1930                1935

Thr Phe  Asp Gly Gln Asn Phe  Lys Leu Thr Gly Ser  Cys Ser Tyr
    1940                1945                1950

Val Leu  Phe Gln Asn Lys Glu  Gln Asp Leu Glu Val  Ile Leu His
    1955                1960                1965

Asn Gly  Ala Cys Ser Pro Gly  Ala Arg Gln Gly Cys  Met Lys Ser
    1970                1975                1980

Ile Glu  Val Lys His Ser Ala  Leu Ser Val Glu Leu  His Ser Asp
    1985                1990                1995

Met Glu  Val Thr Val Asn Gly  Arg Leu Val Ser Val  Pro Tyr Val
    2000                2005                2010

Gly Gly  Asn Met Glu Val Asn  Val Tyr Gly Ala Ile  Met His Glu
    2015                2020                2025

Val Arg  Phe Asn His Leu Gly  His Ile Phe Thr Phe  Thr Pro Gln
    2030                2035                2040

Asn Asn  Glu Phe Gln Leu Gln  Leu Ser Pro Lys Thr  Phe Ala Ser
    2045                2050                2055

Lys Thr  Tyr Gly Leu Cys Gly  Ile Cys Asp Glu Asn  Gly Ala Asn
    2060                2065                2070

Asp Phe  Met Leu Arg Asp Gly  Thr Val Thr Thr Asp  Trp Lys Thr
    2075                2080                2085

Leu Val  Gln Glu Trp Thr Val  Gln Arg Pro Gly Gln  Thr Cys Gln
    2090                2095                2100

Pro Glu  Gln Cys Leu Val Pro  Asp Ser Ser His Cys  Gln Val Leu
    2105                2110                2115

Leu Leu  Pro Leu Phe Ala Glu  Cys His Lys Val Leu  Ala Pro Ala
    2120                2125                2130

Thr Phe  Tyr Ala Ile Cys Gln  Gln Asp Ser Cys His  Gln Glu Gln
    2135                2140                2145
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Val Cys Glu Val Ile Ala Ser Tyr Ala His Leu Cys Arg Thr Asn
2150 2155 2160

Gly Val Cys Val Asp Trp Arg Thr Pro Asp Phe Cys Ala Met Ser
2165 2170 2175

Cys Pro Pro Ser Leu Val Tyr Asn His Cys Glu His Gly Cys Pro
2180 2185 2190

Arg His Cys Asp Gly Asn Val Ser Ser Cys Gly Asp His Pro Ser
2195 2200 2205

Glu Gly Cys Phe Cys Pro Pro Asp Lys Val Met Leu Glu Gly Ser
2210 2215 2220

Cys Val Pro Glu Glu Ala Cys Thr Gln Cys Ile Gly Glu Asp Gly
2225 2230 2235

Val Gln His Gln Phe Leu Glu Ala Trp Val Pro Asp His Gln Pro
2240 2245 2250

Cys Gln Ile Cys Thr Cys Leu Ser Gly Arg Lys Val Asn Cys Thr
2255 2260 2265

Thr Gln Pro Cys Pro Thr Ala Lys Ala Pro Thr Cys Gly Leu Cys
2270 2275 2280

Glu Val Ala Arg Leu Arg Gln Asn Ala Asp Gln Cys Cys Pro Glu
2285 2290 2295

Tyr Glu Cys Val Cys Asp Pro Val Ser Cys Asp Leu Pro Pro Val
2300 2305 2310

Pro His Cys Glu Arg Gly Leu Gln Pro Thr Leu Thr Asn Pro Gly
2315 2320 2325

Glu Cys Arg Pro Asn Phe Thr Cys Ala Cys Arg Lys Glu Glu Cys
2330 2335 2340

Lys Arg Val Ser Pro Pro Ser Cys Pro Pro His Arg Leu Pro Thr
2345 2350 2355

Leu Arg Lys Thr Gln Cys Cys Asp Glu Tyr Glu Cys Ala Cys Asn
2360 2365 2370

Cys Val Asn Ser Thr Val Ser Cys Pro Leu Gly Tyr Leu Ala Ser
2375 2380 2385

Thr Ala Thr Asn Asp Cys Gly Cys Thr Thr Thr Thr Cys Leu Pro
2390 2395 2400

Asp Lys Val Cys Val His Arg Ser Thr Ile Tyr Pro Val Gly Gln
2405 2410 2415

Phe Trp Glu Glu Gly Cys Asp Val Cys Thr Cys Thr Asp Met Glu
2420 2425 2430

Asp Ala Val Met Gly Leu Arg Val Ala Gln Cys Ser Gln Lys Pro
2435 2440 2445

Cys Glu Asp Ser Cys Arg Ser Gly Phe Thr Tyr Val Leu His Glu
2450 2455 2460

Gly Glu Cys Cys Gly Arg Cys Leu Pro Ser Ala Cys Glu Val Val
2465 2470 2475

Thr Gly Ser Pro Arg Gly Asp Ser Gln Ser Ser Trp Lys Ser Val
2480 2485 2490

Gly Ser Gln Trp Glu Asn Pro Cys Leu Ile Asn Glu Cys Val Arg
2495 2500 2505

Val Lys Glu Glu Val Phe Ile Gln Gln Arg Asn Val Ser Cys Pro
2510 2515 2520

Gln Leu Glu Val Pro Val Cys Pro Ser Gly Phe Gln Leu Ser Cys
2525 2530 2535

Lys Thr Ser Ala Cys Cys Pro Ser Cys Arg Cys Glu Arg Met Glu
2540 2545 2550

Ala Cys Met Leu Asn Gly Thr Val Ile Gly Pro Gly Lys Thr Val
2555 2560 2565

Met Ile Asp Val Cys Thr Thr Cys Arg Cys Met Val Gln Val Gly
2570 2575 2580

Val Ile Ser Gly Phe Lys Leu Glu Cys Arg Lys Thr Thr Cys Asn
2585 2590 2595

Pro Cys Pro Leu Gly Tyr Lys Glu Glu Asn Asn Thr Gly Glu Cys
2600 2605 2610

Cys Gly Arg Cys Leu Pro Thr Ala Cys Thr Ile Gln Leu Arg Gly
2615 2620 2625

```
Gly Gln  Ile Met Thr Leu Lys  Arg Asp Glu Thr Leu  Gln Asp Gly
    2630                 2635                 2640

Cys Asp  Thr His Phe Cys Lys  Val Asn Glu Arg Gly  Glu Tyr Phe
    2645                 2650                 2655

Trp Glu  Lys Arg Val Thr Gly  Cys Pro Pro Phe Asp  Glu His Lys
    2660                 2665                 2670

Cys Leu  Ala Glu Gly Gly Lys  Ile Met Lys Ile Pro  Gly Thr Cys
    2675                 2680                 2685

Cys Asp  Thr Cys Glu Glu Pro  Glu Cys Asn Asp Ile  Thr Ala Arg
    2690                 2695                 2700

Leu Gln  Tyr Val Lys Val Gly  Ser Cys Lys Ser Glu  Val Glu Val
    2705                 2710                 2715

Asp Ile  His Tyr Cys Gln Gly  Lys Cys Ala Ser Lys  Ala Met Tyr
    2720                 2725                 2730

Ser Ile  Asp Ile Asn Asp Val  Gln Asp Gln Cys Ser  Cys Cys Ser
    2735                 2740                 2745

Pro Thr  Arg Thr Glu Pro Met  Gln His Cys Thr Asn  Gly Ser Val
    2750                 2755                 2760

Val Tyr  His Glu Val Leu Asn  Ala Met Glu Cys Lys  Cys Ser Pro
    2765                 2770                 2775

Arg Lys  Cys Ser Lys
    2780
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<210> 3
<211> 2050
<212> PRT
<213> Artificial Sequence

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<223> Synthetic Polypeptide

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<223> mature vWF

<400> 3

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Ser Leu Ser Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
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Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
            20                  25                  30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
        35                  40                  45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
    50                  55                  60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
65                  70                  75                  80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
                85                  90                  95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
            100                 105                 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
        115                 120                 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
    130                 135                 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
145                 150                 155                 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
                165                 170                 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
            180                 185                 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
        195                 200                 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
    210                 215                 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
225                 230                 235                 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
                245                 250                 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
            260                 265                 270
```

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly Leu
465 470 475 480

Val Val Pro Pro Thr Asp Ala Pro Val Ser Pro Thr Thr Leu Tyr Val
485 490 495

Glu Asp Ile Ser Glu Pro Pro Leu His Asp Phe Tyr Cys Ser Arg Leu
500 505 510

Leu Asp Leu Val Phe Leu Leu Asp Gly Ser Ser Arg Leu Ser Glu Ala
515 520 525

```
Glu Phe Glu Val Leu Lys Ala Phe Val Val Asp Met Met Glu Arg Leu
    530                 535                 540

Arg Ile Ser Gln Lys Trp Val Arg Val Ala Val Val Glu Tyr His Asp
545                 550                 555                 560

Gly Ser His Ala Tyr Ile Gly Leu Lys Asp Arg Lys Arg Pro Ser Glu
                565                 570                 575

Leu Arg Arg Ile Ala Ser Gln Val Lys Tyr Ala Gly Ser Gln Val Ala
            580                 585                 590

Ser Thr Ser Glu Val Leu Lys Tyr Thr Leu Phe Gln Ile Phe Ser Lys
        595                 600                 605

Ile Asp Arg Pro Glu Ala Ser Arg Ile Thr Leu Leu Leu Met Ala Ser
    610                 615                 620

Gln Glu Pro Gln Arg Met Ser Arg Asn Phe Val Arg Tyr Val Gln Gly
625                 630                 635                 640

Leu Lys Lys Lys Lys Val Ile Val Ile Pro Val Gly Ile Gly Pro His
                645                 650                 655

Ala Asn Leu Lys Gln Ile Arg Leu Ile Glu Lys Gln Ala Pro Glu Asn
            660                 665                 670

Lys Ala Phe Val Leu Ser Ser Val Asp Glu Leu Glu Gln Gln Arg Asp
        675                 680                 685

Glu Ile Val Ser Tyr Leu Cys Asp Leu Ala Pro Glu Ala Pro Pro Pro
    690                 695                 700

Thr Leu Pro Pro Asp Met Ala Gln Val Thr Val Gly Pro Gly Leu Leu
705                 710                 715                 720

Gly Val Ser Thr Leu Gly Pro Lys Arg Asn Ser Met Val Leu Asp Val
                725                 730                 735

Ala Phe Val Leu Glu Gly Ser Asp Lys Ile Gly Glu Ala Asp Phe Asn
            740                 745                 750

Arg Ser Lys Glu Phe Met Glu Glu Val Ile Gln Arg Met Asp Val Gly
        755                 760                 765

Gln Asp Ser Ile His Val Thr Val Leu Gln Tyr Ser Tyr Met Val Thr
    770                 775                 780
```

```
Val Glu Tyr Pro Phe Ser Glu Ala Gln Ser Lys Gly Asp Ile Leu Gln
785                 790                 795                 800

Arg Val Arg Glu Ile Arg Tyr Gln Gly Gly Asn Arg Thr Asn Thr Gly
                805                 810                 815

Leu Ala Leu Arg Tyr Leu Ser Asp His Ser Phe Leu Val Ser Gln Gly
            820                 825                 830

Asp Arg Glu Gln Ala Pro Asn Leu Val Tyr Met Val Thr Gly Asn Pro
        835                 840                 845

Ala Ser Asp Glu Ile Lys Arg Leu Pro Gly Asp Ile Gln Val Val Pro
    850                 855                 860

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

Trp Pro Asn Ala Pro Ile Leu Ile Gln Asp Phe Glu Thr Leu Pro Arg
                885                 890                 895

Glu Ala Pro Asp Leu Val Leu Gln Arg Cys Cys Ser Gly Glu Gly Leu
            900                 905                 910

Gln Ile Pro Thr Leu Ser Pro Ala Pro Asp Cys Ser Gln Pro Leu Asp
        915                 920                 925

Val Ile Leu Leu Leu Asp Gly Ser Ser Ser Phe Pro Ala Ser Tyr Phe
    930                 935                 940

Asp Glu Met Lys Ser Phe Ala Lys Ala Phe Ile Ser Lys Ala Asn Ile
945                 950                 955                 960

Gly Pro Arg Leu Thr Gln Val Ser Val Leu Gln Tyr Gly Ser Ile Thr
                965                 970                 975

Thr Ile Asp Val Pro Trp Asn Val Val Pro Glu Lys Ala His Leu Leu
            980                 985                 990

Ser Leu Val Asp Val Met Gln Arg  Glu Gly Gly Pro Ser  Gln Ile Gly
        995                 1000                1005

Asp Ala  Leu Gly Phe Ala Val  Arg Tyr Leu Thr Ser  Glu Met His
    1010                1015                1020

Gly Ala  Arg Pro Gly Ala Ser  Lys Ala Val Val Ile  Leu Val Thr
    1025                1030                1035
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Asp Val Ser Val Asp Ser Val Asp Ala Ala Ala Asp Ala Ala Arg
1040 1045 1050

Ser Asn Arg Val Thr Val Phe Pro Ile Gly Ile Gly Asp Arg Tyr
1055 1060 1065

Asp Ala Ala Gln Leu Arg Ile Leu Ala Gly Pro Ala Gly Asp Ser
1070 1075 1080

Asn Val Val Lys Leu Gln Arg Ile Glu Asp Leu Pro Thr Met Val
1085 1090 1095

Thr Leu Gly Asn Ser Phe Leu His Lys Leu Cys Ser Gly Phe Val
1100 1105 1110

Arg Ile Cys Met Asp Glu Asp Gly Asn Glu Lys Arg Pro Gly Asp
1115 1120 1125

Val Trp Thr Leu Pro Asp Gln Cys His Thr Val Thr Cys Gln Pro
1130 1135 1140

Asp Gly Gln Thr Leu Leu Lys Ser His Arg Val Asn Cys Asp Arg
1145 1150 1155

Gly Leu Arg Pro Ser Cys Pro Asn Ser Gln Ser Pro Val Lys Val
1160 1165 1170

Glu Glu Thr Cys Gly Cys Arg Trp Thr Cys Pro Cys Val Cys Thr
1175 1180 1185

Gly Ser Ser Thr Arg His Ile Val Thr Phe Asp Gly Gln Asn Phe
1190 1195 1200

Lys Leu Thr Gly Ser Cys Ser Tyr Val Leu Phe Gln Asn Lys Glu
1205 1210 1215

Gln Asp Leu Glu Val Ile Leu His Asn Gly Ala Cys Ser Pro Gly
1220 1225 1230

Ala Arg Gln Gly Cys Met Lys Ser Ile Glu Val Lys His Ser Ala
1235 1240 1245

Leu Ser Val Glu Leu His Ser Asp Met Glu Val Thr Val Asn Gly
1250 1255 1260

Arg Leu Val Ser Val Pro Tyr Val Gly Gly Asn Met Glu Val Asn
1265 1270 1275

Val Tyr Gly Ala Ile Met His Glu Val Arg Phe Asn His Leu Gly
1280 1285 1290

His Ile Phe Thr Phe Thr Pro Gln Asn Asn Glu Phe Gln Leu Gln
1295 1300 1305

Leu Ser Pro Lys Thr Phe Ala Ser Lys Thr Tyr Gly Leu Cys Gly
1310 1315 1320

Ile Cys Asp Glu Asn Gly Ala Asn Asp Phe Met Leu Arg Asp Gly
1325 1330 1335

Thr Val Thr Thr Asp Trp Lys Thr Leu Val Gln Glu Trp Thr Val
1340 1345 1350

Gln Arg Pro Gly Gln Thr Cys Gln Pro Ile Leu Glu Glu Gln Cys
1355 1360 1365

Leu Val Pro Asp Ser Ser His Cys Gln Val Leu Leu Leu Pro Leu
1370 1375 1380

Phe Ala Glu Cys His Lys Val Leu Ala Pro Ala Thr Phe Tyr Ala
1385 1390 1395

Ile Cys Gln Gln Asp Ser Cys His Gln Glu Gln Val Cys Glu Val
1400 1405 1410

Ile Ala Ser Tyr Ala His Leu Cys Arg Thr Asn Gly Val Cys Val
1415 1420 1425

Asp Trp Arg Thr Pro Asp Phe Cys Ala Met Ser Cys Pro Pro Ser
1430 1435 1440

Leu Val Tyr Asn His Cys Glu His Gly Cys Pro Arg His Cys Asp
1445 1450 1455

Gly Asn Val Ser Ser Cys Gly Asp His Pro Ser Glu Gly Cys Phe
1460 1465 1470

Cys Pro Pro Asp Lys Val Met Leu Glu Gly Ser Cys Val Pro Glu
1475 1480 1485

Glu Ala Cys Thr Gln Cys Ile Gly Glu Asp Gly Val Gln His Gln
1490 1495 1500

Phe Leu Glu Ala Trp Val Pro Asp His Gln Pro Cys Gln Ile Cys
1505 1510 1515

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Thr Cys  Leu Ser Gly Arg Lys  Val Asn Cys Thr Thr  Gln Pro Cys
    1520                1525                1530

Pro Thr  Ala Lys Ala Pro Thr  Cys Gly Leu Cys Glu  Val Ala Arg
    1535                1540                1545

Leu Arg  Gln Asn Ala Asp Gln  Cys Cys Pro Glu Tyr  Glu Cys Val
    1550                1555                1560

Cys Asp  Pro Val Ser Cys Asp  Leu Pro Pro Val Pro  His Cys Glu
    1565                1570                1575

Arg Gly  Leu Gln Pro Thr Leu  Thr Asn Pro Gly Glu  Cys Arg Pro
    1580                1585                1590

Asn Phe  Thr Cys Ala Cys Arg  Lys Glu Glu Cys Lys  Arg Val Ser
    1595                1600                1605

Pro Pro  Ser Cys Pro Pro His  Arg Leu Pro Thr Leu  Arg Lys Thr
    1610                1615                1620

Gln Cys  Cys Asp Glu Tyr Glu  Cys Ala Cys Asn Cys  Val Asn Ser
    1625                1630                1635

Thr Val  Ser Cys Pro Leu Gly  Tyr Leu Ala Ser Thr  Ala Thr Asn
    1640                1645                1650

Asp Cys  Gly Cys Thr Thr Thr  Thr Cys Leu Pro Asp  Lys Val Cys
    1655                1660                1665

Val His  Arg Ser Thr Ile Tyr  Pro Val Gly Gln Phe  Trp Glu Glu
    1670                1675                1680

Gly Cys  Asp Val Cys Thr Cys  Thr Asp Met Glu Asp  Ala Val Met
    1685                1690                1695

Gly Leu  Arg Val Ala Gln Cys  Ser Gln Lys Pro Cys  Glu Asp Ser
    1700                1705                1710

Cys Arg  Ser Gly Phe Thr Tyr  Val Leu His Glu Gly  Glu Cys Cys
    1715                1720                1725

Gly Arg  Cys Leu Pro Ser Ala  Cys Glu Val Val Thr  Gly Ser Pro
    1730                1735                1740

Arg Gly  Asp Ser Gln Ser Ser  Trp Lys Ser Val Gly  Ser Gln Trp
    1745                1750                1755
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Ala Ser  Pro Glu Asn Pro Cys  Leu Ile Asn Glu Cys  Val Arg Val
    1760                 1765                 1770

Lys Glu  Glu Val Phe Ile Gln  Gln Arg Asn Val Ser  Cys Pro Gln
    1775                 1780                 1785

Leu Glu  Val Pro Val Cys Pro  Ser Gly Phe Gln Leu  Ser Cys Lys
    1790                 1795                 1800

Thr Ser  Ala Cys Cys Pro Ser  Cys Arg Cys Glu Arg  Met Glu Ala
    1805                 1810                 1815

Cys Met  Leu Asn Gly Thr Val  Ile Gly Pro Gly Lys  Thr Val Met
    1820                 1825                 1830

Ile Asp  Val Cys Thr Thr Cys  Arg Cys Met Val Gln  Val Gly Val
    1835                 1840                 1845

Ile Ser  Gly Phe Lys Leu Glu  Cys Arg Lys Thr Thr  Cys Asn Pro
    1850                 1855                 1860

Cys Pro  Leu Gly Tyr Lys Glu  Glu Asn Asn Thr Gly  Glu Cys Cys
    1865                 1870                 1875

Gly Arg  Cys Leu Pro Thr Ala  Cys Thr Ile Gln Leu  Arg Gly Gly
    1880                 1885                 1890

Gln Ile  Met Thr Leu Lys Arg  Asp Glu Thr Leu Gln  Asp Gly Cys
    1895                 1900                 1905

Asp Thr  His Phe Cys Lys Val  Asn Glu Arg Gly Glu  Tyr Phe Trp
    1910                 1915                 1920

Glu Lys  Arg Val Thr Gly Cys  Pro Pro Phe Asp Glu  His Lys Cys
    1925                 1930                 1935

Leu Ala  Glu Gly Gly Lys Ile  Met Lys Ile Pro Gly  Thr Cys Cys
    1940                 1945                 1950

Asp Thr  Cys Glu Glu Pro Glu  Cys Asn Asp Ile Thr  Ala Arg Leu
    1955                 1960                 1965

Gln Tyr  Val Lys Val Gly Ser  Cys Lys Ser Glu Val  Glu Val Asp
    1970                 1975                 1980

Ile His  Tyr Cys Gln Gly Lys  Cys Ala Ser Lys Ala  Met Tyr Ser
    1985                 1990                 1995
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Ile Asp  Ile Asn Asp Val Gln  Asp Gln Cys Ser Cys  Cys Ser Pro
    2000                2005                2010

Thr Arg  Thr Glu Pro Met Gln  Val Ala Leu His Cys  Thr Asn Gly
    2015                2020                2025

Ser Val  Val Tyr His Glu Val  Leu Asn Ala Met Glu  Cys Lys Cys
    2030                2035                2040

Ser Pro  Arg Lys Cys Ser Lys
    2045                2050
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