METHOD OF PRODUCING RECOMBINANT HIGH MOLECULAR WEIGHT vWF IN CELL CULTURE

CROSS REFERENCES TO APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 61/362,635, filed July 8, 2010, the disclosure of which is hereby incorporated herein by reference in its entirety for all purposes.

BACKGROUND OF THE INVENTION

[0002] The recombinant expression of therapeutic proteins in cell culture (particularly large-scale cell cultures), including eukaryotic cell culture, and more specifically mammalian cell culture, requires the use of special culture media providing nutrient substances for efficient growth of cells. Cell culture media formulations are often supplemented with a range of additives, including fetal calf serum (FCS), animal derived proteins and/or protein hydrolysates of bovine origin as well as protein hydrolysates derived from plants or yeast. One challenge with such cultures is that the amount of protein and the total and specific activity of the protein produced are often variable across different cell cultures, even when the formulation for the cell culture media is not changed. This variability is especially apparent in the case of large-scale manufacturing processes utilizing cell culture volumes of 10 liters to over 20,000 liters. Cell culture media containing hydrolysates are particularly prone to variability from one cell culture to the next, leading to decreased production of total protein as well as decreased total and specific activity.

[0003] One potential reason for the variability seen across different cell cultures is that contaminants in additives such as hydrolysates vary from one batch to the next. In general, serum or serum-derived substances, such as, e.g., albumin, transferrin or insulin, may comprise unwanted agents that can contaminate the cell cultures and the biological products obtained thereof. Furthermore, human serum derived additives have to be tested for all known viruses,

including hepatitis viruses and HIV which can be transmitted via serum. Moreover, bovine serum and products derived thereof bear the risk of BSE contamination. In addition, all serum-derived products can be contaminated by unknown substances. When using serum or protein additives derived from human or animal sources in cell culture, there are numerous problems (e.g., the varying quality in composition of different batches and the risk of contamination with mycoplasma, viruses or BSE), particularly if the cells are used in the manufacture of drugs or vaccines for human administration. Therefore, many attempts have been made to provide efficient host systems and cultivation conditions, which do not require serum or other animal protein compounds.

[0004] Such serum-free media have been developed on the basis of protein extracts derived from plants or yeast. For example, soy hydrolysates are known to be useful for fermentation processes and can enhance the growth of many fastidious organisms, yeasts and fungi. WO 96/26266 describes that papaic digests of soy meal are a source of carbohydrate and nitrogen and many of the components can be used in tissue culture. Franek *et al.* (Biotechnology Progress (2000) 16, 688-692) describe growth and productivity promoting effects of defined soy and wheat hydrolysate peptide fractions.

[0005] WO 96/15231 discloses a serum-free medium composed of a synthetic minimal essential medium and a yeast extract for the propagation of vertebrate cells and a virus production process. A medium formulation composed of a basal cell culture medium comprising a rice peptide and an extract of yeast and an enzymatic digest thereof, and/or a plant lipid for growth of animal cells is disclosed in WO 98/15614. A medium comprising purified soy hydrolysate for the cultivation of recombinant cells is disclosed in WO 01/23527. WO 00/03000 discloses a medium that comprises a soy hydrolysate and a yeast extract, but also requires the presence of recombinant forms of animal proteins, such as growth factors.

[0006] EP-A-0 481 791 describes a biochemically defined culture medium for culturing engineered CHO cells, which is free from protein, lipid and carbohydrate isolated from an animal source, further comprising a recombinant insulin or insulin analogue, 1% to 0.025% w/v papain digested soy peptone and putrescine. WO 98/08934 describes a serum-free eukaryotic cell culture comprising hydrolyzed soy peptides (1-1000 mg/L), 0.01 to 1 mg/L putrescine and a variety of animal-derived components, including albumin, fetuin, various hormones and other

proteins. In this context, it should be noted that putrescine is also known to be comprised in standard media like DMEM/Ham's F12 in a concentration of 0.08 mg/L.

[0007] The plant and/or yeast hydrolysates, however, are undefined mixtures of oligopeptides and other unknown components and contaminants. Moreover, the quality of commercially available lots of hydrolysates varies extremely. As a result, there are large variations in the production of recombinant proteins or viral products (a variation of up to a factor of three) as a function of the lots of hydrolysates used ("lot-to-lot variation"). This drawback affects the proliferation of the cells as well as the protein expression of each cell. US 2007/0212770 describes various animal protein-free and oligopeptide-free, chemically defined culture mediums that are useful for the large-scale production of recombinant protein biopharmaceuticals.

[0008] Hemostasis involves the interaction of various hemostatic reaction routes finally leading to thrombus formation. Thrombi are deposits of blood components on the surface of the vascular wall that mainly consist of aggregated blood platelets and insoluble cross-linked fibrin. Fibrin formation is the result of the restricted proteolysis of fibrinogen by thrombin, a coagulation enzyme. Thrombin is the end product of the coagulation cascade, a succession of zymogen activations occurring on the surfaces of activated blood platelets and leucocytes, and a variety of vascular cells (for a survey, cf. K. G. Mann et al., Blood, 1990, Vol. 76, pp. 1-16).

[0009] An important function in the coagulation cascade resides in the activation of Factor X by the complex of activated Factor IX (Factor IXa) and activated Factor VIII (Factor VIIIa). A deficiency or a dysfunction of the components of this complex is associated with the blood disease known as hemophilia (J. E. Sadler & E. W. Davie: Hemophilia A, Hemophilia B, and von Willebrand's Disease, in G. Stamatoyannopoulos et al., (Eds.): The molecular basis of blood diseases. W.B. Saunders Co., Philadelphia, 1987, pp. 576-602). Hemophilia A is related to a deficiency of Factor VIII activity, whereas Hemophilia B is related to a Factor IX deficiency. Current treatment consists of a replacement therapy using pharmaceutical preparations comprised of the normal coagulation factor. Of these thrombopathies, Hemophilia A occurs more frequently, affecting approximately one out of 10,000 men. Replacement therapy in Hemophilia A patients involves the repeated administration of preparations containing normal Factor VIII by intravenous infusion. The interval between the infusions is a function of the degradation of the Factor VIII activity in blood circulation. The half-life of the Factor VIII

activity after an infusion differs from one individual to another, ranging from 10 to 30 hours. Thus, a prophylactic therapy requires an infusion every two to three days. This constitutes a heavy load on the life of hemophilic patients, in particular, if the venous access has become difficult due to local citratization following frequent needle punctures for intravenous infusions.

[0010] It would be particularly advantageous if the frequency of infusions could be lowered by using Factor VIII having extended half-lives. It is well known in the art that the half-life of the non-activated Factor VIII heterodimer strongly depends on the presence of von Willebrand Factor, which exhibits a strong affinity to Factor VIII (yet not to Factor VIIIa) and serves as a carrier protein (J. E. Sadler and E. W. Davie: Hemophilia A, Hemophilia B and von Willebrand's disease, in G. Stamatoynnopoulos et al. (Eds.): The molecular basis of blood diseases. W.B. Saunders Co., Philadelphia, 1987, pp. 576-602). It is known that patients suffering from von Willebrand's disease type 3, who do not have a detectable von Willebrand Factor in their blood circulation, also suffer from a secondary Factor VIII deficiency. In addition, the half-life of intravenously administered Factor VIII in those patients is 2 to 4 hours, which is considerably shorter than the 10 to 30 hours observed in Hemophilia A patients. From these findings results that Factor VIII tends to a rapid clearance from the blood circulation and that this process is to some extent inhibited by complexation with its natural carrier, von Willebrand Factor.

[0011] Von Willebrand factor (vWF) is a glycoprotein circulating in plasma as a series of multimers typically ranging in size from about 500 to 20,000 kD (or 2 to 40 dimers of vWF). Dimers and multimeric forms of vWF are composed of 250 kD polypeptide subunits linked together by disulfide bonds. vWF mediates the initial platelet adhesion to the sub-endothelium of the damaged vessel wall; only the larger multimers also exhibiting hemostatic activity. Multimerized VWF binds to the platelet surface glycoprotein Gp1bα, through an interaction in the A1 domain of VWF, in order to facilitate platelet adhesion. It is assumed that endothelial cells secret large polymeric forms of vWF and that those forms of vWF which have a low molecular weight (low molecular weight vWF) have arisen from proteolytic cleavage. The multimers having large molecular masses are stored in the Weibel-Palade bodies of the endothelial cells and liberated upon stimulation.

[0012] Reduction of FVIII binding activity, due to either reduced vWF protein levels or lowered FVIII binding affinity, results in one of three types of von Willebrand's Disease. In addition to,

or alternatively, certain types of von Willebrand's disease are characterized by an increase or decrease in the level of Gp1bα-mediated platelet association, namely in Types 2A, 2B, and 2M (summarized in Castaman *et al.*, Disorders of Hemostasis 88(1):94-108 (2003)). As such, the modulation of vWF interactions with both FVIII and Gp1bα is a viable strategy for the treatment of both Haemophilia and von Willebrand's Disease.

[0013] Given the biological importance of vWF, there is a constant need in the art to improve ways for producing vWF for therapeutic applications. It is well known that vWF can be isolated from endogenous sources, such as human blood plasma. The isolated vWF is advantageous in that it has a high specific activity for carrying out its biological function and can, therefore, be used effectively as a therapeutic protein for treating related diseases, such as von Willebrand's disease. Typically, plasma vWF has a specific Ristocetin activity of about 100 mU/μg, but isolation from human blood plasma has disadvantages because, for example, the plasma can contain a variety of viruses, such as HIV and/or hepatitis viruses, which can be transferred to the patient. Furthermore, plasma is a limited resource and, thus, shortages of plasma can be problematic in providing enough vWF for treatment. As such, recombinant methods for producing vWF are advantageous in addressing some of the problems associated with relying on plasma as a source for vWF. For recombinant production, the full length of cDNA of vWF was cloned; the propolypeptide corresponds to amino acid residues 23 to 764 of the full length prepro-vWF (Eikenboom et al (1995) Haemophilia 1, 77 90).

[0014] Unfortunately, vWF is a molecule with complex post-translational modifications. Also, the multimerization of the vWF dimers to large and ultralarge multimers in the Golgi apparatus is a challenge for expression in mammalian cells. For example, high molecular weight vWF expressed in cell culture of, e.g., human (primary) endothelial cells depends on the specific storage of ultralarge vWF molecules in Weibel-Palade bodies. Such cell cultures are not suitable for the production of therapeutic proteins. Other cell culture methods have been reported, and it is known that cell culture conditions can affect the production of vWF in a variety of ways. For instance, high concentrations of ammonium (NH_4^+) have been shown to disturb posttranslational modifications. Mayadas *et al.* (J. Biol. Chem., 264(23):13497-13503, 1989) demonstrated that levels of 25 mM ammonium resulted in reduced vWF multimerization in endothethial cells, which also negatively affects the specific Ristocetin activity of recombinant vWF. Reduction of

multimerization is generally associated in reduction of activity, particularly specific Ristocetin activity, of recombinant vWF.

[0015] It still remains difficult to predict which parameters can positively or negatively affect production of a particular protein, especially complex glycoproteins like Factor VIII and vWF. For example, certain components of a cell culture medium have been shown to affect production of Factor VIII. As disclosed in US Patent No. 5,804,420, the addition of polyol, copper, and other trace metals can positively affect production yield of Factor VIII. As also described in WO 2009/086309, cell culture processes using copper in have been shown to improve production of Factor VIII. Expression of vWF in recombinant CHO cells has also been reported by Mignot *et al.* (1989). However, none of these examples provide information regarding the specific activity of vWF or its multimeric distribution.

[0016] The ADAMTS (a disintegrin and metalloproteinase with thrombospondin type I motifs) proteins are a family of metalloproteinases containing a number of conserved domains, including a zinc-dependant catalytic domain, a cystein-rich domain, a disintegrin-like domain, and at least one, and in most cases multiple, thrombospondin type I repeats (for review, *see* Nicholson *et al.*, BMC Evol Biol. 2005 Feb 4;5(1):11). These proteins, which are evolutionarily related to the ADAM and MMP families of metalloproteinases (Jones GC, Curr Pharm Biotechnol. 2006 Feb;7(1):25-31), are secreted enzymes that have been linked to a number of diseases and conditions including thrombotic thrombocytopenic purpura (TTP) (Moake JL, Semin Hematol. 2004 Jan;41(1):4-14), connective tissue disorders, cancers, inflammation (Nicholson *et al.*), and severe plasmodium falciparum malaria (Larkin *et al.*, PLoS Pathog. 2009 Mar;5(3):e1000349). Because of these associations, the ADAMTS enzymes have been recognized as potential therapeutic targets for a number of pathologies (Jones GC, Curr Pharm Biotechnol. 2006 Feb;7(1):25-31). Accordingly, methods of producing large yields of ADAMTS proteins having high specific activities, which are free of contaminants such as viruses, BSE, and pathogens like *Mycoplasma* bacteria, are needed.

[0017] One ADAMTS family member, ADAMTS13, cleaves von Willebrand factor (vWF) between residues Tyr 1605 and Met 1606, a function responsible for the degradation of large vWF multimers *in vivo*. Loss of ADAMTS13 activity has been linked to a number of conditions, such as TTP (Moake JL, Semin Hematol. 2004 Jan;41(1):4-14), acute and chronic inflammation

(Chauhan *et al.*, J Exp Med. 2008 Sep 1;205(9):2065-74), and most recently, severe plasmodium falciparum malaria (Larkin *et al.*, PLoS Pathog. 2009 Mar;5(3):e1000349).

[0018] The ADAMTS13 protease is a 190 kDa glycosylated protein produced predominantly by the liver (Levy *et al.*, Nature. 2001; 413:488-494; Fujikawa *et al.*, Blood. 2001; 98:1662-1666; Zheng *et al.*, J Biol Chem. 2001; 276:41059-41063; Soejima *et al.*, J Biochem (Tokyo). 2001; 130:475-480; and Gerritsen *et al.*, Blood. 2001; 98:1654-1661). Much like the higher order rVWF multimers, recombinant expression of the large ADAMTS13 in mammalian cell culture presents many challenges.

[0019] Therefore, there is a need to provide cell culture conditions, particularly large-scale manufacturing culture conditions, that provide consistent total protein yield and/or consistent total and specific activity of the proteins produced between different cell cultures. Consistency among cultures in large-scale manufacturing processes is of importance in the manufacture of therapeutic proteins. There is also a need for cell culture conditions for large-scale production of rVWF with a multimeric distribution and specific Ristocetin activity comparable or higher than VWF as it is present in normal human plasma. Similarly, as ADAMTS proteins have been implicated in a number of diseases and conditions, there is a need in the art for methods of large scale production of recombinant ADAMTS proteins having high specific activities, which are suitable for pharmaceutical formulation and administration. The present invention satisfies these and other needs in the art for the production of recombinant Von Willebrand Factor and recombinant ADAMTS13.

BRIEF SUMMARY OF INVENTION

[0020] In certain aspects, the present invention is based on the surprising finding that supplementation of cell culture media used to express recombinant Von Willebrand Factor (rVWF) and recombinant ADAMTS13 (rA13) results in significantly improved protein expression and enzymatic activity.

[0021] In a first aspect, the present invention provides a method for producing a recombinant Von Willebrand Factor (rVWF) composition, the method comprising the steps of: (a) providing a basal cell culture media; (b) supplementing the basal cell culture media with copper to provide a

final copper concentration of at least 2.4 µg/L; (c) providing one or more cells comprising a nucleic acid encoding a rVWF protein; (d) culturing the one or more cells in the copper supplemented cell culture media such that rVWF is expressed and excreted from the cells into a culture supernatant; and (e) recovering at least a portion of the culture supernatant, wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 30 mU/µg rVWF.

[0022] In one embodiment of the methods provided above, the method further comprises a step of supplementing the basal cell culture media with a hydrolysate prior to culturing the one or more cells.

[0023] In one embodiment of the methods provided above, the hydrolysate is a plant hydrolysate. In a specific embodiment, the hydrolysate is a soy hydrolysate.

[0024] In one embodiment of the methods provided above, the basal cell culture media is an animal protein free culture media.

[0025] In one embodiment of the methods provided above, the basal cell culture media is a protein free culture media.

[0026] In one embodiment of the methods provided above, the basal cell culture media is a chemically defined culture media.

[0027] In one embodiment of the methods provided above, the final copper concentration of the copper supplemented basal cell culture media is at least 4 µg/L copper.

[0028] In one embodiment of the methods provided above, the final copper concentration of the copper supplemented basal cell culture media is between 2.4 µg/L and 20 µg/L copper.

[0029] In one embodiment of the methods provided above, the copper supplementing the basal cell culture media is provided as a copper salt, a copper chelate, or a combination thereof.

[0030] In one embodiment of the methods provided above, the copper salt is selected from the group consisting of copper sulfate, copper acetate, copper carbonate, copper chloride, copper hydroxide, copper nitrate, and copper oxide.

[0031] In one embodiment of the methods provided above, the one or more cells are mammalian cells. In a specific embodiment, the mammalian cells are CHO cells.

[0032] In one embodiment of the methods provided above, culturing the one or more cells comprises batch cultivation of the cells.

[0033] In one embodiment of the methods provided above, culturing the one or more cells comprises continuous cultivation of the cells. In a specific embodiment, the continuous cultivation of cells is performed in chemostatic mode. In another specific embodiment, the continuous cultivation of cells is performed in perfusion mode.

[0034] In one embodiment of the methods provided above, the one or more cells is cultured in at least 100 L of the supplemented basal cell culture media.

[0035] In one embodiment of the methods provided above, the cell density is maintained at less than $2.5x10^6$ cells per mL during the step of culturing the one or more cells.

[0036] In one embodiment of the methods provided above, the cell density is maintained at less than $2.0x10^6$ cells per mL during the step of culturing the one or more cells.

[0037] In one embodiment of the methods provided above, the cell density is maintained at less than $1.5x10^6$ cells per mL during the step of culturing the one or more cells.

[0038] In one embodiment of the methods provided above, the step of recovering at least a portion of the culture supernatant comprises filtration or centrifugation to remove cells from the portion of culture supernatant.

[0039] In one embodiment of the methods provided above, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 40 mU/μg rVWF. In a specific embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more specific embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 60 mU/μg rVWF. In a more specific embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF. In a yet more specific embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 80 mU/μg rVWF.

[0040] In one embodiment of the methods provided above, at least 10% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers. In a specific embodiment, at least 15% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 20% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 25% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 30% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers.

[0041] In one embodiment of the methods provided above, the supernatant contains high molecular weight VWF multimers of 14 to 22 dimers.

[0042] In one embodiment of the methods provided above, the $NH4^+$ content of the culture supernatant is maintained at a concentration below 10 mM.

[0043] In one embodiment of the methods provided above, the $NH4^+$ content of the culture supernatant is maintained at a concentration below 4 mM.

[0044] In one embodiment of the methods provided above, rVWF is co-expressed with recombinant Factor VIII (rFVIII). In a specific embodiment, the method further comprises a step of purifying rVWF away from at least 50% of the rFVIII present in the recovered supernatant. In one embodiment, the ratio of rVWF to rFVIII after the purification step is at least 10:1.

[0045] In one embodiment of the methods provided above, the method further comprises a rVWF enrichment step.

[0046] In a second aspect, the present invention provides a recombinant Von Willebrand Factor (rVWF) composition prepared by a method provided herein.

[0047] In one embodiment of the compositions provided above, the composition further comprises recombinant Factor VIII (rFVIII). In a specific embodiment, the ratio of rVWF to rFVIII is at least 10:1.

[0048] In one embodiment of the compositions provided above, the composition is formulated for pharmaceutical administration. In a specific embodiment, the composition is formulated for intravenous administration.

[0049] In a third aspect, the present invention provides a cell culture supernatant comprising recombinant Von Willebrand Factor (rVWF), wherein the supernatant is prepared by a method provided herein.

[0050] In a fourth aspect, the present invention provides a cell culture supernatant comprising recombinant Von Willebrand Factor (rVWF), wherein at least 10% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers. In a specific embodiment, at least 15% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 20% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 25% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 30% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers. In yet another specific embodiment of the supernatants provided above, the supernatant is prepared according to a method provided herein.

[0051] In a fifth aspect, the present invention provides a cell culture supernatant comprising recombinant Von Willebrand Factor (rVWF), wherein the supernatant contains at least 0.4 IU ristocetin cofactor activity per mL. In a specific embodiment, the supernatant contains at least 0.5 IU ristocetin cofactor activity per mL. In another specific embodiment, the supernatant contains at least 0.6 IU ristocetin cofactor activity per mL. In another specific embodiment, the supernatant contains at least 0.7 IU ristocetin cofactor activity per mL. In yet another specific embodiment of the supernatants provided above, the supernatant is prepared according to a method provided herein.

[0052] In a sixth aspect, the present invention provides a method for producing a recombinant ADAMTS13 (rA13) composition, the method comprising the steps of: (a) providing a basal cell culture media; (b) supplementing the basal cell culture media with copper to provide a final copper concentration of at least 1.0 μg/L; (c) providing one or more cells comprising a nucleic acid encoding a rA13 protein; (d) culturing the one or more cells in the copper supplemented cell culture media such that rA13 is expressed and excreted from the cells into a culture supernatant; and (e) recovering at least a portion of the culture supernatant, wherein at least 1500 Units

FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant.

[0053] In one embodiment of the methods provided above, the basal cell culture media is an animal protein free culture media.

[0054] In one embodiment of the methods provided above, the basal cell culture media is a protein free culture media.

[0055] In one embodiment of the methods provided above, the basal cell culture media is a chemically defined culture media.

[0056] In one embodiment of the methods provided above, the final copper concentration of the supplemented basal cell culture media is at least 1 μg/L copper.

[0057] In one embodiment of the methods provided above, the final copper concentration of the supplemented basal cell culture media is at least 2 μg/L copper.

[0058] In one embodiment of the methods provided above, the final copper concentration of the supplemented basal cell culture media is at least 4 μg/L copper.

[0059] In one embodiment of the methods provided above, the final copper concentration of the supplemented basal cell culture media is between 1 μg/L and 6 μg/L copper.

[0060] In one embodiment of the methods provided above, the final copper concentration of the supplemented basal cell culture media is between 2 μg/L and 4 μg/L copper.

[0061] In one embodiment of the methods provided above, copper supplementing the basal cell culture media is provided as a copper salt, a copper chelate, or a combination thereof. In a specific embodiment, the copper salt is selected from the group consisting of copper sulfate, copper acetate, copper carbonate, copper chloride, copper hydroxide, copper nitrate, and copper oxide.

[0062] In one embodiment of the methods provided above, the one or more cells are mammalian cells. In a specific embodiment, the mammalian cells are CHO cells.

[0063] In one embodiment of the methods provided above, culturing the one or more cells comprises batch cultivation of the cells.

[0064] In one embodiment of the methods provided above, culturing the one or more cells comprises continuous cultivation of the cells. In a specific embodiment, the continuous cultivation of cells is performed in chemostatic mode. In another specific embodiment, the continuous cultivation of cells is performed in perfusion mode.

[0065] In one embodiment of the methods provided above, the one or more cells is cultured in at least 100 L of the supplemented basal cell culture media.

[0066] In one embodiment of the methods provided above, the cell density is maintained at less than $4.0x10^6$ cells per mL during the step of culturing the one or more cells.

[0067] In one embodiment of the methods provided above, the cell density is maintained at less than $3.5x10^6$ cells per mL during the step of culturing the one or more cells.

[0068] In one embodiment of the methods provided above, the cell density is maintained at less than $3.0x10^6$ cells per mL during the step of culturing the one or more cells.

[0069] In one embodiment of the methods provided above, the cell density is maintained at less than $2.5x10^6$ cells per mL during the step of culturing the one or more cells.

[0070] In one embodiment of the methods provided above, the cell density is maintained at less than $2.0x10^6$ cells per mL during the step of culturing the one or more cells.

[0071] In one embodiment of the methods provided above, the cell density is maintained at less than $1.5x10^6$ cells per mL during the step of culturing the one or more cells.

[0072] In one embodiment of the methods provided above, the step of recovering at least a portion of the culture supernatant comprises filtration or centrifugation to remove cells from the portion of culture supernatant.

[0073] In one embodiment of the methods provided above, at least 2000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant.

[0074] In one embodiment of the methods provided above, at least 2500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant.

[0075] In one embodiment of the methods provided above, the recovered supernatant has a rA13 specific FRETS-VWF73 activity of at least 800 mU/μg.

[0076] In a preferred embodiment of the methods provided above, the recovered supernatant has a rA13 specific FRETS-VWF73 activity of at least 1200 mU/μg.

[0077] In a more preferred embodiment of the methods provided above, the recovered supernatant has a rA13 specific FRETS-VWF73 activity of at least 1600 mU/μg.

[0078] In one embodiment of the methods provided above, the $NH4^+$ content of the culture supernatant is maintained at a concentration below 10 mM.

[0079] In one embodiment of the methods provided above, the $NH4^+$ content of the culture supernatant is maintained at a concentration below 5 mM.

[0080] In one embodiment of the methods provided above, the $NH4^+$ content of the culture supernatant is maintained at a concentration below 4 mM.

[0081] In one embodiment of the methods provided above, the method further comprises a rA13 enrichment step.

[0082] In a seventh aspect, the present invention provides a cell culture supernatant comprising recombinant ADAMTS13 (rA13), wherein the supernatant is prepared by a method provided herein.

[0083] In an eighth aspect, the present invention provides a cell culture supernatant comprising recombinant Von Willebrand Factor (rVWF), wherein the supernatant contains at least 5 U FRETS-VWF73 activity per mL. In a specific embodiment, the supernatant contains at least 6 U FRETS-VWF73 activity per mL. In another specific embodiment, the supernatant contains at least 7 U FRETS-VWF73 activity per mL. In another specific embodiment, the supernatant contains at least 8 U FRETS-VWF73 activity per mL. In another specific embodiment, the supernatant contains at least 9 U FRETS-VWF73 activity per mL. In another specific embodiment, the supernatant contains at least 10 U FRETS-VWF73 activity per mL. In yet another specific embodiment of the supernatants provided above, the supernatant is prepared according to a method provided herein.

[0084] In a ninth aspect, the present invention provides a cell culture supernatant comprising recombinant Von Willebrand Factor (rVWF), wherein the supernatant contains at least 2 μg rA13 per mL. In a specific embodiment, the supernatant contains at least 3 μg rA13 per mL. In another specific embodiment, the supernatant contains at least 4 μg rA13 per mL. In another specific embodiment, the supernatant contains at least 5 μg rA13 per mL. In another specific embodiment, the supernatant contains at least 6 μg rA13 per mL. In yet another specific embodiment of the supernatants provided above, the supernatant is prepared according to a method provided herein.

[0085] In a tenth aspect, the present invention provides a recombinant ADAMTS13 (rA13) composition prepared by a method according to any one of the methods described above.

[0086] In one embodiment of the compositions provided above, the composition is formulated for pharmaceutical administration. In a specific embodiment, the composition is formulated for intravenous administration.

BRIEF DESCRIPTION OF DRAWINGS

[0087] Figure 1. (**1A**) Low resolution (1 %) agarose gel electrophoresis of rVWF expressed in mammalian cell culture in the presence of low (1.0 μg/L) and high (4.3 μg/L) copper concentration, as described in Example 2. Note that culture Day 3 is equivalent to batch day 1 of Table **7** and Table **8**. (**1B**) The relative amounts of VWF multimers having 1 to 10 dimers (band number 1) and more than 10 dimers (band number 2), as indicated by the bands defined in Figure 1A, was quantitated by densitometric analysis.

[0088] Figure 2. (**2A**) Interval plot of the average rVWF specific activity present in rVWF cell culture supernatants grown at high and low cell densities in the presence of high or low levels of copper. (**2B**) Interval plot of the average NH_4^+ concentration found in rVWF cell culture supernatants grown at high and low cell densities in the presence of high or low levels of copper.

[0089] Figure 3. Supernatants from cell cultures expressing recombinant ADAMTS13 in the presence of increasing levels of copper were investigated by SDS-PAGE analysis. rA13 was visualized after SDS-PAGE by (**3A**) silver staining and (**3B**) anti-A13 western blotting.

[0090] Figure 4. Plot of volumetric productivity (P Frets) data versus copper concentration showing an extrapolation (solid line) of optimal copper concentration effect on rA13 productivity.

[0091] Figure 5A-K. Bar graphs from continuous suspension (chemostat) cell cultures expressing rA13 over a culture time of 8 weeks comparing the effects of basal levels of copper (0.66 µg/L) to that of cultures supplemented to a final concentration of 2 µg/L copper. Each bar represents the mean data of one week of chemostatic culturing. The legend refers to the particular week represented in the data.

[0092] Figure 6. (**6A**) Low resolution (1 %) agarose gel electrophoresis of rVWF expressed in mammalian cell culture in the presence of low (1.0 µg/L) and high (4.3 µg/L) copper concentration under high and low cell densities as described in Example 3. Note that culture Day 8 and 17 ("CST8" and "CST17") is equivalent to Day 8 and Day 17 of Table 10 to Table 13. (**6B**) The relative amounts of VWF multimers having 1 to 10 dimers and more than 10 dimers, as indicated by the bands defined in Figure 6A, was quantitated by densitometric analysis.

DETAILED DESCRIPTION OF INVENTION

I. Introduction

[0093] Recombinant vWF (rVWF) and recombinant ADAMTS13 (rA13) can be produced by expression in large-scale mammalian cell cultures. However, the activity of these proteins when produced using standard cell culture conditions often varies across cell cultures, even when the general formulations of the media are not changed, and the specific activities of the recombinant proteins are often not equal to that of vWF and rA13 derived from blood plasma. In addition, rVWF expressed in mammalian cell cultures tends to produce protein compositions with low (under 10%) percentages of higher order multimers (higher order multimers include molecules containing more than 10 VWF dimers). These drawbacks in standard production methods of rVWF and rA13 are particularly problematic when developing cultures for large scale production (i.e., from 10 to over 20,000 liter cultures).

[0094] One potential source of the variability often seen in different cell culture batches is the presence of contaminants in components of the cell culture media. These contaminants may be present in different amounts in different batches, leading to variable results in the production of rVWF and rA13. After investigating the different contaminants found in various cell culture media additives, the present inventors found that the presence of hydrolysates leads to variation in copper concentrations in the cell media. Further investigation provided the surprising result that supplementing copper concentrations in cell media to produce a total copper concentration of at least about 1 μg/L to about 20 μg/L consistently increased the total and specific activity of rVWF and rA13 and/or could also lead to increased total protein yield. Thus, the present invention provides methods and compositions for high yield production of rVWF and rA13 proteins with high specific activity.

[0095] In one aspect, the present invention provides cell culture methods and compositions for producing large quantities of rVWF and rA13 with activity that is comparable to or higher than that seen with plasma derived vWF (pdVWF) or plasma derived ADAMTS13 (pdA13). In further aspects, the rVWF and rA13 proteins produced in accordance with the present invention show consistently higher activity than proteins produced using standard cell culture methods in media that has not been supplemented with copper or other supplements described in further detail herein. Advantageously, in certain embodiments of the methods and compositions provided herein, the rVWF and rA13 proteins produced in accordance with the present invention show consistently higher specific activity (*i.e.*, U/mg protein) than proteins produced using standard cell culture methods in media that has not been supplemented with copper or other supplements described in further detail herein. Likewise, the methods provided herein for the production of rVWF and rA13 provide higher yields of activity per volume culture (*i.e.*, U/L/D), as compared to standard cell culture methods utilizing media that has not been supplemented with copper or other supplements described in further detail herein.

[0096] In a further aspect, the present invention provides cell culture methods in which a basal cell culture medium is supplemented with copper to result in a total concentration of at least about 1 μg/L. In other embodiments, the basal cell culture medium is supplemented with copper to result in a total concentration of at least about 2 μg/L. In yet other embodiments, the basal cell culture medium is supplemented with copper to result in a total concentration of at least about 1

μg/L to about 20 μg/L. In some embodiments, the total concentration of copper is from about 1.5 - 4.5 μg/L. In certain embodiments, the cell culture medium is supplemented to result in about 1, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8, 3, 3.2, 3.4, 3.6, 3.8, 4, 4.2, 4.4, 4.6, 4.8, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 μg/L copper, or more. Basal cell culture media generally have a trace copper concentration of under 1 μg/L.

[0097] In some embodiments, the present invention provides cell culture methods in which a basal cell culture medium is supplemented with from about 1.0 to about 20 μg/L copper for production of rVWF. In further embodiments, the basal cell culture medium is supplemented with from about 1.5-15, 2.0-10, 2.5-8, 3.0-6, 4.0 -5.0 μg/L copper for production of rVWF. In still further embodiments, the basal cell culture medium may, in addition to the supplemented copper, also include one or more hydrolysates.

[0098] In other embodiments, the present invention provides cell culture methods in which a basal cell culture medium is supplemented with from about 1.5 to about 4 μg/L copper for production of rA13. In further embodiments, the basal cell culture medium is supplemented with from about 1.6 - 3.8, 1.7 - 3.6, 1.8 - 3.4, 1.9 - 3.2, 2.0 - 3.0, 2.1 - 2.8, 2.2 - 2.6, 2.3 - 2.4 μg/L copper for production of rA13. In still further embodiments, the basal cell culture medium may, in addition to the supplemented copper, also include one or more hydrolysates. In yet further embodiments, the basal cell culture medium includes, in addition to copper and/or one or more hydrolysates, about 1.0 to about 30 μM zinc. In still further embodiments, the basal cell culture medium further includes, in addition to copper and/or one or more hydrolysates and/or zinc, between about 0.5 to about 5.0 mM calcium.

[0099] In a still further aspect and in accordance with any of the above, the present invention provides cell culture methods in which the ammonium levels of the cell culture solution are low (under 10 mM). In certain embodiments, cell culture methods of the present invention utilize cell culture media having over 1, 2, 3, 4, or 5 μg/L copper in combination with low levels of ammonium.

[0100] One of the advantages of the methods and compositions of the present invention is that they are amenable to large scale cell culture production. These large scale cell cultures are at least 10 L, 50 L, 100 L, 150 L, 200 L, 250 L, 500 L, 750 L, 1,000 L, 1,500 L, 2,000 L, 5,000 L, 10,000 L, or 20,000 liter cultures.

[0101] In certain aspects, the methods of the invention do not necessarily result in a higher amount of recombinant protein overall, but the recombinant protein (either rVWF or rA13) that is produced shows higher total and specific activity than is seen in proteins produced using standard cell cultures, particularly as compared to proteins produced in cell cultures in which the cell culture medium has not been supplemented with additional copper. In further aspects, the rVWF and rA13 proteins produced in cells cultured in copper supplemented media show consistently increased activity per liter of cell culture as compared to cells cultured in basal cell culture media that has not been supplemented with copper. In still further aspects, the copper supplemented media of the present invention result in increased protein yield, increased number of cells in the culture, and/or increased total activity per liter of culture as compared to media that has not been supplemented with copper.

[0102] Still further advantages of methods and compositions of the invention is that they result a population of proteins containing a high percentage (over 10%) of highly multimerized rVWF.

[0103] Although much of the discussion herein regarding ADAMTS proteins is in terms of ADAMTS13 (A13), it will be appreciated that because all ADAMTS proteins share a common core domain architecture and common structure-function relationships, the methods and compositions described herein are applicable for production of any ADAMTS proteins, not only rA13.

II. Definitions

[0104] As used herein, "recombinant vWF" includes vWF obtained via recombinant DNA technology. In certain embodiments, vWF proteins of the invention can comprise a construct, for example, prepared as in WO 1986/06096 published on Oct. 23, 1986 and U.S. patent application Ser. No. 07/559,509, filed on Jul. 23, 1990, in the name of Ginsburg *et al.*, which is incorporated herein by reference with respect to the methods of producing recombinant vWF. The vWF in the present invention can include all potential forms, including the monomeric and multimeric forms. It should also be understood that the present invention encompasses different forms of vWF to be used in combination. For example, the vWF of the present invention may include different multimers, different derivatives and both biologically active derivatives and derivatives not biologically active.

[0105] The term "recombinant" when used with reference, *e.g.*, to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

[0106] In the context of the present invention, the recombinant vWF embraces any member of the vWF family from, for example, a mammal such as a primate, human, monkey, rabbit, pig, rodent, mouse, rat, hamster, gerbil, canine, feline, and biologically active derivatives thereof. In a preferred embodiment, the recombinant VWF is human VWF. Mutant and variant vWF proteins having activity are also embraced, as are functional fragments and fusion proteins of the vWF proteins. Furthermore, the vWF of the invention may further comprise tags that facilitate purification, detection, or both. The vWF described herein may further be modified with a therapeutic moiety or a moiety suitable imaging *in vitro* or *in vivo*.

[0107] The terms "highly multimeric vWF," "high molecular weight vWF," and "HMW VWF" may be used interchangeably and refer to covalently attached vWF multimers containing more than 10 VWF dimers. In certain embodiments, HMW VWF contains at least 11 VWF dimers, or at least 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or more VWF dimers.

[0108] As used herein, an "ADAMTS protein" refers to a polypeptide of the disintegrin and metalloproteinase with thrombospondin type I motifs family of metalloproteinases. Members of this family include the human proteins ADAMTS1 (NM_006988), ADAMTS2 (NM_014244; NM_021599), ADAMTS3 (NM_014243), ADAMTS4 (NM_005099), ADAMTS5 (NM_007038), ADAMTS6 (NM_014273), ADAMTS7 (NM_0142727), ADAMTS8 (NM_007037), ADAMTS9 (NM_182920; NM_182921; NM_020249), ADAMTS10 (NM_030957), ADAMTS12 (NM_030955), ADAMTS13 (NM_139025; NM_139026; NM_139027; NM_139028), ADAMTS14 (NM_139155; NM_080722), ADAMTS15 (NM_139055), ADAMTS16 (NM_139056), ADAMTS17 (NM_139057), ADAMTS18 (NM_199355; NM_139054), ADAMTS19 (NM_133638), and ADAMTS20 (NM_025003, NM_175851). ADAMTS proteins include both full-length proteins and partial polypeptides that display at least partial biological activity, for example, at least 10%, 20%, 30%, 40%, 50%, 60%,

70%, 80%, 90%, or more of the activity demonstrated by the full-length protein, in particular the protease activity demonstrated by the full length protein. In certain instances, an ADAMTS protein will be post-translationally modified either *in vivo* or *in vitro*, for example, by enzymatic or chemical means. It is understood that the ADAMTS proteins of the present invention include alternatively spliced isoforms, conservatively modified proteins, substantially identical proteins, homologues, and the like.

[0109] In the context of the present invention, an ADAMTS protein embraces any member of the ADAMTS family from, for example, a mammal such as a primate, human, monkey, rabbit, pig, rodent, mouse, rat, hamster, gerbil, canine, feline, and biologically active derivatives thereof. Mutant and variant ADAMTS proteins having activity are also embraced, as are functional fragments and fusion proteins of the ADAMTS proteins. Furthermore, the ADAMTS proteins of the invention may further comprise tags that facilitate purification, detection, or both. The ADAMTS proteins described herein may further be modified with a therapeutic moiety or a moiety suitable imaging *in vitro* or *in vivo*.

[0110] As used herein, an "ADAMTS13 protein" refers to any protein or polypeptide with ADAMTS13 activity, particularly the ability to cleave the peptide bond between residues Tyr-842 and Met-843 of VWF. In an exemplary embodiment, an ADAMTS13 protein refers to a polypeptide comprising an amino acid sequence that is highly similar to that of NP_620594 (ADAMTS13 isoform 1, preproprotein) or amino acids 75 to 1427 of NP_620594 (ADAMTS13 isoform 1, mature polypeptide). In another embodiment, an ADAMTS13 protein refers to a polypeptide comprising an amino acid sequence that is highly similar to that of NP_620596 (ADAMTS13 isoform 2, preproprotein) or amino acids 75 to 1371 of NP_620594 (ADAMTS13 isoform 2, mature polypeptide). In yet another embodiment, ADAMTS13 proteins include polypeptides comprising an amino acid sequence highly similar to that of NP_620595 (ADAMTS13 isoform 3, preproprotein) or amino acids 75 to 1340 of NP_620595 (ADAMTS13 isoform 1, mature polypeptide). As used herein, an ADAMTS13 protein includes natural variants with vWF cleaving activity and artificial constructs with vWF cleaving activity. As used in the present invention, ADAMTS13 encompasses any natural variants, alternative sequences, isoforms or mutant proteins that retain some basal activity. Examples of ADAMTS13 mutations found in the human population include, without limitation, R7W, V88M,

H96D, R102C, R193W, T196I, H234Q, A250V, R268P, W390C, R398H, Q448E, Q456H, P457L, C508Y, R528G, P618A, R625H, I673F, R692C, A732V, S903L, C908Y, C951G, G982R, C1024G, A1033T, R1095W, R1123C, C1213Y, T1226I, G1239V, R1336W, many of which have been found associated with thrombotic thrombocytopenic purpura (TTP). ADAMTS13 proteins also includes polypeptides containing post-translational modifications. For example, ADAMTS13 has been shown to be modified by N-acetylglucosamine (GlcNAc) at residues 614, 667, and 1354, and it has been predicted that residues 142, 146, 552, 579, 707, 828, and 1235 may also be modified in this fashion.

[0111] Proteolytically active recombinant ADAMTS13 may be prepared by expression in mammalian cell cultures, as described in Plaimauer *et al.*, (2002, Blood. 15;100(10):3626-32) and US 2005/0266528, the disclosures of which are herein incorporated by reference in their entireties for all purposes. Methods for the expression of recombinant ADAMTS13 in cell culture are disclosed in Plaimauer B, Scheiflinger F. (Semin Hematol. 2004 Jan;41(1):24-33 and US 2011/0086413, the disclosures of which are herein incorporated by reference in their entireties for all purposes.

[0112] As used herein, the term "biologically active derivative", when used in the context of an ADAMTS protein, also embraces polypeptides obtained via recombinant DNA technology. This may include any method 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 prokaryotic or eukaryotic cells by transfection, *i.e.*, via electroporation or microinjection, (iii) cultivating said transformed cells, *e.g.*, in a continuous or batch-wise manner, (iv) expressing an ADAMTS protein, *e.g.*, constitutively or upon induction, and (v) isolating said ADAMTS protein, *e.g.*, from the culture medium or by harvesting the transformed cells, in order to (vi) obtain substantially purified recombinant ADAMTS protein, *e.g.*, via ion exchange chromatography, size exclusion chromatography, affinity chromatography, hydrophobic interaction chromatography, and the like. The term "biologically active derivative" includes also chimeric molecules such as e.g. an ADAMTS protein, or functional fragment thereof, in combination with a second polypeptide, *e.g.*, an immunoglobulin Fc domain or an albumin domain, in order to improve the biological/pharmacological properties such as *e.g.*, half life of the ADAMTS protein in the circulation system of a mammal, particularly a human.

[0113] The terms "isolated," "purified," or "biologically pure" refer to material that is substantially or essentially free from components that normally accompany it as found in its native state. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. In one embodiment, rVWF is the predominant species present in a preparation is substantially purified. In another embodiment, rA13 is the predominant species present in a preparation is substantially purified. The term "purified" in some embodiments denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. In other embodiments, it means that the nucleic acid or protein is at least 50% pure, more preferably at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more pure. "Purify" or "purification" in other embodiments means removing at least one contaminant from the composition to be purified. In this sense, purification does not require that the purified compound be homogenous, *e.g.*, 100% pure.

[0114] The biological activity of vWF can be measured by known *in vitro* assays. For example, the Ristocetin Cofactor assay is based on the agglutination of fresh or formalin-fixed platelets induced by the antibiotic ristocetin in the presence of vWF. The degree of platelet agglutination depends on the vWF concentration and can be measured by the turbidimetric method, e.g. by use of an aggregometer (Weiss et al., J. Clin. Invest. 52: 2708-2716, 1973; Macfarlane et al., Thromb. Diath. Haemorrh. 34: 306-308, 1975). As provided herein, the specific Ristocetin Cofactor activity of the vWF of the present invention is described in terms of mU/μg of vWF, as measured using *in vitro* assays.

[0115] As used herein, "one unit of ADAMTS activity" is defined as the amount of activity in 1 mL of pooled normal human plasma, regardless of the assay being used. For example, when the ADAMTS protein is ADAMTS13, one unit of ADAMTS13 FRETS-VWF73 activity is the amount of activity needed to cleave the same amount of FRETS-VWF73 substrate (Kokame *et al.*, Br J Haematol. 2005 Apr; 129(1):93-100) as is cleaved by one mL of pooled normal human plasma. Conveniently, ADAMTS13 activity may be determined by functional assays, such as functional assays employing modified von Willebrand factor peptides as substrate for ADAMTS13 (Tripodi *et al. J Thromb Haemost*. 2008 Sep;6(9): 1534-41). A preferred method of determining recombinant human ADAMTS13 activity is disclosed in Gerritsen *et al.* (Assay

of von Willebrand factor (vWF)-cleaving protease based on decreased collagen binding affinity of degraded vWF: a tool for the diagnosis of thrombotic thrombocytopenic purpura (TTP). Thromb Haemost 1999; 82: 1386- 1389). In one embodiment, to be considered as a ADAMTS13 protein as defined above, a polypeptide or protein must have at least 1% of the vWF cleaving activity of native ADAMTS13. In other embodiments, an ADAMTS13 protein will contain at least 10% of the activity of native ADAMTS13. In yet other embodiments, an ADAMTS13 protein will contain at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of the activity of native ADAMTS13. The quantity of an ADAMTS13 protein may also be determined by measurement of an ADAMTS13 antigen, for example using the ELISA method disclosed in Rieger *et al.,* (2006, *Thromb Haemost.* 2006 95(2):212- 20). A well established convention in the art is that 1 mL of pooled normal human plasma contains 1 μg of ADAMTS13. Thus, the convention in the art is that 1 μg of plasma-derived ADAMTS13 has one unit of ADAMTS13 activity.

[0116] The terms "cell culture solution," "cell culture medium or media," and "cell culture supernatant" refer to aspects of cell culture processes generally well known in the art. In the context of the present invention, a cell culture solution can include cell culture media and cell culture supernatant. The cell culture media are externally added to the cell culture solution, optionally together with supplements, to provide nutrients and other components for culturing cells expressing rVWF or rA13. The cell culture supernatant refers to a cell culture solution comprising the nutrients and other components from the cell culture medium as well as products released, metabolized, and/or excreted from the cells during culture, but not the cells themselves. Thus in one context, a cell culture supernatant can refer to the liquid phase of a cell culture solution (*i.e.*, the cell culture solution excluding the cells). For example, the ammonium concentration of a culture supernatant generally refers to the ammonium concentration present in the cell culture solution. In other contexts, a cell culture supernatant refers to a cell culture solution from which the cells have been removed (*i.e.*, a recovered cell culture supernatant).

[0117] As used herein, the terms "vitamin B3," "nicotinamide," "niacinamide," "niacin," and "nicotinic acid" may be used interchangeably to refer to any member of the B3 family of vitamins. Accordingly, any member of this family may be used to supplement medium used in the methods of the present invention.

[0118] As used herein, the term "chemically defined medium" or "chemically defined media" refers to a synthetic growth medium in which the identity and concentration of all the components are known. Chemically defined mediums do not contain bacterial, yeast, animal, or plant extracts, although they may or may not include individual plant or animal-derived components (*e.g.*, proteins, polypeptides, *etc.*). Non-limiting examples of commercially available chemically defined mediums include, various Dulbecco's Modified Eagle's (DME) mediums (Sigma-Aldrich Co; SAFC Biosciences, Inc), Ham's Nutrient Mixture (Sigma-Aldrich Co; SAFC Biosciences, Inc), combinations thereof, and the like. Methods of preparing chemically defined culture mediums are known in the art, for example in U.S. Patent Numbers 6,171,825 and 6,936,441, WO 2007/077217, and U.S. Patent Application Publication Numbers 2008/0009040 and 2007/0212770, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

[0119] As used herein, the term "oligopeptide-free culture medium" or "oligopeptide-free culture media" refers to a protein-free medium that does not comprise oligopeptides, such as, e.g., oligopeptides derived from a protein hydrolysate. In one embodiment, the medium does not comprise oligopeptides having twenty or more amino acids. In one embodiment of the present invention, the medium does not comprise oligopeptides having fifteen or more amino acids. In another embodiment of the invention, the medium does not comprise oligopeptides having ten or more amino acids. In one embodiment the medium does not comprise oligopeptides having seven or more amino acids. In another embodiment the medium does not comprise oligopeptides having five or more amino acids. In still another embodiment the medium does not comprise oligopeptides having three or more amino acids. According to a further embodiment of the present invention, the medium does not comprise oligopeptides having two or more amino acids. Methods of preparing oligopeptide-free culture medium are known in the art, for example in U.S. Patent Numbers 6,171,825 and 6,936,441, WO 2007/077217, and U.S. Patent Application Publication Numbers 2008/0009040 and 2007/0212770, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

[0120] As used herein, the term "serum-free culture medium" or "serum-free culture media" refers to a culture medium that is not supplemented with an animal serum. Although oftentimes serum-free mediums are chemically defined mediums, serum-free mediums may be

supplemented with discrete animal or plant proteins or protein fractions. Methods of preparing serum-free culture medium are known in the art, for example in U.S. Patent Numbers 6,171,825 and 6,936,441, WO 2007/077217, and U.S. Patent Application Publication Numbers 2008/0009040 and 2007/0212770, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

[0121] As used herein, the term "animal protein-free culture medium" or "animal protein-free culture media" refers to a culture medium that is not supplemented with an animal serum, protein, or protein fraction. Although oftentimes animal protein-free culture mediums are chemically defined mediums, animal protein-free culture mediums may contain plant or yeast hydrolysates. Methods of preparing animal protein-free culture medium are known in the art, for example in U.S. Patent Numbers 6,171,825 and 6,936,441, WO 2007/077217, and U.S. Patent Application Publication Numbers 2008/0009040 and 2007/0212770, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

[0122] As used herein, the term "basal cell culture medium" or "basal cell culture media" refers to a chemically defined culture medium, an oligopeptide-free culture medium, a serum-free culture medium, or an animal protein-free culture medium that has not been supplemented with a hydrolysate, *e.g.*, a plant or yeast hydrolysate. basal mediums are well known in the art, e.g., DMEM, Ham's F12, DMEM/Ham's F12, Medium 199, McCoy, or RPMI. The basal medium can include a number of ingredients, including amino acids, vitamins, organic and inorganic salts, and sources of carbohydrate. Each ingredient can be present in an amount that supports the cultivation of a cell, such amounts being generally known to a person skilled in the art. The medium can include auxiliary substances, such as buffer substances, e.g., sodium bicarbonate, antioxidants, stabilizers to counteract mechanical stress, or protease inhibitors. If necessary, a non-ionic surfactant such as copolymers and/or mixtures of polyethylene glycols and polypropylene glycols can be added.

III. Cell Culture Media and Cell Culture Supernatant

[0123] One aspect of the present invention relates to cell culture media for producing rVWF and/or rA13 that have increased activity as compared to rVWF and rA13 produced with basal cell culture media. In one aspect, the present invention relates to cell culture media for producing rVWF and/or rA13 in which basal cell culture media is supplemented with one or

more additional substances. In specific embodiments and as is discussed in further detail below, cell culture conditions of the present invention include basal cell culture media supplemented to have at least 1.0 μg/L copper. In further embodiments, cell culture media of use and supernatants derived from the processes in the present invention also comprise low levels (under 10 mM) of ammonium. In a particular embodiment, the cell culture conditions used for expressing rVWF and/or rA13 are controlled such that the cell culture supernatant maintains a low level of ammonium, *i.e.*, less than 10 mM and preferably less than 5 mM.

[0124] The culture media of the present invention can be based on a suitable basal medium well known in the art, e.g., DMEM, Ham's F12, DMEM/Ham's F12, Medium 199, McCoy, or RPMI. The basal medium can include a number of ingredients, including amino acids, vitamins, organic and inorganic salts, and sources of carbohydrate. Each ingredient can be present in an amount that supports the cultivation of a cell, such amounts being generally known to a person skilled in the art. The medium can include auxiliary substances, such as buffer substances, e.g., sodium bicarbonate, antioxidants, stabilizers to counteract mechanical stress, or protease inhibitors. If necessary, a non-ionic surfactant such as copolymers and/or mixtures of polyethylene glycols and polypropylene glycols can be added.

[0125] Generally, basal media contain less than 1 μg/L of copper -- for example, DMEM/Ham's F12 has a copper concentration of about 0.3 μg/L. Such concentrations of copper do not provide enough copper ions to support production of rVWF and rA13 proteins of the present invention, which show high specific activity.

[0126] Copper can be provided to cell culture media of the present invention through a variety of ways, such as through addition of a medium supplement. In some embodiments, the medium supplement can contain hydrolysate, which can be provided to increase the copper concentration in the media. Hydrolysates can include any hydrolysate well known in the art, such as plant hydrolysates, soy hydrolysates, and wheat gluten hydrolysate. In certain embodiments, addition of hydrolysate can contribute an increased copper concentration of about 0.2 to about 10 μg/L of Cu^{2+}. In some embodiments, the amount of copper provided by the hydrolysate can depend on the amount of copper in the hydrolysate as well as the amount of hydrolysate added. The copper content of a hydrolysate can be determined by elemental analysis, *e.g.*, atom adsorption

spectroscopy (GFAA: graphite furnace atomic adsorption), or mass spectroscopy methods (*e.g.*, ICP-MS).

[0127] In certain embodiments, copper can be provided to the culture media alone or in addition to hydrolysate by providing a medium supplement including a suitable copper salt or copper chelate. Suitable copper salts can include but are not limited to copper sulfate, copper acetate, copper carbonate, copper chloride, copper hydroxide, copper nitrate, and copper oxide. Suitable copper chelators can include but are not limited to albumin, ethylenediaminetetraacetic acid (EDTA), polyamine chelating agents, ethylenediamine, diethylenetriamine, triethylenetetramine, triethylenediamine, tetraethylenepentamine, aminoethylethanolamine, aminoethylpiperazine, pentaethylenehexamine, triethylenetetramine-hydrochloride, tetraethylenepentamine-hydrochloride, pentaethylenehexamine-hydrochloride, tetraethylpentamine, captopril, penicilamine, N,N'-bis(3-aminopropyl)-1,3-propanediamine, N,N,Bis (2 animoethyl) 1,3 propane diamine, 1,7-dioxa-4,10-diazacyclododecane, 1,4,8,11-tetraaza cyclotetradecane-5,7-dione, 1,4,7-triazacyclononane trihydrochloride, 1-oxa-4,7,10-triazacyclododecane, 1,4,8,12-tetraaza cyclopentadecane, and 1,4,7,10-tetraaza cyclododecane.

[0128] In certain embodiments, basal cell culture media is supplemented with copper to result in a total copper concentration of about 1.0 to about 20 µg/L. In a specific embodiment, the basal cell media is supplemented with copper to a final concentration of between about 1.0 to about 10 µg/L. In further embodiments, the basal cell culture media is supplemented to result in a final copper concentration of about 1.0 - 5.0, 1.2 - 4.0, 1.3 - 3.0, 1.4 - 2.9, 1.5 - 2.8, 1.6 - 2.7, 1.7 - 2.6, 1.8 - 2.5, 1.9 - 2.4, 2.0 - 2.3, 2.1 - 2.2 µg/L copper. In further embodiments, the basal cell culture media used in methods of the present invention are supplemented to result in about 1.2 - 9.5, 1.4 - 9, 1.6 - 8.5, 1.8 - 8, 2.0 - 7.5, 2.2 - 7, 2.4 - 6.5, 2.6 - 6.0, 2.8 - 5.5, 3.0 - 5.0, 3.2 - 4.5, 3.4 - 4, and 2 - 4 µg/L copper. In yet other embodiments, the basal cell culture media used in methods of the present invention are supplemented to result in about 1-6, 2-5, 3-4 µg/L copper. In one embodiment, the basal cell culture media is supplemented with copper to result in a total copper concentration of at least 1 µg/L. In another embodiment, the basal cell culture media is supplemented with copper to result in a total copper concentration of at least 2 µg/L. In yet another embodiment, the basal cell culture media is supplemented with copper to result in a total copper concentration of at least 4 µg/L. In certain embodiments, the basal cell culture media is

supplemented with copper to result in a total copper concentration of at least 1 μg/L, or at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 μg/L copper, or more. In certain embodiments and as discussed in further detail herein, cultures for producing rA13 may contain from about 2 – 4 μg/L of copper, whereas cultures for producing rVWF may contain at least 2 μg/L of copper.

[0129] The concentrations indicated above are the respective concentrations of pure copper, in cupric form (Cu^{2+}). If a copper derivative, e.g., a hydrated salt, or a compound comprising copper, e.g., a copper chelator, is used, the amount of derivative or chelator is added such that the final concentration of the copper is in the ranges described herein. For example, 2 μg/L of $CuSO_4 \cdot 5H_2O$ is equivalent to a copper concentration of about 0.51 μg/L (without sulfate and $5H_2O$).

[0130] Advantageously, it has been found that the use of cell culture medium in cell culture processes resulting in low ammonium (NH_4^+) concentrations in the cell culture solution (*i.e.*, in the culture supernatant) results in the expression of recombinant VWF and/or rA13 with higher specific activities. Accordingly, in certain embodiments, the NH_4^+ concentration of the supernatant is no higher than 10 mM. In a preferred embodiment, the NH_4^+ concentration of the supernatant is no higher than 5 mM. In a preferred embodiment, the NH_4^+ concentration of the supernatant is no higher than 4 mM. In yet other embodiments, the NH_4^+ concentration of the supernatant is no higher than 10 mM, 9 mM, 8 mM, 7 mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, or less.

[0131] Accordingly, in certain embodiments, the methods and compositions provided herein rely on the use of basal cell culture media supplemented with copper (*e.g.*, to a final concentration of at least 2 μg/L) used in a process that results in an NH_4^+ concentration of no higher than 10 mM in the supernatant. In yet other embodiments, the basal cell culture medium is supplemented to provide a final copper and ammonium concentration according to any one of variations 1 to 440, as provided in **Table 1**.

Table 1. Exemplary embodiments of copper and ammonium concentrations present in culture media and supernatant useful for the expression of a recombinant proteins as provided herein.

		Ammonium Concentration										
		N.D.	NMT 10mM	NMT 9mM	NMT 8mM	NMT 7mM	NMT 6mM	NMT 5mM	NMT 4mM	NMT 3mM	NMT 2mM	NMT 1mM

Copper Concentration												
	AL 1 µg/L	Var. 1	Var. 41	Var. 81	Var. 121	Var. 161	Var. 201	Var. 241	Var. 281	Var. 321	Var. 361	Var. 401
	AL 2 µg/L	Var. 2	Var. 42	Var. 82	Var. 122	Var. 162	Var. 202	Var. 242	Var. 282	Var. 322	Var. 362	Var. 402
	AL 3 µg/L	Var. 3	Var. 43	Var. 83	Var. 123	Var. 163	Var. 203	Var. 243	Var. 283	Var. 323	Var. 363	Var. 403
	AL 4 µg/L	Var. 4	Var. 44	Var. 84	Var. 124	Var. 164	Var. 204	Var. 244	Var. 284	Var. 324	Var. 364	Var. 404
	AL 5 µg/L	Var. 5	Var. 45	Var. 85	Var. 125	Var. 165	Var. 205	Var. 245	Var. 285	Var. 325	Var. 365	Var. 405
	AL 6 µg/L	Var. 6	Var. 46	Var. 86	Var. 126	Var. 166	Var. 206	Var. 246	Var. 286	Var. 326	Var. 366	Var. 406
	AL 7 µg/L	Var. 7	Var. 47	Var. 87	Var. 127	Var. 167	Var. 207	Var. 247	Var. 287	Var. 327	Var. 367	Var. 407
	AL 8 µg/L	Var. 8	Var. 48	Var. 88	Var. 128	Var. 168	Var. 208	Var. 248	Var. 288	Var. 328	Var. 368	Var. 408
	AL 9 µg/L	Var. 9	Var. 49	Var. 89	Var. 129	Var. 169	Var. 209	Var. 249	Var. 289	Var. 329	Var. 369	Var. 409
	AL 10 µg/L	Var. 10	Var. 50	Var. 90	Var. 130	Var. 170	Var. 210	Var. 250	Var. 290	Var. 330	Var. 370	Var. 410
	About 1 µg/L	Var. 11	Var. 51	Var. 91	Var. 131	Var. 171	Var. 211	Var. 251	Var. 291	Var. 331	Var. 371	Var. 411
	About 1.5 µg/L	Var. 12	Var. 52	Var. 92	Var. 132	Var. 172	Var. 212	Var. 252	Var. 292	Var. 332	Var. 372	Var. 412
	About 2 µg/L	Var. 13	Var. 53	Var. 93	Var. 133	Var. 173	Var. 213	Var. 253	Var. 293	Var. 333	Var. 373	Var. 413
	About 2.5 µg/L	Var. 14	Var. 54	Var. 94	Var. 134	Var. 174	Var. 214	Var. 254	Var. 294	Var. 334	Var. 374	Var. 414
	About 3 µg/L	Var. 15	Var. 55	Var. 95	Var. 135	Var. 175	Var. 215	Var. 255	Var. 295	Var. 335	Var. 375	Var. 415
	About 3.5 µg/L	Var. 16	Var. 56	Var. 96	Var. 136	Var. 176	Var. 216	Var. 256	Var. 296	Var. 336	Var. 376	Var. 416
	About 4 µg/L	Var. 17	Var. 57	Var. 97	Var. 137	Var. 177	Var. 217	Var. 257	Var. 297	Var. 337	Var. 377	Var. 417
	About 4.5 µg/L	Var. 18	Var. 58	Var. 98	Var. 138	Var. 178	Var. 218	Var. 258	Var. 298	Var. 338	Var. 378	Var. 418
	About 5 µg/L	Var. 19	Var. 59	Var. 99	Var. 139	Var. 179	Var. 219	Var. 259	Var. 299	Var. 339	Var. 379	Var. 419
	About 5.5 µg/L	Var. 20	Var. 60	Var. 100	Var. 140	Var. 180	Var. 220	Var. 260	Var. 300	Var. 340	Var. 380	Var. 420
	About 6 µg/L	Var. 21	Var. 61	Var. 101	Var. 141	Var. 181	Var. 221	Var. 261	Var. 301	Var. 341	Var. 381	Var. 421
	About 7 µg/L	Var. 22	Var. 62	Var. 102	Var. 142	Var. 182	Var. 222	Var. 262	Var. 302	Var. 342	Var. 382	Var. 422
	About 8 µg/L	Var. 23	Var. 63	Var. 103	Var. 143	Var. 183	Var. 223	Var. 263	Var. 303	Var. 343	Var. 383	Var. 423
	About 9 µg/L	Var. 24	Var. 64	Var. 104	Var. 144	Var. 184	Var. 224	Var. 264	Var. 304	Var. 344	Var. 384	Var. 424

	About 10 µg/L	Var. 25	Var. 65	Var. 105	Var. 145	Var. 185	Var. 225	Var. 265	Var. 305	Var. 345	Var. 385	Var. 425
	1-20 µg/L	Var. 26	Var. 66	Var. 106	Var. 146	Var. 186	Var. 226	Var. 266	Var. 306	Var. 346	Var. 386	Var. 426
	2-20 µg/L	Var. 27	Var. 67	Var. 107	Var. 147	Var. 187	Var. 227	Var. 267	Var. 307	Var. 347	Var. 387	Var. 427
	1-10 µg/L	Var. 28	Var. 68	Var. 108	Var. 148	Var. 188	Var. 228	Var. 268	Var. 308	Var. 348	Var. 388	Var. 428
	2-10 µg/L	Var. 29	Var. 69	Var. 109	Var. 149	Var. 189	Var. 229	Var. 269	Var. 309	Var. 349	Var. 389	Var. 429
	1-6 µg/L	Var. 30	Var. 70	Var. 110	Var. 150	Var. 190	Var. 230	Var. 270	Var. 310	Var. 350	Var. 390	Var. 430
	2-6 µg/L	Var. 31	Var. 71	Var. 111	Var. 151	Var. 191	Var. 231	Var. 271	Var. 311	Var. 351	Var. 391	Var. 431
	3-6 µg/L	Var. 32	Var. 72	Var. 112	Var. 152	Var. 192	Var. 232	Var. 272	Var. 312	Var. 352	Var. 392	Var. 432
	4-6 µg/L	Var. 33	Var. 73	Var. 113	Var. 153	Var. 193	Var. 233	Var. 273	Var. 313	Var. 353	Var. 393	Var. 433
	1-5 µg/L	Var. 34	Var. 74	Var. 114	Var. 154	Var. 194	Var. 234	Var. 274	Var. 314	Var. 354	Var. 394	Var. 434
	2-5 µg/L	Var. 35	Var. 75	Var. 115	Var. 155	Var. 195	Var. 235	Var. 275	Var. 315	Var. 355	Var. 395	Var. 435
	3-5 µg/L	Var. 36	Var. 76	Var. 116	Var. 156	Var. 196	Var. 236	Var. 276	Var. 316	Var. 356	Var. 396	Var. 436
	4-5 µg/L	Var. 37	Var. 77	Var. 117	Var. 157	Var. 197	Var. 237	Var. 277	Var. 317	Var. 357	Var. 397	Var. 437
	1-4 µg/L	Var. 38	Var. 78	Var. 118	Var. 158	Var. 198	Var. 238	Var. 278	Var. 318	Var. 358	Var. 398	Var. 438
	2-4 µg/L	Var. 39	Var. 79	Var. 119	Var. 159	Var. 199	Var. 239	Var. 279	Var. 319	Var. 359	Var. 399	Var. 439
	3-4 µg/L	Var. 40	Var. 80	Var. 120	Var. 160	Var. 200	Var. 240	Var. 280	Var. 320	Var. 360	Var. 400	Var. 440

*ND = not defined
*NMT = no more than
*AL = at least

[0132] In some embodiments, the copper supplemented media of the present invention are produced by supplementing basal media that is animal protein-free and/or chemically defined. Methods of preparing animal protein-free and chemically defined culture mediums are known in the art, for example in U.S. Patent Numbers 6,171,825 and 6,936,441, WO 2007/077217, and U.S. Patent Application Publication Numbers 2008/0009040 and 2007/0212770, the disclosures of which are incorporated herein by reference in their entireties for all purposes. In one embodiment, the basal culture medium used in the methods described herein is animal protein-free or oligopeptide-free medium. In certain embodiments, the culture medium may be chemically defined. In certain embodiments, the culture media may contain at least one polyamine at a concentration of about 0.5 mg/L to about 10 mg/L.

[0133] In further embodiments and in addition to any of the description provided above, culture media of the invention are provided in which a basal medium is supplemented with copper and at least one of calcium, zinc, and/or vitamin B3. In certain embodiments, the medium may be an animal protein-free, oligopeptide-free, or chemically defined medium. In certain embodiments, the animal protein-free or oligopeptide free medium is prepared as taught in U.S. Patent Numbers 6,171,825 and 6,936,441, WO 2007/077217, and U.S. Patent Application Publication Numbers 2008/0009040 and 2007/0212770, the disclosures of which are incorporated herein by reference in their entireties for all purposes, both of which are incorporated herein by reference in their entireties for all purposes, and supplemented with additional copper and optionally one or more of calcium, zinc, and vitamin B3. In a specific embodiment, the chemically defined culture medium may be similar to a Dulbecco's Modified Eagle's Media / Ham's F12 1:1 mixture (DMEM/Ham's F12), which has been supplemented with additional copper and optionally calcium, zinc, and/or vitamin B3, in order to increase the specific activity of rVWF or rA13 expressed in a cell cultured in the medium. In yet other embodiments, the culture medium is animal component free. In another embodiment, the culture medium contains protein, *e.g.*, animal protein from serum such as fetal calf serum. In another embodiment, the culture has recombinant proteins exogenously added. In another embodiment, the proteins are from a certified pathogen free animal.

[0134] In certain embodiments, the culture media contains at least one polyamine at a concentration of at or about between 0.5 mg/L and 30 mg/L. In another embodiment, the culture medium contains at least one polyamine at or about between 0.5 mg/L and 10 mg/L. In one embodiment, the culture medium contains at least one polyamine at or about between 2 mg/L and 8 mg/L. In certain embodiments the polyamine is from the group of ornithine, putrescine, spermine or spermidine, or the like. In a preferred embodiment, the polyamine is putrescine. In a specific embodiment, the culture medium contains at or about between 2 mg/L and 8 mg/L putrescine.

[0135] In one embodiment, the culture media contains at least one polyamine at a concentration of at or about between 0.5 mg/L and 30 mg/L and a copper and ammonium combination according to any one of variations 1 to 440, as set forth in **Table 1**. In another embodiment, the culture medium contains at least one polyamine at or about between 0.5 mg/L and 10 mg/L and a

copper and ammonium combination according to any one of variations 1 to 440, as set forth in **Table 1**. In one embodiment, the culture medium contains at least one polyamine at or about between 2 mg/L and 8 mg/L and a copper and ammonium combination according to any one of variations 1 to 440, as set forth in **Table 1**. In certain embodiments the polyamine is from the group of ornithine, putrescine, spermine or spermidine, or the like. In a preferred embodiment, the polyamine is putrescine. In a specific embodiment, the culture medium contains at or about between 2 mg/L and 8 mg/L putrescine and a copper and ammonium combination according to any one of variations 1 to 440, as set forth in **Table 1**.

[0136] In further aspects, in addition to copper, cell culture media of use in the present invention may further include one or more of: additional calcium, zinc, one or more vitamins, and any combination thereof.

[0137] Generally, any calcium salt may be used to supplement the media of the invention, non-limiting examples of acceptable salts include $CaCl_2$, $CaCl_2$, $CaFPO_3 \cdot 2H_2O$, CaI_2, $CaBr_2$, $(C_2H_3O_2)_2Ca$, $(CHO_2)_2Ca$, $(C_6H_7O_6)_2Ca$, $(C_6H_5O_7)_2Ca_3 \cdot 2H_2O$, and the like. In certain embodiments, a pharmaceutically acceptable salt of calcium is used to supplement the culture mediums of the invention.

[0138] Generally, any zinc salt may be used to supplement the media of the invention, non-limiting examples of acceptable salts include, $ZnSO_4 \cdot 7H_2O$, $ZnSO_3 \cdot 2H_2O$, $(C_6H_5O_7)_2Zn_3 \cdot 2H_2O$, $ZnBr_2$, $ZnBr_2 \cdot 2H_2O$, $ZnCl_2$, Zn $(NO_3)_2 \cdot 6H_2O$, Zn $(H_2PO_4)_2 \cdot H_2O$, $(C_2H_3O_2)_2Zn \cdot 2H_2O$, and the like. In certain embodiments, a pharmaceutically acceptable salt of zinc is used to supplement the culture mediums of the invention. In other embodiments, a zinc containing peptide or protein preparation, for example insulin, may be used to the supplement the culture provided herein.

[0139] In still further aspects, basal cell media supplemented with copper and one or more of the additional materials discussed above may further be used in cultures with low ammonium levels in the supernatant. In certain embodiments, supplemented cell culture media of use in the present invention result in ammonium levels in the cell culture solution of under 10 mM. In further embodiments, the supplemented cell culture media of the present invention is used with cell culture ammonium levels from about 0.5 – 9.5, 1.0 – 9.0, 1.5 – 8.5, 2.0 – 8.0, 2.5 – 7.5, 3.0 – 7.0, 3.5 – 6.5, 4.0 – 6.0, 4.5 – 5.5 mM.

[0140] In one embodiment, the copper and ammonium concentrations of a cell culture media and a cell culture supernatant are maintained for an extended period of time during the manufacturing process. In a specific embodiment, the copper and ammonium concentrations of a cell culture are maintained for the duration of a manufacturing process, *i.e.*, during the time in which rVWF or rA13 is being expressed and recovered from a large-scale cell culture. In certain embodiments, the copper and ammonium concentrations are maintained in the culture solution at a level according to any one of variations 1 to 440, as set forth in **Table 1**. In a preferred embodiment the copper and ammonium concentrations are maintained for the entire time of such a production process.

[0141] In some embodiments, the culture medium provided by the invention may be provided in a liquid or a dry or powder form. The medium may be pre-aliquoted in an amount suitable for single use or provided in a larger quantity that may be used for more than one cell-culture. Generally, the medium of the invention will be provided in a sterile fashion.

[0142] Specific details of cell culture media of use for producing rVWF or rA13 are discussed below. Although the following are discussed with respect to either rVWF or rA13, it will be appreciated that any of the discussion provided below with respect to rVWF is applicable for rA13, and vice versa.

A. Recombinant VWF Cell Culture Media

[0143] One aspect of the present invention relates to a cell culture solution for producing recombinant vWF, more specifically, high molecular weight vWF having a high specific activity, which are further described herein. In one embodiment, the present invention provides a cell culture solution for producing high molecular weight, recombinant vWF, comprising a cell culture medium comprising a copper concentration of at least about 2.4 μg/L and a plurality of cells expressing highly multimeric vWF comprising about 14 to about 22 dimers and a specific Ristocetin co-factor activity of at least about 30 mU/μg.

[0144] In one embodiment, the cell culture solution further comprises an ammonium concentration of less than 10 mM. In a preferred embodiment, the cell culture comprises an ammonium concentration of no more than 5 mM. In yet other embodiments, the cell culture comprises an ammonium concentration of no more than 10 mM, or no more than 9 mM, 8 mM, 7

mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, or less. In yet other embodiments, the cell culture has a copper and ammonium concentration according to any one of variations 1 to 440, as set forth in **Table 1**. In certain embodiments, the ammonium concentration of the cell culture is maintained for an extended period at a concentration as provided above. For example, in one embodiment, the ammonium concentration is maintained at a low concentration for at least 3 days, or at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 5 mM for at least 7 days. In another specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 4 mM for at least 7 days. In a specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 5 mM for at least 14 days. In another specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 4 mM for at least 14 days. In yet another embodiment, the ammonium concentration of the cell culture is maintained at a low level for the duration of the process (*i.e.*, for the entire time the culture is being used to produce rVWF).

[0145] In one embodiment of the invention, the cell culture media can comprise a copper concentration of at least about 2.4 μg/L, in another embodiment at least about 3 μg/L, in yet another embodiment at least about 4 μg/L, in yet another embodiment at least about 8 μg/L, in yet another embodiment at least about 10 μg/L, in yet another embodiment at least about 15 μg/L, and in a further embodiment at least about 20 μg/L.

[0146] In other embodiments, the copper concentration in the cell culture media of the present invention can range from about 2.4 μg/L to about 20 μg/L, in another embodiment from about 2.4 μg/L to about 15 μg/L, in yet another embodiment from about 2.4 μg/L to about 10 μg/L, in yet another embodiment from about 2.4 μg/L to about 8 μg/L, in yet another embodiment from about 2.4 μg/L to about 6 μg/L, in yet another embodiment from about 2.4 μg/L to about 4 μg/L, in yet another embodiment from about 4 μg/L to about 20 μg/L, in yet another embodiment from about 4 μg/L to about 15 μg/L, in yet another embodiment from about 4 μg/L to about 10 μg/L, in yet another embodiment from about 4 μg/L to about 8 μg/L, and in a further embodiment from about 4 μg/L to about 6 μg/L.

[0147] The present invention also provides kits for the expression or production of rVWF, the kits comprising a culture medium suitable for the expression of rVWF having high specific activity.

B. ADAMTS13 (A13) Cell Culture Media

[0148] In one aspect, the present invention provides culture media that are useful for the expression of ADAMTS proteins having high specific activities. Advantageously, it has been found that by supplementing a culture medium with copper, that the activities of recombinant ADAMTS (*e.g.*, rADAMTS13) enzymes expressed in cells cultured in the supplemented medium are greatly enhanced, while the enzymes are expressed at levels as high, if not higher, than cells cultured in non-supplemented mediums.

[0149] In one aspect, the present invention provides cell culture media supplemented with copper for the expression of recombinant ADAMTS13 protein with high specific activity. In one embodiment, the media are supplemented to result in a total copper concentration of from about 2 to about 4 μg/L. In further embodiments, the media are supplemented to results in a total copper concentration of from about 1 - 3, 2 - 3, 3 - 4 μg/L. In one embodiment, the media contain a copper concentration of at least 1 μg/L. In another embodiment, the media contains at least 2 μg/L copper. In another embodiment, the media contains at least 4 μg/L copper. In other embodiments, the media contains between 2 μg/L and 20 μg/L copper. In another embodiment, the media contains between 1 μg/L and 6 μg/L copper. In another embodiment, the media contains between 2 μg/L and 5 μg/L copper. In another embodiment, the media contains between 3 μg/L and 4 μg/L copper. In yet other embodiments, the media contains at least 1 μg/L copper, or at least 2 μg/L, 3 μg/L, 4 μg/L, 5 μg/L, 6 μg/L, 7 μg/L, 8 μg/L, 9 μg/L, 10 μg/L, 11 μg/L, 12 μg/L, 13 μg/L, 14 μg/L, 15 μg/L, 16 μg/L, 17 μg/L, 18 μg/L, 19 μg/L, 20 μg/L, or higher concentrations of copper.

[0150] In one embodiment, the cell culture solution further comprises an ammonium concentration of less than 10 mM. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 5 mM. In yet other embodiments, the cell culture solution comprises an ammonium concentration of no more than 10 mM, or no more than 9 mM, 8 mM, 7 mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, or less. In yet other embodiments, the cell culture solution has a copper and ammonium concentration according to

any one of variations 1 to 440, as set forth in **Table 1**. In certain embodiments, the ammonium concentration of the cell culture is maintained for an extended period at a concentration as provided above. For example, in one embodiment, the ammonium concentration is maintained at a low concentration for at least 3 days, or at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 5 mM for at least 7 days. In another specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 4 mM for at least 7 days. In a specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 5 mM for at least 14 days. In another specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 4 mM for at least 14 days. In yet another embodiment, the ammonium concentration of the cell culture is maintained at a low level for the duration of the process (*i.e.*, for the entire time the culture is being used to produce rA13).

[0151] In one embodiment, a culture medium is provided for the expression of a recombinant ADAMTS protein (*e.g.*, rADAMTS13) containing at least 1 μg/L copper and at least 2 μM zinc. In other embodiments, the media contains at least 2 μg/L copper or at least 4 μg/L copper. In another embodiment wherein the media is supplemented with copper, the culture medium also contains at least at or about 5 μM zinc. In one embodiment, the culture medium also contains at or about between 2 μM and 12 μM zinc. In another embodiment, the culture medium also contains at or about between 5 μM and 12 μM zinc. In yet other embodiments, the culture medium also may contain at least at or about 2 μM, or at least at or about 3 μM, 4 μM, 5 μM, 6 μM, 7 μM, 8 μM, 9 μM, 10 μM, 11 μM, 12 μM, 13 μM, 14 μM, 15 μM, 20 μM, 25 μM, 30 μM, or more zinc. In one embodiment, the culture medium contains a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**.

Table 2. Exemplary embodiments of copper and zinc concentrations present in culture media useful for the expression of a recombinant ADAMTS13 protein.

		Zinc Concentration										
		AL 2 μM	AL 3 μM	AL 4 μM	AL 5 μM	AL 6 μM	AL 7 μM	AL 8 μM	AL 9 μM	AL 10 μM	2-12 μM	5-12 μM
C	AL 1 μg/L	Var. 441	Var. 481	Var. 521	Var. 561	Var. 601	Var. 641	Var. 681	Var. 721	Var. 761	Var. 801	Var. 841

	AL 2 µg/L	Var. 442	Var. 482	Var. 522	Var. 562	Var. 602	Var. 642	Var. 682	Var. 722	Var. 762	Var. 802	Var. 842
	AL 3 µg/L	Var. 443	Var. 483	Var. 523	Var. 563	Var. 603	Var. 643	Var. 683	Var. 723	Var. 763	Var. 803	Var. 843
	AL 4 µg/L	Var. 444	Var. 484	Var. 524	Var. 564	Var. 604	Var. 644	Var. 684	Var. 724	Var. 764	Var. 804	Var. 844
	AL 5 µg/L	Var. 445	Var. 485	Var. 525	Var. 565	Var. 605	Var. 645	Var. 685	Var. 725	Var. 765	Var. 805	Var. 845
	AL 6 µg/L	Var. 446	Var. 486	Var. 526	Var. 566	Var. 606	Var. 646	Var. 686	Var. 726	Var. 766	Var. 806	Var. 846
	AL 7 µg/L	Var. 447	Var. 487	Var. 527	Var. 567	Var. 607	Var. 647	Var. 687	Var. 727	Var. 767	Var. 807	Var. 847
	AL 8 µg/L	Var. 448	Var. 488	Var. 528	Var. 568	Var. 608	Var. 648	Var. 688	Var. 728	Var. 768	Var. 808	Var. 848
	AL 9 µg/L	Var. 449	Var. 489	Var. 529	Var. 569	Var. 609	Var. 649	Var. 689	Var. 729	Var. 769	Var. 809	Var. 849
	AL 10 µg/L	Var. 450	Var. 490	Var. 530	Var. 570	Var. 610	Var. 650	Var. 690	Var. 730	Var. 770	Var. 810	Var. 850
	About 1 µg/L	Var. 451	Var. 491	Var. 531	Var. 571	Var. 611	Var. 651	Var. 691	Var. 731	Var. 771	Var. 811	Var. 851
	About 1.5 µg/L	Var. 452	Var. 492	Var. 532	Var. 572	Var. 612	Var. 652	Var. 692	Var. 732	Var. 772	Var. 812	Var. 852
	About 2 µg/L	Var. 453	Var. 493	Var. 533	Var. 573	Var. 613	Var. 653	Var. 693	Var. 733	Var. 773	Var. 813	Var. 853
	About 2.5 µg/L	Var. 454	Var. 494	Var. 534	Var. 574	Var. 614	Var. 654	Var. 694	Var. 734	Var. 774	Var. 814	Var. 854
	About 3 µg/L	Var. 455	Var. 495	Var. 535	Var. 575	Var. 615	Var. 655	Var. 695	Var. 735	Var. 775	Var. 815	Var. 855
	About 3.5 µg/L	Var. 456	Var. 496	Var. 536	Var. 576	Var. 616	Var. 656	Var. 696	Var. 736	Var. 776	Var. 816	Var. 856
	About 4 µg/L	Var. 457	Var. 497	Var. 537	Var. 577	Var. 617	Var. 657	Var. 697	Var. 737	Var. 777	Var. 817	Var. 857
	About 4.5 µg/L	Var. 458	Var. 498	Var. 538	Var. 578	Var. 618	Var. 658	Var. 698	Var. 738	Var. 778	Var. 818	Var. 858
	About 5 µg/L	Var. 459	Var. 499	Var. 539	Var. 579	Var. 619	Var. 659	Var. 699	Var. 739	Var. 779	Var. 819	Var. 859
	About 5.5 µg/L	Var. 460	Var. 500	Var. 540	Var. 580	Var. 620	Var. 660	Var. 700	Var. 740	Var. 780	Var. 820	Var. 860
	About 6 µg/L	Var. 461	Var. 501	Var. 541	Var. 581	Var. 621	Var. 661	Var. 701	Var. 741	Var. 781	Var. 821	Var. 861
	About 7 µg/L	Var. 462	Var. 502	Var. 542	Var. 582	Var. 622	Var. 662	Var. 702	Var. 742	Var. 782	Var. 822	Var. 862
	About 8 µg/L	Var. 463	Var. 503	Var. 543	Var. 583	Var. 623	Var. 663	Var. 703	Var. 743	Var. 783	Var. 823	Var. 863
	About 9 µg/L	Var. 464	Var. 504	Var. 544	Var. 584	Var. 624	Var. 664	Var. 704	Var. 744	Var. 784	Var. 824	Var. 864
	About 10 µg/L	Var. 465	Var. 505	Var. 545	Var. 585	Var. 625	Var. 665	Var. 705	Var. 745	Var. 785	Var. 825	Var. 865

	1-20 µg/L	Var. 466	Var. 506	Var. 546	Var. 586	Var. 626	Var. 666	Var. 706	Var. 746	Var. 786	Var. 826	Var. 866
	2-20 µg/L	Var. 467	Var. 507	Var. 547	Var. 587	Var. 627	Var. 667	Var. 707	Var. 747	Var. 787	Var. 827	Var. 867
	1-10 µg/L	Var. 468	Var. 508	Var. 548	Var. 588	Var. 628	Var. 668	Var. 708	Var. 748	Var. 788	Var. 828	Var. 868
	2-10 µg/L	Var. 469	Var. 509	Var. 549	Var. 589	Var. 629	Var. 669	Var. 709	Var. 749	Var. 789	Var. 829	Var. 869
	1-6 µg/L	Var. 470	Var. 510	Var. 550	Var. 590	Var. 630	Var. 670	Var. 710	Var. 750	Var. 790	Var. 830	Var. 870
	2-6 µg/L	Var. 471	Var. 511	Var. 551	Var. 591	Var. 631	Var. 671	Var. 711	Var. 751	Var. 791	Var. 831	Var. 871
	3-6 µg/L	Var. 472	Var. 512	Var. 552	Var. 592	Var. 632	Var. 672	Var. 712	Var. 752	Var. 792	Var. 832	Var. 872
	4-6 µg/L	Var. 473	Var. 513	Var. 553	Var. 593	Var. 633	Var. 673	Var. 713	Var. 753	Var. 793	Var. 833	Var. 873
	1-5 µg/L	Var. 474	Var. 514	Var. 554	Var. 594	Var. 634	Var. 674	Var. 714	Var. 754	Var. 794	Var. 834	Var. 874
	2-5 µg/L	Var. 475	Var. 515	Var. 555	Var. 595	Var. 635	Var. 675	Var. 715	Var. 755	Var. 795	Var. 835	Var. 875
	3-5 µg/L	Var. 476	Var. 516	Var. 556	Var. 596	Var. 636	Var. 676	Var. 716	Var. 756	Var. 796	Var. 836	Var. 876
	4-5 µg/L	Var. 477	Var. 517	Var. 557	Var. 597	Var. 637	Var. 677	Var. 717	Var. 757	Var. 797	Var. 837	Var. 877
	1-4 µg/L	Var. 478	Var. 518	Var. 558	Var. 598	Var. 638	Var. 678	Var. 718	Var. 758	Var. 798	Var. 838	Var. 878
	2-4 µg/L	Var. 479	Var. 519	Var. 559	Var. 599	Var. 639	Var. 679	Var. 719	Var. 759	Var. 799	Var. 839	Var. 879
	3-4 µg/L	Var. 480	Var. 520	Var. 560	Var. 600	Var. 640	Var. 680	Var. 720	Var. 760	Var. 800	Var. 840	Var. 880

*AL = at least

[0152] In one embodiment, the cell culture solution further comprises a low ammonium concentration. In one embodiment, the cell culture solution comprises an ammonium concentration of less than 10 mM and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In a specific embodiment, the ammonium is maintained at a concentration of no more than 10 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 6 mM and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In a specific embodiment, the ammonium is maintained at a concentration of no more than 6 mM for at least 7 days. In another preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 5 mM and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In a specific embodiment, the ammonium is maintained at a concentration of no more than 5 mM for at least 7 days. In another preferred embodiment, the cell culture solution comprises an ammonium

concentration of no more than 4 mM and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In a specific embodiment, the ammonium is maintained at a concentration of no more than 4 mM for at least 7 days. In yet other embodiments, the cell culture solution comprises an ammonium concentration of no more than 10 mM, or no more than 9 mM, 8 mM, 7 mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, or less and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In yet another specific embodiment, the ammonium concentration of the cell culture is maintained at a low level for the duration of the process (*i.e.*, for the entire time the culture is being used to produce rA13).

[0153] In one embodiment, a culture medium is provided for the expression of a recombinant ADAMTS protein (*e.g.*, rADAMTS13) containing at least 1 μg/L copper and at least at or about 0.5 mM calcium. In other embodiments, the media contains at least 2 μg/L copper or at least 4 μg/L copper. In another embodiment wherein the media is supplemented with copper, the culture medium also contains at least 1.5 mM calcium. In one embodiment, the culture medium contains at or about between 0.5 mM and 1.5 mM calcium. In yet other embodiments, the culture medium may contain at least at or about 0.5 mM, or at least at or about 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1.0 mM, 1.1 mM, 1.2 mM, 1.3 mM, 1.4 mM, 1.5 mM, 1.6 mM, 1.7 mM, 1.8 mM, 1.9 mM, 2.0 mM, 2.25 mM, 2.5 mM, 2.75 mM, 3.0 mM, 3.5 mM, 4.0 mM, 4.5 mM, 5.0 mM, or more calcium. In one embodiment, the culture medium contains a copper and calcium concentration according to any one of variations 881 to 1320, as set forth in **Table 3**.

Table 3. Exemplary embodiments of copper and calcium concentrations present in culture media useful for the expression of a recombinant ADAMTS13 protein.

		Calcium Concentration										
		AL 0.5 mM	AL 0.75 mM	AL 1.0 mM	AL 1.25 mM	AL 1.5 mM	AL 2.0 mM	AL 2.5 mM	AL 3.0 mM	AL 4 mM	AL 5 mM	0.5-1.5 mM
Copper Concentration	AL 1 μg/L	Var. 881	Var. 921	Var. 961	Var. 1001	Var. 1041	Var. 1081	Var. 1121	Var. 1161	Var. 1201	Var. 1241	Var. 1281
	AL 2 μg/L	Var. 882	Var. 922	Var. 962	Var. 1002	Var. 1042	Var. 1082	Var. 1122	Var. 1162	Var. 1202	Var. 1242	Var. 1282
	AL 3 μg/L	Var. 883	Var. 923	Var. 963	Var. 1003	Var. 1043	Var. 1083	Var. 1123	Var. 1163	Var. 1203	Var. 1243	Var. 1283
	AL 4 μg/L	Var. 884	Var. 924	Var. 964	Var. 1004	Var. 1044	Var. 1084	Var. 1124	Var. 1164	Var. 1204	Var. 1244	Var. 1284
	AL 5 μg/L	Var. 885	Var. 925	Var. 965	Var. 1005	Var. 1045	Var. 1085	Var. 1125	Var. 1165	Var. 1205	Var. 1245	Var. 1285

	AL 6 µg/L	Var. 886	Var. 926	Var. 966	Var. 1006	Var. 1046	Var. 1086	Var. 1126	Var. 1166	Var. 1206	Var. 1246	Var. 1286
	AL 7 µg/L	Var. 887	Var. 927	Var. 967	Var. 1007	Var. 1047	Var. 1087	Var. 1127	Var. 1167	Var. 1207	Var. 1247	Var. 1287
	AL 8 µg/L	Var. 888	Var. 928	Var. 968	Var. 1008	Var. 1048	Var. 1088	Var. 1128	Var. 1168	Var. 1208	Var. 1248	Var. 1288
	AL 9 µg/L	Var. 889	Var. 929	Var. 969	Var. 1009	Var. 1049	Var. 1089	Var. 1129	Var. 1169	Var. 1209	Var. 1249	Var. 1289
	AL 10 µg/L	Var. 890	Var. 930	Var. 970	Var. 1010	Var. 1050	Var. 1090	Var. 1130	Var. 1170	Var. 1210	Var. 1250	Var. 1290
	About 1 µg/L	Var. 891	Var. 931	Var. 971	Var. 1011	Var. 1051	Var. 1091	Var. 1131	Var. 1171	Var. 1211	Var. 1251	Var. 1291
	About 1.5 µg/L	Var. 892	Var. 932	Var. 972	Var. 1012	Var. 1052	Var. 1092	Var. 1132	Var. 1172	Var. 1212	Var. 1252	Var. 1292
	About 2 µg/L	Var. 893	Var. 933	Var. 973	Var. 1013	Var. 1053	Var. 1093	Var. 1133	Var. 1173	Var. 1213	Var. 1253	Var. 1293
	About 2.5 µg/L	Var. 894	Var. 934	Var. 974	Var. 1014	Var. 1054	Var. 1094	Var. 1134	Var. 1174	Var. 1214	Var. 1254	Var. 1294
	About 3 µg/L	Var. 895	Var. 935	Var. 975	Var. 1015	Var. 1055	Var. 1095	Var. 1135	Var. 1175	Var. 1215	Var. 1255	Var. 1295
	About 3.5 µg/L	Var. 896	Var. 936	Var. 976	Var. 1016	Var. 1056	Var. 1096	Var. 1136	Var. 1176	Var. 1216	Var. 1256	Var. 1296
	About 4 µg/L	Var. 897	Var. 937	Var. 977	Var. 1017	Var. 1057	Var. 1097	Var. 1137	Var. 1177	Var. 1217	Var. 1257	Var. 1297
	About 4.5 µg/L	Var. 898	Var. 938	Var. 978	Var. 1018	Var. 1058	Var. 1098	Var. 1138	Var. 1178	Var. 1218	Var. 1258	Var. 1298
	About 5 µg/L	Var. 899	Var. 939	Var. 979	Var. 1019	Var. 1059	Var. 1099	Var. 1139	Var. 1179	Var. 1219	Var. 1259	Var. 1299
	About 5.5 µg/L	Var. 900	Var. 940	Var. 980	Var. 1020	Var. 1060	Var. 1100	Var. 1140	Var. 1180	Var. 1220	Var. 1260	Var. 1300
	About 6 µg/L	Var. 901	Var. 941	Var. 981	Var. 1021	Var. 1061	Var. 1101	Var. 1141	Var. 1181	Var. 1221	Var. 1261	Var. 1301
	About 7 µg/L	Var. 902	Var. 942	Var. 982	Var. 1022	Var. 1062	Var. 1102	Var. 1142	Var. 1182	Var. 1222	Var. 1262	Var. 1302
	About 8 µg/L	Var. 903	Var. 943	Var. 983	Var. 1023	Var. 1063	Var. 1103	Var. 1143	Var. 1183	Var. 1223	Var. 1263	Var. 1303
	About 9 µg/L	Var. 904	Var. 944	Var. 984	Var. 1024	Var. 1064	Var. 1104	Var. 1144	Var. 1184	Var. 1224	Var. 1264	Var. 1304
	About 10 µg/L	Var. 905	Var. 945	Var. 985	Var. 1025	Var. 1065	Var. 1105	Var. 1145	Var. 1185	Var. 1225	Var. 1265	Var. 1305
	1-20 µg/L	Var. 906	Var. 946	Var. 986	Var. 1026	Var. 1066	Var. 1106	Var. 1146	Var. 1186	Var. 1226	Var. 1266	Var. 1306
	2-20 µg/L	Var. 907	Var. 947	Var. 987	Var. 1027	Var. 1067	Var. 1107	Var. 1147	Var. 1187	Var. 1227	Var. 1267	Var. 1307
	1-10 µg/L	Var. 908	Var. 948	Var. 988	Var. 1028	Var. 1068	Var. 1108	Var. 1148	Var. 1188	Var. 1228	Var. 1268	Var. 1308
	2-10 µg/L	Var. 909	Var. 949	Var. 989	Var. 1029	Var. 1069	Var. 1109	Var. 1149	Var. 1189	Var. 1229	Var. 1269	Var. 1309

	1-6 µg/L	Var. 910	Var. 950	Var. 990	Var. 1030	Var. 1070	Var. 1110	Var. 1150	Var. 1190	Var. 1230	Var. 1270	Var. 1310
	2-6 µg/L	Var. 911	Var. 951	Var. 991	Var. 1031	Var. 1071	Var. 1111	Var. 1151	Var. 1191	Var. 1231	Var. 1271	Var. 1311
	3-6 µg/L	Var. 912	Var. 952	Var. 992	Var. 1032	Var. 1072	Var. 1112	Var. 1152	Var. 1192	Var. 1232	Var. 1272	Var. 1312
	4-6 µg/L	Var. 913	Var. 953	Var. 993	Var. 1033	Var. 1073	Var. 1113	Var. 1153	Var. 1193	Var. 1233	Var. 1273	Var. 1313
	1-5 µg/L	Var. 914	Var. 954	Var. 994	Var. 1034	Var. 1074	Var. 1114	Var. 1154	Var. 1194	Var. 1234	Var. 1274	Var. 1314
	2-5 µg/L	Var. 915	Var. 955	Var. 995	Var. 1035	Var. 1075	Var. 1115	Var. 1155	Var. 1195	Var. 1235	Var. 1275	Var. 1315
	3-5 µg/L	Var. 916	Var. 956	Var. 996	Var. 1036	Var. 1076	Var. 1116	Var. 1156	Var. 1196	Var. 1236	Var. 1276	Var. 1316
	4-5 µg/L	Var. 917	Var. 957	Var. 997	Var. 1037	Var. 1077	Var. 1117	Var. 1157	Var. 1197	Var. 1237	Var. 1277	Var. 1317
	1-4 µg/L	Var. 918	Var. 958	Var. 998	Var. 1038	Var. 1078	Var. 1118	Var. 1158	Var. 1198	Var. 1238	Var. 1278	Var. 1318
	2-4 µg/L	Var. 919	Var. 959	Var. 999	Var. 1039	Var. 1079	Var. 1119	Var. 1159	Var. 1199	Var. 1239	Var. 1279	Var. 1319
	3-4 µg/L	Var. 920	Var. 960	Var. 1000	Var. 1040	Var. 1080	Var. 1120	Var. 1160	Var. 1200	Var. 1240	Var. 1280	Var. 1320

*AL = at least

[0154] In one embodiment, the cell culture solution further comprises a low ammonium concentration. In one embodiment, the cell culture solution comprises an ammonium concentration of less than 10 mM and a copper and calcium concentration according to any one of variations 881 to 1320, as set forth in **Table 3**. In a specific embodiment, the ammonium is maintained at a concentration of no more than 10 mM for at least 7 days. In a preferred embodiment, the cell culture medium comprises an ammonium concentration of no more than 6 mM and a copper and calcium concentration according to any one of variations 881 to 1320, as set forth in **Table 3**. In a specific embodiment, the ammonium is maintained at a concentration of no more than 6 mM for at least 7 days. In another preferred embodiment, the cell culture medium comprises an ammonium concentration of no more than 5 mM and a copper and calcium concentration according to any one of variations 881 to 1320, as set forth in **Table 3**. In a specific embodiment, the ammonium is maintained at a concentration of no more than 5 mM for at least 7 days. In another preferred embodiment, the cell culture medium comprises an ammonium concentration of no more than 4 mM and a copper and calcium concentration according to any one of variations 881 to 1320, as set forth in **Table 3**. In a specific embodiment, the ammonium is maintained at a concentration of no more than 4 mM for at least 7 days. In yet other embodiments, the cell culture medium comprises an ammonium concentration

of no more than 10 mM, or no more than 9 mM, 8 mM, 7 mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, or less and a copper and calcium concentration according to any one of variations 881 to 1320, as set forth in **Table 3**. In yet another specific embodiment, the ammonium concentration of the cell culture is maintained at a low level for the duration of the process (*i.e.*, for the entire time the culture is being used to produce rA13).

[0155] In one embodiment, the cell culture medium is supplemented with copper, zinc and calcium. In a specific embodiment, the culture medium has a calcium concentration of at least 0.5 mM and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In another specific embodiment, the culture medium has a calcium concentration of at least 1.5 mM and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In another specific embodiment, the culture medium has a calcium concentration between 0.5 mM and 1.5 mM and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In yet other embodiments, the culture medium has a calcium concentration of at least 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1.0 mM, 1.1 mM, 1.2 mM, 1.3 mM, 1.4 mM, 1.5 mM, 1.6 mM, 1.7 mM, 1.8 mM, 1.9 mM, 2.0 mM, 2.25 mM, 2.5 mM, 2.75 mM, 3.0 mM, 3.5 mM, 4.0 mM, 4.5 mM, 5.0 mM, or more, and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**.

[0156] In one embodiment, a culture medium is provided for the expression of a recombinant ADAMTS protein (*e.g.*, rADAMTS13) containing at least 1 μg/L copper and at least 2 mg/L nicotinamide (vitamin B3). In other embodiments, the media contains at least 2 μg/L copper or at least 4 μg/L copper. In another embodiment wherein the media is supplemented with copper, the culture medium also contains at least 7 mg/L nicotinamide (vitamin B3). In one embodiment, the culture medium contains at or about between 2 mg/L and 10 mg/L nicotinamide (vitamin B3). In yet other embodiments, the culture medium may contain at least at or about 2 mg/L, 3 mg/L, 4 mg/L, 5 mg/L, 6 mg/L, 7 mg/L, 8 mg/L, 9 mg/L, 10 mg/L, 15 mg/L, 20 mg/L, or higher concentrations of nicotinamide (vitamin B3). In one embodiment, the culture medium contains a copper and nicotinamide concentration according to any one of variations 1321 to 1760, as set forth in **Table 4**.

Table 4. Exemplary embodiments of copper and nicotinamide concentrations present in culture media useful for the expression of a recombinant ADAMTS13 protein.

		Calcium Concentration										
		AL 2 mg/mL	AL 3 mg/mL	AL 4 mg/mL	AL 5 mg/mL	AL 6 mg/mL	AL 7 mg/mL	AL 8 mg/mL	AL 9 mg/mL	AL 10 mg/mL	AL 15 mg/mL	2-10 mg/mL
Copper Concentration	AL 1 µg/L	Var. 1321	Var. 1361	Var. 1401	Var. 1441	Var. 1481	Var. 1521	Var. 1561	Var. 1601	Var. 1641	Var. 1681	Var. 1721
	AL 2 µg/L	Var. 1322	Var. 1362	Var. 1402	Var. 1442	Var. 1482	Var. 1522	Var. 1562	Var. 1602	Var. 1642	Var. 1682	Var. 1722
	AL 3 µg/L	Var. 1323	Var. 1363	Var. 1403	Var. 1443	Var. 1483	Var. 1523	Var. 1563	Var. 1603	Var. 1643	Var. 1683	Var. 1723
	AL 4 µg/L	Var. 1324	Var. 1364	Var. 1404	Var. 1444	Var. 1484	Var. 1524	Var. 1564	Var. 1604	Var. 1644	Var. 1684	Var. 1724
	AL 5 µg/L	Var. 1325	Var. 1365	Var. 1405	Var. 1445	Var. 1485	Var. 1525	Var. 1565	Var. 1605	Var. 1645	Var. 1685	Var. 1725
	AL 6 µg/L	Var. 1326	Var. 1366	Var. 1406	Var. 1446	Var. 1486	Var. 1526	Var. 1566	Var. 1606	Var. 1646	Var. 1686	Var. 1726
	AL 7 µg/L	Var. 1327	Var. 1367	Var. 1407	Var. 1447	Var. 1487	Var. 1527	Var. 1567	Var. 1607	Var. 1647	Var. 1687	Var. 1727
	AL 8 µg/L	Var. 1328	Var. 1368	Var. 1408	Var. 1448	Var. 1488	Var. 1528	Var. 1568	Var. 1608	Var. 1648	Var. 1688	Var. 1728
	AL 9 µg/L	Var. 1329	Var. 1369	Var. 1409	Var. 1449	Var. 1489	Var. 1529	Var. 1569	Var. 1609	Var. 1649	Var. 1689	Var. 1729
	AL 10 µg/L	Var. 1330	Var. 1370	Var. 1410	Var. 1450	Var. 1490	Var. 1530	Var. 1570	Var. 1610	Var. 1650	Var. 1690	Var. 1730
	About 1 µg/L	Var. 1331	Var. 1371	Var. 1411	Var. 1451	Var. 1491	Var. 1531	Var. 1571	Var. 1611	Var. 1651	Var. 1691	Var. 1731
	About 1.5 µg/L	Var. 1332	Var. 1372	Var. 1412	Var. 1452	Var. 1492	Var. 1532	Var. 1572	Var. 1612	Var. 1652	Var. 1692	Var. 1732
	About 2 µg/L	Var. 1333	Var. 1373	Var. 1413	Var. 1453	Var. 1493	Var. 1533	Var. 1573	Var. 1613	Var. 1653	Var. 1693	Var. 1733
	About 2.5 µg/L	Var. 1334	Var. 1374	Var. 1414	Var. 1454	Var. 1494	Var. 1534	Var. 1574	Var. 1614	Var. 1654	Var. 1694	Var. 1734
	About 3 µg/L	Var. 1335	Var. 1375	Var. 1415	Var. 1455	Var. 1495	Var. 1535	Var. 1575	Var. 1615	Var. 1655	Var. 1695	Var. 1735
	About 3.5 µg/L	Var. 1336	Var. 1376	Var. 1416	Var. 1456	Var. 1496	Var. 1536	Var. 1576	Var. 1616	Var. 1656	Var. 1696	Var. 1736
	About 4 µg/L	Var. 1337	Var. 1377	Var. 1417	Var. 1457	Var. 1497	Var. 1537	Var. 1577	Var. 1617	Var. 1657	Var. 1697	Var. 1737
	About 4.5 µg/L	Var. 1338	Var. 1378	Var. 1418	Var. 1458	Var. 1498	Var. 1538	Var. 1578	Var. 1618	Var. 1658	Var. 1698	Var. 1738

	About 5 µg/L	Var. 1339	Var. 1379	Var. 1419	Var. 1459	Var. 1499	Var. 1539	Var. 1579	Var. 1619	Var. 1659	Var. 1699	Var. 1739
	About 5.5 µg/L	Var. 1340	Var. 1380	Var. 1420	Var. 1460	Var. 1500	Var. 1540	Var. 1580	Var. 1620	Var. 1660	Var. 1700	Var. 1740
	About 6 µg/L	Var. 1341	Var. 1381	Var. 1421	Var. 1461	Var. 1501	Var. 1541	Var. 1581	Var. 1621	Var. 1661	Var. 1701	Var. 1741
	About 7 µg/L	Var. 1342	Var. 1382	Var. 1422	Var. 1462	Var. 1502	Var. 1542	Var. 1582	Var. 1622	Var. 1662	Var. 1702	Var. 1742
	About 8 µg/L	Var. 1343	Var. 1383	Var. 1423	Var. 1463	Var. 1503	Var. 1543	Var. 1583	Var. 1623	Var. 1663	Var. 1703	Var. 1743
	About 9 µg/L	Var. 1344	Var. 1384	Var. 1424	Var. 1464	Var. 1504	Var. 1544	Var. 1584	Var. 1624	Var. 1664	Var. 1704	Var. 1744
	About 10 µg/L	Var. 1345	Var. 1385	Var. 1425	Var. 1465	Var. 1505	Var. 1545	Var. 1585	Var. 1625	Var. 1665	Var. 1705	Var. 1745
	1-20 µg/L	Var. 1346	Var. 1386	Var. 1426	Var. 1466	Var. 1506	Var. 1546	Var. 1586	Var. 1626	Var. 1666	Var. 1706	Var. 1746
	2-20 µg/L	Var. 1347	Var. 1387	Var. 1427	Var. 1467	Var. 1507	Var. 1547	Var. 1587	Var. 1627	Var. 1667	Var. 1707	Var. 1747
	1-10 µg/L	Var. 1348	Var. 1388	Var. 1428	Var. 1468	Var. 1508	Var. 1548	Var. 1588	Var. 1628	Var. 1668	Var. 1708	Var. 1748
	2-10 µg/L	Var. 1349	Var. 1389	Var. 1429	Var. 1469	Var. 1509	Var. 1549	Var. 1589	Var. 1629	Var. 1669	Var. 1709	Var. 1749
	1-6 µg/L	Var. 1350	Var. 1390	Var. 1430	Var. 1470	Var. 1510	Var. 1550	Var. 1590	Var. 1630	Var. 1670	Var. 1710	Var. 1750
	2-6 µg/L	Var. 1351	Var. 1391	Var. 1431	Var. 1471	Var. 1511	Var. 1551	Var. 1591	Var. 1631	Var. 1671	Var. 1711	Var. 1751
	3-6 µg/L	Var. 1352	Var. 1392	Var. 1432	Var. 1472	Var. 1512	Var. 1552	Var. 1592	Var. 1632	Var. 1672	Var. 1712	Var. 1752
	4-6 µg/L	Var. 1353	Var. 1393	Var. 1433	Var. 1473	Var. 1513	Var. 1553	Var. 1593	Var. 1633	Var. 1673	Var. 1713	Var. 1753
	1-5 µg/L	Var. 1354	Var. 1394	Var. 1434	Var. 1474	Var. 1514	Var. 1554	Var. 1594	Var. 1634	Var. 1674	Var. 1714	Var. 1754
	2-5 µg/L	Var. 1355	Var. 1395	Var. 1435	Var. 1475	Var. 1515	Var. 1555	Var. 1595	Var. 1635	Var. 1675	Var. 1715	Var. 1755
	3-5 µg/L	Var. 1356	Var. 1396	Var. 1436	Var. 1476	Var. 1516	Var. 1556	Var. 1596	Var. 1636	Var. 1676	Var. 1716	Var. 1756
	4-5 µg/L	Var. 1357	Var. 1397	Var. 1437	Var. 1477	Var. 1517	Var. 1557	Var. 1597	Var. 1637	Var. 1677	Var. 1717	Var. 1757
	1-4 µg/L	Var. 1358	Var. 1398	Var. 1438	Var. 1478	Var. 1518	Var. 1558	Var. 1598	Var. 1638	Var. 1678	Var. 1718	Var. 1758
	2-4 µg/L	Var. 1359	Var. 1399	Var. 1439	Var. 1479	Var. 1519	Var. 1559	Var. 1599	Var. 1639	Var. 1679	Var. 1719	Var. 1759
	3-4 µg/L	Var. 1360	Var. 1400	Var. 1440	Var. 1480	Var. 1520	Var. 1560	Var. 1600	Var. 1640	Var. 1680	Var. 1720	Var. 1760

*AL = at least

[0157] In one embodiment, the cell culture solution further comprises a low ammonium concentration. In one embodiment, the culture medium comprises an ammonium concentration

of less than 10 mM and a copper and nicotinamide concentration according to any one of variations 1321 to 1760, as set forth in **Table 4**. In a specific embodiment, the ammonium is maintained at a concentration of no more than 10 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 6 mM and a copper and nicotinamide concentration according to any one of variations 1321 to 1760, as set forth in **Table 4**. In a specific embodiment, the ammonium is maintained at a concentration of no more than 6 mM for at least 7 days. In another preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 5 mM and a copper and nicotinamide concentration according to any one of variations 1321 to 1760, as set forth in **Table 4**. In a specific embodiment, the ammonium is maintained at a concentration of no more than 5 mM for at least 7 days. In another preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 4 mM and a copper and nicotinamide concentration according to any one of variations 1321 to 1760, as set forth in **Table 4**. In a specific embodiment, the ammonium is maintained at a concentration of no more than 4 mM for at least 7 days. In yet other embodiments, the cell culture solution comprises an ammonium concentration of no more than 10 mM, or no more than 9 mM, 8 mM, 7 mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, or less and a copper and nicotinamide concentration according to any one of variations 1321 to 1760, as set forth in **Table 4**. In yet another specific embodiment, the ammonium concentration of the cell culture is maintained at a low level for the duration of the process (*i.e.*, for the entire time the culture is being used to produce rA13).

[0158] In one embodiment, the cell culture medium is supplemented with copper, zinc and nicotinamide. In a specific embodiment, the culture medium has a nicotinamide concentration of at least 2 mg/mL and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In another specific embodiment, the culture medium has a nicotinamide concentration of at least 7 mg/mL mM and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In another specific embodiment, the culture medium has a nicotinamide concentration between 2 mg/mL and 10 mg/mL and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In yet other embodiments, the culture medium has a nicotinamide concentration of at least 2 mg/mL, 3 mg/mL, 4 mg/mL, 5 mg/mL, 6 mg/mL, 7 mg/mL, 8 mg/mL, 9 mg/mL, 10 mg/mL, 11 mg/mL, 12 mg/mL, 13 mg/mL, 14 mg/mL, 15 mg/mL, or more, and a

copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**.

[0159] In one embodiment, the cell culture medium is supplemented with copper, calcium and nicotinamide. In a specific embodiment, the culture medium has a nicotinamide concentration of at least 2 mg/mL and a copper and calcium concentration according to any one of variations 881 to 1320, as set forth in **Table 3**. In another specific embodiment, the culture medium has a nicotinamide concentration of at least 7 mg/mL mM and a copper and calcium concentration according to any one of variations 881 to 1320, as set forth in **Table 3**. In another specific embodiment, the culture medium has a nicotinamide concentration between 2 mg/mL and 10 mg/mL and a copper and calcium concentration according to any one of variations 881 to 1320, as set forth in **Table 3**. In yet other embodiments, the culture medium has a nicotinamide concentration of at least 2 mg/mL, 3 mg/mL, 4 mg/mL, 5 mg/mL, 6 mg/mL, 7 mg/mL, 8 mg/mL, 9 mg/mL, 10 mg/mL, 11 mg/mL, 12 mg/mL, 13 mg/mL, 14 mg/mL, 15 mg/mL, or more, and a copper and calcium concentration according to any one of variations 881 to 1320, as set forth in **Table 3**.

IV. Methods for the Production of Blood Factors Having High Specific Activity

A. Cell Cultivation Methods

[0160] The present invention provides methods for large-scale production of recombinant proteins (such as rVWF and rA13). In certain embodiments, such large-scale production methods utilize stirred/agitated tank reactors for manufacture of these therapeutic recombinant proteins.

[0161] In certain embodiments, the methods of the present invention can comprise the use of a cell culture system operated under a batch or continuous mode of operation. For example, when batch cell cultures are utilized, they may be operated under single batch, fed-batch, or repeated-batch mode. Likewise, continuous cell cultures may be operated under, for example, perfusion, turbidostat or chemostat mode. Batch and continuous cell cultivation may be performed under either suspension or adherence conditions. When operated under suspension conditions, the cells will be freely suspended and mixed within the culture medium. Alternatively, under adherence

conditions, the cells will be bound to a solid phase, for example, a microcarrier, a porous microcarrier, disk carrier, ceramic cartridge, hollow fiber, flat sheet, gel matrix, and the like.

[0162] A batch culture is typically a large scale cell culture in which a cell inoculum is cultured to a maximum density in a tank or fermenter, and harvested and processed as a single batch. A fed-batch culture it typically a batch culture which is supplied with either fresh nutrients (*e.g.*, growth-limiting substrates) or additives (*e.g.*, precursors to products). The feed solution is usually highly concentrated to avoid dilution of the bioreactor. In a repeated-batch culture, the cells are placed in a culture medium and grown to a desired cell density. To avoid the onset of a decline phase and cell death, the culture is then diluted with complete growth medium before the cells reach their maximum concentration. The amount and frequency of dilution varies widely and depends on the growth characteristics of the cell line and convenience of the culture process. The process can be repeated as many times as required and, unless cells and medium are discarded at subculture, the volume of culture will increase stepwise as each dilution is made. The increasing volume may be handled by having a reactor of sufficient size to allow dilutions within the vessel or by dividing the diluted culture into several vessels. The rationale of this type of culture is to maintain the cells in an exponentially growing state. Serial subculture is characterized in that the volume of culture is always increasing stepwise, there can be multiple harvests, the cells continue to grow and the process can continue for as long as desired. In certain embodiments, a recombinant ADAMTS protein (*e.g.*, rADAMTS13) may be recovered after harvesting the supernatant of a batch culture. In other embodiments, a recombinant VWF may be recovered after harvesting the supernatant of a batch culture.

[0163] A continuous culture can be a suspension culture that is continuously supplied with nutrients by the inflow of fresh medium, wherein the culture volume is usually kept constant by the concomitant removal of spent medium. In chemostat and turbidostat methods, the extracted medium contains cells. Thus, the cells remaining in the cell culture vessel must grow to maintain a steady state. In the chemostat method, the growth rate is typically controlled by controlling the dilution rate, *i.e.*, the rate at which fresh medium is added. The growth rate of the cells in the culture may be controlled, for example, at a sub-maximal growth rate, by alteration of the dilution rate. In contrast, in the turbidostat method, the dilution rate is set to permit the maximum growth rate that the cells can achieve at the given operating conditions, such as pH

and temperature. In certain embodiments, the rVWF or rA13 is recovered after harvesting the supernatant of a continuous culture. An exemplary method for continuous cell cultivation is described in WO/2011/012725 (Grillberger et al.), the content of which is hereby incorporated by reference in its entirety for all purposes.

[0164] In a perfusion culture, the extracted medium is depleted of cells, which are retained in the culture vessel, for example, by filtration or by centrifugal methods that lead to the reintroduction of the cells into the culture. However, typically membranes used for filtration do not retain 100% of cells, and so a proportion is removed when the medium is extracted. It may not be crucial to operate perfusion cultures at very high growth rates, as the majority of the cells are retained in the culture vessel. In certain embodiments, the rVWF or rA13 is recovered after harvesting the supernatant of a perfusion culture.

[0165] Stirred-tank reactor system can be used for batch and continuous cell cultures operated under suspension or adherent modes. Generally, the stirred-tank reactor system can be operated as any conventional stirred-tank reactor with any type of agitator such as a Rushton, hydrofoil, pitched blade, or marine.

[0166] In certain embodiments, the cell-culture methods of the invention may comprise the use of a microcarrier. In some embodiments, the cell-cultures of the embodiments can be performed in large bioreactors under conditions suitable for providing high volume-specific culture surface areas to achieve high cell densities and protein expression. One means for providing such growth conditions is to use microcarriers for cell-culture in stirred tank bioreactors. The concept of cell-growth on microcarriers was first described by van Wezel (van Wezel, A.L., Nature 216:64-5 (1967)) and allows for cell attachment on the surface of small solid particles suspended in the growth medium. These methods provide for high surface-to-volume ratios and thus allow for efficient nutrient utilization. Furthermore, for expression of secreted proteins in eukaryotic cell lines, the increased surface-to-volume ratio allows for higher levels of secretion and thus higher protein yields in the supernatant of the culture. Finally, these methods allow for the easy scale-up of eukaryotic expression cultures.

[0167] The cells expressing vWF and/or rA13 can be bound to a spherical or a porous microcarrier during cell culture growth. The microcarrier can be a microcarrier selected from the group of microcarriers based on dextran, collagen, plastic, gelatine and cellulose and others as

described in Butler (1988. In: Spier & Griffiths, Animal Cell Biotechnology 3:283-303). It is also possible to grow the cells to a biomass on spherical microcarriers and subculture the cells when they have reached final fermenter biomass and prior to production of the expressed protein on a porous microcarrier or vice versa. Suitable spherical microcarriers can include smooth surface microcarriers, such as Cytodex™ 1, Cytodex™ 2, and Cytodex™ 3 (GE Healthcare) and macroporous microcarriers such as Cytopore™ 1, Cytopore™ 2, Cytoline™ 1, and Cytoline™ 2 (GE Healthcare).

[0168] As described above, the present invention includes cell culture media having an increased copper concentration. It is understood that all of the embodiments and concentrations described in the "Cell Culture Media" section above can be applied to the methods of the present invention described herein.

[0169] In certain embodiments, the culture can be maintained for at least about 7 days, or at least about 14 days, 21 days, 28 days, or at least about 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, or at least about 2 months, or 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 months or longer. The cell density at which a cell-culture is maintained at for production of a recombinant vWF or recombinant rA13 protein will depend upon the culture-conditions and medium used for protein expression. One of skill in the art will readily be able to determine the optimal cell density for a cell-culture producing rVWF or rA13. In one embodiment, the culture is maintained at a cell density of between about $0.5x10^6$ and $4x10^7$ cells/mL for an extended period of time. In other embodiments, the cell density is maintained at a concentration of between about $1.0x10^6$ and about $1.0x10^7$ cells/mL for an extended period of time. In other embodiments, the cell density is maintained at a concentration of between about $1.0x10^6$ and about $4.0x10^6$ cells/mL for an extended period of time. In other embodiments, the cell density is maintained at a concentration of between about $1.0x10^6$ and about $4.0x10^6$ cells/mL for an extended period of time. In yet other embodiments, the cell density may be maintained at a concentration between about $2.0x10^6$ and about $4.0x10^6$ cells/mL, or between about $1.0x10^6$ and about $2.5x10^6$ cells/mL, or between about $1.5x10^6$ and about $3.5x10^6$ cells/mL, or any other similar range, for an extended period of time.

[0170] In one embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $4.0x10^6$ cells/mL for at least 7 days. In a specific

embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $4.0x10^6$ cells/mL for at least 14 days. In a more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $4.0x10^6$ cells/mL for at least 21 days. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $4.0x10^6$ cells/mL for at least 28 days. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $4.0x10^6$ cells/mL for at least 5 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $4.0x10^6$ cells/mL for at least 6 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $4.0x10^6$ cells/mL for at least 7 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $4.0x10^6$ cells/mL for at least 8 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $4.0x10^6$ cells/mL for at least 9 weeks.

[0171] In one embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.5x10^6$ cells/mL for at least 7 days. In a specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.5x10^6$ cells/mL for at least 14 days. In a more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.5x10^6$ cells/mL for at least 21 days. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.5x10^6$ cells/mL for at least 28 days. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.5x10^6$ cells/mL for at least 5 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.5x10^6$ cells/mL for at least 6 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.5x10^6$ cells/mL for at least 7 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a

concentration of no more than $3.5x10^6$ cells/mL for at least 8 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.5x10^6$ cells/mL for at least 9 weeks.

[0172] In one embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.0x10^6$ cells/mL for at least 7 days. In a specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.0x10^6$ cells/mL for at least 14 days. In a more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.0x10^6$ cells/mL for at least 21 days. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.0x10^6$ cells/mL for at least 28 days. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.0x10^6$ cells/mL for at least 5 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.0x10^6$ cells/mL for at least 6 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.0x10^6$ cells/mL for at least 7 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.0x10^6$ cells/mL for at least 8 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $3.0x10^6$ cells/mL for at least 9 weeks.

[0173] In one embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.5x10^6$ cells/mL for at least 7 days. In a specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.5x10^6$ cells/mL for at least 14 days. In a more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.5x10^6$ cells/mL for at least 21 days. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.5x10^6$ cells/mL for at least 28 days. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a

concentration of no more than $2.5x10^6$ cells/mL for at least 5 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.5x10^6$ cells/mL for at least 6 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.5x10^6$ cells/mL for at least 7 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.5x10^6$ cells/mL for at least 8 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.5x10^6$ cells/mL for at least 9 weeks.

[0174] In another embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.0x10^6$ cells/mL for at least 7 days. In a specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.0x10^6$ cells/mL for at least 14 days. In a more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.0x10^6$ cells/mL for at least 21 days. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.0x10^6$ cells/mL for at least 28 days. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.0x10^6$ cells/mL for at least 5 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.0x10^6$ cells/mL for at least 6 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.0x10^6$ cells/mL for at least 7 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.0x10^6$ cells/mL for at least 8 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $2.0x10^6$ cells/mL for at least 9 weeks.

[0175] In one embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $1.5x10^6$ cells/mL for at least 7 days. In a specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a

concentration of no more than $1.5x10^6$ cells/mL for at least 14 days. In a more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $1.5x10^6$ cells/mL for at least 21 days. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $1.5x10^6$ cells/mL for at least 28 days. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $1.5x10^6$ cells/mL for at least 5 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $1.5x10^6$ cells/mL for at least 6 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $1.5x10^6$ cells/mL for at least 7 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $1.5x10^6$ cells/mL for at least 8 weeks. In a yet more specific embodiment, the cell density of a continuous cell culture provided herein is maintained at a concentration of no more than $1.5x10^6$ cells/mL for at least 9 weeks.

[0176] The following provides specific details on methods for producing rVWF and rA13. As will be appreciated, although the conditions are presented specifically for rVWF or rA13, the conditions for rVWF can be used for producing rA13 and vice versa.

B. Methods of Producing High Molecular Weight Recombinant vWF

[0177] In another aspect, the present invention further relates to methods for producing vWF under cell culture conditions comprising a cell culture medium having an increased copper concentration. In certain embodiments, the culture also comprises a low ammonium concentration. As used herein, the term "cell culture" and "cell culture solution" are used interchangeably.

[0178] In one embodiment, the present invention provides a method of producing a high molecular weight, recombinant vWF, comprising: a) providing a culture of cells; b) introducing a nucleic acid sequence coding for vWF; c) selecting the cells carrying the nucleic acid sequence; and, d) expressing vWF in the cells under cell culture conditions comprising a cell culture medium comprising a copper concentration of at least about 2.4 μg/L and a cell culture supernatant comprising an ammonium concentration less than about 10 mM, wherein the vWF is

highly multimeric vWF comprising about 14 to about 22 dimers and a specific Ristocetin activity of at least about 30 mU/μg. In further embodiments, the multimeric rVWF produced using methods of the present invention comprise about 10-30, 12-28, 14-26, 16-24, 18-22, 20-21 dimers. In still further embodiments, the rVWF produced in accordance with the present invention has a specific activity of at least about 20, 22.5, 25, 27.5, 30, 32.5, 35, 37.5, 40, 42.5, 45, 47.5, 50, 52.5, 55, 57.5, 60, 62.5, 65, 67.5, 70, 72.5, 75, 77.5, 80, or more mU/μg. In a specific embodiment, the cell density of the continuous cell culture for production of rVWF is maintained at a concentration of no more than $2.5x10^6$ cells/mL for an extended period. In other specific embodiments, the cell density is maintained at no more than $2.0x10^6$ cells/mL, $1.5x10^6$ cells/mL, $1.0x10^6$ cells/mL, $0.5x10^6$ cells/mL, or less. In one embodiment, the cell density is maintained at between $1.5x10^6$ cells/mL and $2.5x10^6$ cells/mL.

[0179] In one embodiment, the present invention provides a method for producing a recombinant Von Willebrand Factor (rVWF) composition, the method comprising the steps of: (a) providing a basal cell culture media; (b) supplementing the basal cell culture media with copper to provide a final copper concentration of at least 2.0 μg/L; (c) providing one or more cells comprising a nucleic acid encoding a rVWF protein; (d) culturing the one or more cells in the copper supplemented cell culture media such that rVWF is expressed and excreted from the cells into a culture supernatant; and (e) recovering at least a portion of the culture supernatant, wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 30 mU/μg rVWF. In one embodiment, the cell culture solution further comprises an ammonium concentration of less than 10 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 10 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 5 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 5 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 4 mM. In another specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 4 mM for at least 7 days. In yet other embodiments, the cell culture solution comprises an ammonium concentration of no more than 10 mM, or no more than 9 mM, 8 mM, 7 mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, or less. In yet

another embodiment, the ammonium concentration of the cell culture is maintained at a low level for the duration of the process (*i.e.*, for the entire time the culture is being used to produce rVWF). In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0180] In one embodiment of the method described above, the basal cell culture media is supplemented with copper to provide a final copper concentration of at least 2.4 μg/L. In one embodiment, the cell culture solution further comprises an ammonium concentration of less than 10 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 10 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 5 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 5 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 4 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 4 mM for at least 7 days. In yet other embodiments, the cell culture solution comprises an ammonium concentration of no more than 10 mM, or no more than 9 mM, 8 mM, 7 mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, or less. In yet another embodiment, the ammonium concentration of the cell culture solution is maintained at a low level for the duration of the process (*i.e.*, for the entire time the culture is being used to produce rVWF). In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0181] In one embodiment of the method described above, the basal cell culture media is supplemented with copper to provide a final copper concentration of at least 3 μg/L. In one embodiment, the cell culture solution further comprises an ammonium concentration of less than 10 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 10 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no

more than 5 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 5 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 4 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 4 mM for at least 7 days. In yet other embodiments, the cell culture solution comprises an ammonium concentration of no more than 10 mM, or no more than 9 mM, 8 mM, 7 mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, or less. In yet another embodiment, the ammonium concentration of the cell culture solution is maintained at a low level for the duration of the process (*i.e.*, for the entire time the culture is being used to produce rVWF). In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0182] In one embodiment of the method described above, the basal cell culture media is supplemented with copper to provide a final copper concentration of at least 4 μg/L. In one embodiment, the cell culture solution further comprises an ammonium concentration of less than 10 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 10 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 5 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 5 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 4 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 4 mM for at least 7 days. In yet other embodiments, the cell culture media comprises an ammonium concentration of no more than 10 mM, or no more than 9 mM, 8 mM, 7 mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, or less. In yet another embodiment, the ammonium concentration of the cell culture solution is maintained at a low level for the duration of the process (*i.e.*, for the entire time the culture is being used to produce rVWF). In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at

least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0183] In one embodiment of the method described above, the basal cell culture media is supplemented with copper to provide a final copper concentration of about 4.3 μg/L. In one embodiment, the cell culture solution further comprises an ammonium concentration of less than 10 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 10 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 5 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 5 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 4 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 4 mM for at least 7 days. In yet other embodiments, the cell culture solution comprises an ammonium concentration of no more than 10 mM, or no more than 9 mM, 8 mM, 7 mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, or less. In yet another embodiment, the ammonium concentration of the cell culture solution is maintained at a low level for the duration of the process (*i.e.*, for the entire time the culture is being used to produce rVWF). In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0184] In one embodiment of the method described above, the basal cell culture media is supplemented with copper to provide a final copper concentration of between 2 μg/L and 20 μg/L. In one embodiment, the cell culture solution further comprises an ammonium concentration of less than 10 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 10 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 5 mM. In another specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 5 mM for at least 7 days. In a preferred embodiment, the cell culture solution

comprises an ammonium concentration of no more than 4 mM. In another specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 4 mM for at least 7 days. In yet other embodiments, the cell culture solution comprises an ammonium concentration of no more than 10 mM, or no more than 9 mM, 8 mM, 7 mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, or less. In yet another embodiment, the ammonium concentration of the cell culture is maintained at a low level for the duration of the process (*i.e.*, for the entire time the culture is being used to produce rVWF). In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0185] In one embodiment of the method described above, the basal cell culture media is supplemented with copper to provide a final copper concentration of between 3 μg/L and 10 μg/L. In one embodiment, the cell culture solution further comprises an ammonium concentration of less than 10 mM. In another specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 10 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 5 mM. In another specific embodiment, the ammonium concentration of the cell culture solution is maintained at an ammonium concentration of no more than 5 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 4 mM. In another specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 4 mM for at least 7 days. In yet other embodiments, the cell culture solution comprises an ammonium concentration of no more than 10 mM, or no more than 9 mM, 8 mM, 7 mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, or less. In yet another embodiment, the ammonium concentration of the cell culture solution is maintained at a low level for the duration of the process (*i.e.*, for the entire time the culture is being used to produce rVWF). In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0186] In one embodiment of the method described above, the basal cell culture media is supplemented with copper to provide a final copper concentration of between 4 μg/L and 7.5 μg/L. In one embodiment, the cell culture solution further comprises an ammonium concentration of less than 10 mM. In another specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 10 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 5 mM. In another specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 5 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 4 mM. In another specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 4 mM for at least 7 days. In yet other embodiments, the cell culture solution comprises an ammonium concentration of no more than 10 mM, or no more than 9 mM, 8 mM, 7 mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, or less. In yet another embodiment, the ammonium concentration of the cell culture is maintained at a low level for the duration of the process (*i.e.*, for the entire time the culture is being used to produce rVWF). In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0187] In one embodiment, the present invention provides a method for producing a recombinant Von Willebrand Factor (rVWF) composition, the method comprising the steps of: (a) providing a basal cell culture media; (b) supplementing the basal cell culture media with copper; (c) providing one or more cells comprising a nucleic acid encoding a rVWF protein; (d) culturing the one or more cells in the copper supplemented cell culture media such that rVWF is expressed and excreted from the cells into a culture supernatant; and (e) recovering at least a portion of the culture supernatant, wherein the NH_4^+ concentration of the supernatant is maintained at a low level for at least 7 days, and further wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 30 mU/μg rVWF. In a specific embodiment, the copper concentration and NH_4^+ concentration of the cell culture solution is maintained at a concentration according to any one of variations 1 to 440, as set forth in **Table 1**. In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg

rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0188] In one embodiment of the method described above, the copper concentration and NH_4^+ concentration of the cell culture is maintained at a concentration according to any one of variations 1 to 440, as set forth in **Table 1** for at least 14 days. In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0189] In one embodiment of the method described above, the copper concentration and NH_4^+ concentration of the cell culture is maintained at a concentration according to any one of variations 1 to 440, as set forth in **Table 1** for at least 21 days. In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0190] In one embodiment of the method described above, the copper concentration and NH_4^+ concentration of the cell culture is maintained at a concentration according to any one of variations 1 to 440, as set forth in **Table 1** for at least 28 days. In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0191] In one embodiment of the method described above, the copper concentration and NH_4^+ concentration of the cell culture is maintained at a concentration according to any one of variations 1 to 440, as set forth in **Table 1** for at least 5 weeks. In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0192] In one embodiment of the method described above, the copper concentration and NH_4^+ concentration of the cell culture is maintained at a concentration according to any one of variations 1 to 440, as set forth in **Table 1** for at least 6 weeks. In a preferred embodiment, the

recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0193] In one embodiment of the method described above, the copper concentration and NH_4^+ concentration of the cell culture is maintained at a concentration according to any one of variations 1 to 440, as set forth in **Table 1** for at least 7 weeks. In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0194] In one embodiment of the method described above, the copper concentration and NH_4^+ concentration of the cell culture is maintained at a concentration according to any one of variations 1 to 440, as set forth in **Table 1** for at least 8 weeks. In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0195] In one embodiment of the method described above, the copper concentration and NH_4^+ concentration of the cell culture is maintained at a concentration according to any one of variations 1 to 440, as set forth in **Table 1** for at least 9 weeks. In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0196] In one embodiment, the present invention provides a method for producing a recombinant Von Willebrand Factor (rVWF) composition, the method comprising the steps of: (a) providing a basal cell culture media; (b) supplementing the basal cell culture media with copper to provide a final copper concentration of at least 2.0 μg/L; (c) providing one or more cells comprising a nucleic acid encoding a rVWF protein; (d) culturing the one or more cells in the copper supplemented cell culture media such that rVWF is expressed and excreted from the cells into a culture supernatant; (e) monitoring the ammonium concentration of the culture supernatant; and (f) recovering at least a portion of the culture supernatant, wherein culture supernatant comprising an ammonium concentration of more than 10 mM is not used for producing the

rVWF composition, and further wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 30 mU/μg rVWF. In certain embodiments, the final copper concentration of the supplemented basal culture media is at least 2.4 μg/L, 3 μg/L, 4 μg/L, 5 μg/L, 6 μg/L, 7 μg/L, 8 μg/L, 9 μg/L, 10 μg/L, 15 μg/L, 20 μg/L, or higher. In other embodiments, the final copper concentration of the supplemented basal culture media is between 2-20 μg/L, 2-10 μg/L, 3-8 μg/L, or 4-6 μg/L. In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0197] In one embodiment of the method described above, culture supernatant comprising an ammonium concentration of more than 6 mM is not used for producing the rVWF composition. In certain embodiments, the final copper concentration of the supplemented basal culture media is at least 2.4 μg/L, 3 μg/L, 4 μg/L, 5 μg/L, 6 μg/L, 7 μg/L, 8 μg/L, 9 μg/L, 10 μg/L, 15 μg/L, 20 μg/L, or higher. In other embodiments, the final copper concentration of the supplemented basal culture media is between 2-20 μg/L, 2-10 μg/L, 3-8 μg/L, or 4-6 μg/L. In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0198] In one embodiment of the method described above, culture supernatant comprising an ammonium concentration of more than 5 mM is not used for producing the rVWF composition. In certain embodiments, the final copper concentration of the supplemented basal culture media is at least 2.4 μg/L, 3 μg/L, 4 μg/L, 5 μg/L, 6 μg/L, 7 μg/L, 8 μg/L, 9 μg/L, 10 μg/L, 15 μg/L, 20 μg/L, or higher. In other embodiments, the final copper concentration of the supplemented basal culture media is between 2-20 μg/L, 2-10 μg/L, 3-8 μg/L, or 4-6 μg/L. In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0199] In one embodiment of the method described above, culture supernatant comprising an ammonium concentration of more than 4 mM is not used for producing the rVWF composition. In certain embodiments, the final copper concentration of the supplemented basal culture media

is at least 2.4 μg/L, 3 μg/L, 4 μg/L, 5 μg/L, 6 μg/L, 7 μg/L, 8 μg/L, 9 μg/L, 10 μg/L, 15 μg/L, 20 μg/L, or higher. In other embodiments, the final copper concentration of the supplemented basal culture media is between 2-20 μg/L, 2-10 μg/L, 3-8 μg/L, or 4-6 μg/L. In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/μg rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/μg rVWF.

[0200] Recombinant vWF can be produced by expression in a suitable eukaryotic host system. Examples of eukaryotic cells include, without limitation, mammalian cells, such as CHO, COS, HEK 293, BHK, SK-Hep, and HepG2; insect cells, e.g., SF9 cells, SF21 cells, S2 cells, and High Five cells; and yeast cells, e.g., *Saccharomyces* or *Schizosaccharomyces* cells. In one embodiment, the vWF can be expressed in yeast cells, insect cells, avian cells, mammalian cells, and the like. For example, in a human cell line, a hamster cell line, or a murine cell line. In one particular embodiment, the cell line is a CHO, BHK, or HEK cell line. Typically, mammalian cells, e.g., CHO cells from a continuous cell line, can be used to express the vWF of the present invention.

[0201] In certain embodiments, the nucleic acid sequence comprising a sequence coding for vWF can be a vector. The vector can be delivered by a virus or can be a plasmid. The nucleic acid sequence coding for the protein can be a specific gene or a biologically functional part thereof. In one embodiment, the protein is at least a biologically active part of vWF.

[0202] A wide variety of vectors can be used for the expression of the vWF and can be selected from eukaryotic expression vectors. Examples of vectors for eukaryotic expression include: (i) for expression in yeast, vectors such as pAO, pPIC, pYES, pMET, using promoters such as AOX1, GAP, GAL1, AUG1, etc; (ii) for expression in insect cells, vectors such as pMT, pAc5, pIB, pMIB, pBAC, etc., using promoters such as PH, p10, MT, Ac5, OpIE2, gp64, polh, etc., and (iii) for expression in mammalian cells, vectors such as pSVL, pCMV, pRc/RSV, pcDNA3, pBPV, etc., and vectors derived from viral systems such as vaccinia virus, adeno-associated viruses, herpes viruses, retroviruses, etc., using promoters such as CMV, SV40, EF-1, UbC, RSV, ADV, BPV, and β-actin. An exemplary vector for expressing rVWF is described by Kaufman *et al.* (Mol Cell Biol. 1989 Mar;9(3):1233-42), the content of which is hereby incorporated by reference in its entirety for all purposes.

[0203] In some embodiments of the present invention, the nucleic acid sequence further comprises other sequences suitable for a controlled expression of a protein such as promoter sequences, enhancers, TATA boxes, transcription initiation sites, polylinkers, restriction sites, poly-A-sequences, protein processing sequences, selection markers, and the like which are generally known to a person of ordinary skill in the art.

[0204] In addition to cell culture media comprising an increased copper concentration, the cell culture conditions of the present invention can include an ammonium concentration of less than about 25 mM throughout an entire upstream process in culture systems. In one embodiment, the cell culture conditions include an ammonium concentration of less than about 25 mM, in another embodiment less than about 20 mM, in yet another embodiment less than about 15 mM, in yet another embodiment less than about 10 mM, and in a further embodiment less than about 5 mM.

[0205] In some embodiments, the ammonium concentrations of the present invention are kept constant throughout the entire upstream process of the cell culture system. The cells used according to the present invention can be cultivated, e.g., by methods that are modified as compared to conventional batch-cultivation and feed-batch-cultivation, each of which are generally known in the field. However, such conventional techniques can produce high concentrations of ammonium at the end of the culture. The methods of the present invention overcome this problem by employing production systems that can provide a continuous supply of culture media through techniques such as, e.g., perfusion or chemostat cultures. Following culture of the host cells, the vWF can be recovered from the spent medium using standard methodologies, such as ultrafiltration or centrifugation. If desired, the vWF can be purified by, e.g., ion exchange and/or size exclusion chromatography and the like.

[0206] A continuous culture (e.g., a perfusion or chemostat culture) can be a suspension culture that is continuously supplied with nutrients by the inflow of fresh medium, wherein the culture volume is usually constant. Similarly, continuous fermentation can refer to a process in which cells or micro-organisms are maintained in culture in the exponential growth phase by the continuous addition of fresh medium that is exactly balanced by the removal of cell suspension from the bioreactor. Furthermore, a stirred-tank reactor system can be used for suspension, perfusion, chemostatic, and/or microcarrier cultures. Generally, the stirred-tank reactor system

can be operated as any conventional stirred-tank reactor with any type of agitator such as a Rushton, hydrofoil, pitched blade, or marine.

C. Methods of Producing Recombinant ADAMTS13 (A13)

[0207] In another aspect, the present invention further relates to methods for producing rA13 under cell culture conditions comprising a cell culture medium having an increased copper concentration. In certain embodiments, the culture also comprises a low ammonium concentration.

[0208] In one embodiment, the present invention provides a method for producing a recombinant ADAMTS13 (rA13) composition, the method comprising the steps of: (a) providing a basal cell culture media; (b) supplementing the basal cell culture media with copper to provide a final copper concentration of at least 1.0 μg/L; (c) providing one or more cells comprising a nucleic acid encoding a rA13 protein; (d) culturing the one or more cells in the copper supplemented cell culture media such that rA13 is expressed and excreted from the cells into a culture supernatant; and (e) recovering at least a portion of the culture supernatant, wherein at least 1500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day (*i.e.*, 1500 Units FRETS-VWF73 activity per day per liter cell culture; P FRETS) is present in the recovered culture supernatant. In certain embodiments, the final copper concentration of the supplemented basal culture media is at least 2 μg/L, 3 μg/L, 4 μg/L, 5 μg/L, 6 μg/L, or higher. In other embodiments, the final copper concentration of the supplemented basal culture media is between 1-6 μg/L, 2-5 μg/L, 2-4 μg/L, or 3-4 μg/L. In a preferred embodiment, at least 2000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In a more preferred embodiment, at least 2500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In a most preferred embodiment, at least 3000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In certain embodiments, the methods provide sustainably improved volumetric FRETS-VWF73 productivity (P FRETS). For example, in certain embodiments, at least 1500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media is recovered daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a preferred embodiment, at least 2000 Units FRETS-VWF73 activity per liter

supplemented basal cell culture media is recovered daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a more preferred embodiment, at least 2500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media is recovered daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a most preferred embodiment, at least 3000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media is recovered daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a specific embodiment, the cell density of the continuous cell culture for production of rA13 is maintained at a concentration of no more than $4.0x10^6$ cells/mL for an extended period. In other specific embodiments, the cell density is maintained at no more than $3.5x10^6$ cells/mL, $3.0x10^6$ cells/mL, $2.5x10^6$ cells/mL, $2.0x10^6$ cells/mL, $1.5x10^6$ cells/mL, $1.0x10^6$ cells/mL, or less. In one embodiment, the cell density is maintained at between $3.0x10^6$ cells/mL and $4.0x10^6$ cells/mL.

[0209] In one embodiment of the methods described above, the recovered supernatant has at least 4 Units FRETS-VWF73 activity per mL supernatant (FRETS). In a preferred embodiment, the recovered supernatant has at least 6 Units FRETS-VWF73 activity per mL supernatant. In a more preferred embodiment, the recovered supernatant has at least 8 Units FRETS-VWF73 activity per mL supernatant. In a most preferred embodiment, the recovered supernatant has at least 10 Units FRETS-VWF73 activity per mL supernatant. In certain embodiments, the methods provide sustainably improved FRETS production. For example, in certain embodiments, supernatant having at least 4 Units FRETS-VWF73 activity per mL are recovered daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a preferred embodiment, supernatant having at least 6 Units FRETS-VWF73 activity per mL are recovered daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a more preferred embodiment, supernatant having at least 8 Units FRETS-VWF73 activity per mL are recovered daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a most preferred embodiment, supernatant having at least 10 Units FRETS-VWF73 activity per mL are recovered daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days.

[0210] In one embodiment of the methods described above, the cell culture results in the production of at least 800 mU of FRETS-VWF73 activity per 10^6 cells present in the culture per

day (*i.e.*, q FRETS). In a preferred embodiment, the cell culture results in the production of at least 1 U of FRETS-VWF73 activity per 10^6 cells present in the culture per day. In a more preferred embodiment, the cell culture results in the production of at least 1.2 U of FRETS-VWF73 activity per 10^6 cells present in the culture per day. In a most preferred embodiment, the cell culture results in the production of at least 1.4 U of FRETS-VWF73 activity per 10^6 cells present in the culture per day. In certain embodiments, the methods provide sustainably improved cell-specific FRETS-VWF73 productivity (q FRETS). For example, in certain embodiments, the cell culture results in the production of at least 800 mU of FRETS-VWF73 activity per 10^6 cells present in the culture daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a preferred embodiment, the cell culture results in the production of at least 1 U of FRETS-VWF73 activity per 10^6 cells present in the culture daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a more preferred embodiment, the cell culture results in the production of at least 1.2 U of FRETS-VWF73 activity per 10^6 cells present in the culture daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a most preferred embodiment, the cell culture results in the production of at least 1.4 U of FRETS-VWF73 activity per 10^6 cells present in the culture daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days.

[0211] In one embodiment of the methods described above, the cell culture results in the production of at least 1 mg rA13, as measured by ELISA, per liter culture per day (P ELISA). In a preferred embodiment, the cell culture results in the production of at least 1.5 mg rA13, as measured by ELISA, per liter culture per day. In a more preferred embodiment, the cell culture results in the production of at least 2 mg rA13, as measured by ELISA, per liter culture per day. In certain embodiments, the methods provide sustainably improved rA13 production. For example, in certain embodiments, the cell culture results in the production of at least 1 mg rA13, as measured by ELISA, per liter culture daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a preferred embodiment, the cell culture results in the production of at least 1.5 mg rA13, as measured by ELISA, per liter culture daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a more preferred embodiment, the cell culture results in the production of at least 2 mg rA13, as measured by ELISA, per liter culture daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days.

[0212] In one embodiment of the methods described above, the cell culture results in the production of at least 0.5 μg rA13, as measured by ELISA, per 10^6 cells present in the culture per day (*i.e.*, q ELISA). In a preferred embodiment, the cell culture results in the production of at least 0.7 μg rA13, as measured by ELISA, per 10^6 cells present in the culture per day. In a more preferred embodiment, the cell culture results in the production of at least 0.9 μg rA13, as measured by ELISA, per 10^6 cells present in the culture per day. In certain embodiments, the methods provide sustainably improved q ELISA production. For example, in certain embodiments, the cell culture results in the production of at least 0.5 μg rA13, as measured by ELISA, per 10^6 cells present in the culture daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a preferred embodiment, the cell culture results in the production of at least 0.7 μg rA13, as measured by ELISA, per 10^6 cells present in the culture daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a more preferred embodiment, the cell culture results in the production of at least 0.9 μg rA13, as measured by ELISA, per 10^6 cells present in the culture daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days.

[0213] In one embodiment of the methods described above, the recovered supernatant has at least 3 μg rA13, as measured by ELISA, per mL supernatant. In a preferred embodiment, the recovered supernatant has at least 4 μg rA13, as measured by ELISA, per mL supernatant. In a more preferred embodiment, the recovered supernatant has at least 5 μg rA13, as measured by ELISA, per mL supernatant. In a most preferred embodiment, the recovered supernatant has at least 6 μg rA13, as measured by ELISA, per mL supernatant. In certain embodiments, the methods provide sustainably improved rA13 production. For example, in certain embodiments, supernatant having at least 3 μg rA13 per mL are recovered daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a preferred embodiment, supernatant having at least 4 μg rA13 per mL are recovered daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a more preferred embodiment, supernatant having at least 5 μg rA13 per mL are recovered daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days. In a most preferred embodiment, supernatant having at least 6 μg rA13 per mL are recovered daily for at least 7 days, or at least 14, 21, 28, 35, 42, 49, 56, 63, 70, or more days.

[0214] In one embodiment of the methods described above, the cell culture solution further comprises an ammonium concentration of less than 10 mM. In another specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 10 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 5 mM. In another specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 5 mM for at least 7 days. In a preferred embodiment, the cell culture solution comprises an ammonium concentration of no more than 4 mM. In another specific embodiment, the ammonium concentration of the cell culture is maintained at an ammonium concentration of no more than 4 mM for at least 7 days. In yet other embodiments, the cell culture solution comprises an ammonium concentration of no more than 10 mM, or no more than 9 mM, 8 mM, 7 mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, or less. In yet another embodiment, the ammonium concentration of the cell culture is maintained at a low level for the duration of the process (*i.e.*, for the entire time the culture is being used to produce rA13). In a particular embodiment, the culture solution has a copper and ammonium concentration according to any one of variations 1 to 440, as provided in **Table 1**.

[0215] In one embodiment, the present invention provides a method for producing a recombinant ADAMTS13 (rA13) composition, the method comprising the steps of: (a) providing a basal cell culture media; (b) supplementing the basal cell culture media with copper and zinc; (c) providing one or more cells comprising a nucleic acid encoding a rA13 protein; (d) culturing the one or more cells in the copper supplemented cell culture media such that rA13 is expressed and excreted from the cells into a culture supernatant; and (e) recovering at least a portion of the culture supernatant, wherein at least 1500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In one embodiment, the culture medium contains at least 1 μg/L copper and at least 2 μM zinc. In other embodiments, the media contains at least 2 μg/L copper or at least 4 μg/L copper. In one embodiment wherein the media is supplemented with copper, the culture medium also contains at least at or about 5 μM zinc. In one embodiment, the culture medium also contains at or about between 2 μM and 12 μM zinc. In another embodiment, the culture medium also contains at or about between 5 μM and 12 μM zinc. In yet other embodiments, the culture medium also may

contain at least at or about 2 μM, or at least at or about 3 μM, 4 μM, 5 μM, 6 μM, 7 μM, 8 μM, 9 μM, 10 μM, 11 μM, 12 μM, 13 μM, 14 μM, 15 μM, 20 μM, 25 μM, 30 μM, or more zinc. In one embodiment, the culture medium contains a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In a preferred embodiment, at least 2000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In a more preferred embodiment, at least 2500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In a most preferred embodiment, at least 3000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant.

[0216] In one embodiment, the present invention provides a method for producing a recombinant ADAMTS13 (rA13) composition, the method comprising the steps of: (a) providing a basal cell culture media; (b) supplementing the basal cell culture media with copper and calcium; (c) providing one or more cells comprising a nucleic acid encoding a rA13 protein; (d) culturing the one or more cells in the copper supplemented cell culture media such that rA13 is expressed and excreted from the cells into a culture supernatant; and (e) recovering at least a portion of the culture supernatant, wherein at least 1500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In one embodiment, the culture medium contains at least 1 μg/L copper and at least 0.5 mM calcium. In other embodiments, the media contains at least 2 μg/L copper or at least 4 μg/L copper. In another embodiment wherein the media is supplemented with copper, the culture medium also contains at least 1.5 mM calcium. In one embodiment, the culture medium contains at or about between 0.5 mM and 1.5 mM calcium. In yet other embodiments, the culture medium may contain at least at or about 0.5 mM, or at least at or about 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1.0 mM, 1.1 mM, 1.2 mM, 1.3 mM, 1.4 mM, 1.5 mM, 1.6 mM, 1.7 mM, 1.8 mM, 1.9 mM, 2.0 mM, 2.25 mM, 2.5 mM, 2.75 mM, 3.0 mM, 3.5 mM, 4.0 mM, 4.5 mM, 5.0 mM, or more calcium. In one embodiment, the culture medium contains a copper and calcium concentration according to any one of variations 881 to 1320, as set forth in **Table 3**. In a preferred embodiment, at least 2000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In a more preferred

embodiment, at least 2500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In a most preferred embodiment, at least 3000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant.

[0217] In one embodiment, the present invention provides a method for producing a recombinant ADAMTS13 (rA13) composition, the method comprising the steps of: (a) providing a basal cell culture media; (b) supplementing the basal cell culture media with copper, zinc, and calcium; (c) providing one or more cells comprising a nucleic acid encoding a rA13 protein; (d) culturing the one or more cells in the copper supplemented cell culture media such that rA13 is expressed and excreted from the cells into a culture supernatant; and (e) recovering at least a portion of the culture supernatant, wherein at least 1500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In one embodiment, the culture medium has a calcium concentration of at least 0.5 mM and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In another specific embodiment, the culture medium has a calcium concentration of at least 1.5 mM and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In another specific embodiment, the culture medium has a calcium concentration between 0.5 mM and 1.5 mM and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In yet other embodiments, the culture medium has a calcium concentration of at least 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1.0 mM, 1.1 mM, 1.2 mM, 1.3 mM, 1.4 mM, 1.5 mM, 1.6 mM, 1.7 mM, 1.8 mM, 1.9 mM, 2.0 mM, 2.25 mM, 2.5 mM, 2.75 mM, 3.0 mM, 3.5 mM, 4.0 mM, 4.5 mM, 5.0 mM, or more, and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In a preferred embodiment, at least 2000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In a more preferred embodiment, at least 2500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In a most preferred embodiment, at least 3000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant.

[0218] In one embodiment, the present invention provides a method for producing a recombinant ADAMTS13 (rA13) composition, the method comprising the steps of: (a) providing a basal cell culture media; (b) supplementing the basal cell culture media with copper and nicotinamide; (c) providing one or more cells comprising a nucleic acid encoding a rA13 protein; (d) culturing the one or more cells in the copper supplemented cell culture media such that rA13 is expressed and excreted from the cells into a culture supernatant; and (e) recovering at least a portion of the culture supernatant, wherein at least 1500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In one embodiment, the culture medium contains at least 1 μg/L copper and at least 2 mg/L nicotinamide (vitamin B3). In other embodiments, the media contains at least 2 μg/L copper or at least 4 μg/L copper. In another embodiment wherein the media is supplemented with copper, the culture medium also contains at least 7 mg/L nicotinamide (vitamin B3). In one embodiment, the culture medium contains at or about between 2 mg/L and 10 mg/L nicotinamide (vitamin B3). In yet other embodiments, the culture medium may contain at least at or about 2 mg/L, 3 mg/L, 4 mg/L, 5 mg/L, 6 mg/L, 7 mg/L, 8 mg/L, 9 mg/L, 10 mg/L, 15 mg/L, 20 mg/L, or higher concentrations of nicotinamide (vitamin B3). In one embodiment, the culture medium contains a copper and nicotinamide concentration according to any one of variations 1321 to 1760, as set forth in **Table 4**. In a preferred embodiment, at least 2000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In a more preferred embodiment, at least 2500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In a most preferred embodiment, at least 3000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant.

[0219] In one embodiment, the present invention provides a method for producing a recombinant ADAMTS13 (rA13) composition, the method comprising the steps of: (a) providing a basal cell culture media; (b) supplementing the basal cell culture media with copper, zinc, and nicotinamide; (c) providing one or more cells comprising a nucleic acid encoding a rA13 protein; (d) culturing the one or more cells in the copper supplemented cell culture media such that rA13 is expressed and excreted from the cells into a culture supernatant; and (e) recovering at least a portion of the culture supernatant, wherein at least 1500 Units FRETS-VWF73 activity per liter

supplemented basal cell culture media per day is present in the recovered culture supernatant. In one embodiment, the cell culture medium has a nicotinamide concentration of at least 2 mg/mL and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In another specific embodiment, the culture medium has a nicotinamide concentration of at least 7 mg/mL mM and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In another specific embodiment, the culture medium has a nicotinamide concentration between 2 mg/mL and 10 mg/mL and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In yet other embodiments, the culture medium has a nicotinamide concentration of at least 2 mg/mL, 3 mg/mL, 4 mg/mL, 5 mg/mL, 6 mg/mL, 7 mg/mL, 8 mg/mL, 9 mg/mL, 10 mg/mL, 11 mg/mL, 12 mg/mL, 13 mg/mL, 14 mg/mL, 15 mg/mL, or more, and a copper and zinc concentration according to any one of variations 441 to 880, as set forth in **Table 2**. In a preferred embodiment, at least 2000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In a more preferred embodiment, at least 2500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In a most preferred embodiment, at least 3000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant.

[0220] In one embodiment, the present invention provides a method for producing a recombinant ADAMTS13 (rA13) composition, the method comprising the steps of: (a) providing a basal cell culture media; (b) supplementing the basal cell culture media with copper, calcium, and nicotinamide; (c) providing one or more cells comprising a nucleic acid encoding a rA13 protein; (d) culturing the one or more cells in the copper supplemented cell culture media such that rA13 is expressed and excreted from the cells into a culture supernatant; and (e) recovering at least a portion of the culture supernatant, wherein at least 1500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In one embodiment, the cell culture medium has a nicotinamide concentration of at least 2 mg/mL and a copper and calcium concentration according to any one of variations 881 to 1320, as set forth in **Table 3**. In another specific embodiment, the culture medium has a nicotinamide concentration of at least 7 mg/mL mM and a copper and calcium concentration according to any one of variations 881 to 1320, as set forth in **Table 3**. In another specific embodiment, the

culture medium has a nicotinamide concentration between 2 mg/mL and 10 mg/mL and a copper and calcium concentration according to any one of variations 881 to 1320, as set forth in **Table 3**. In yet other embodiments, the culture medium has a nicotinamide concentration of at least 2 mg/mL, 3 mg/mL, 4 mg/mL, 5 mg/mL, 6 mg/mL, 7 mg/mL, 8 mg/mL, 9 mg/mL, 10 mg/mL, 11 mg/mL, 12 mg/mL, 13 mg/mL, 14 mg/mL, 15 mg/mL, or more, and a copper and calcium concentration according to any one of variations 881 to 1320, as set forth in **Table 3**. In a preferred embodiment, at least 2000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In a more preferred embodiment, at least 2500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant. In a most preferred embodiment, at least 3000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant.

[0221] Recombinant ADAMTS proteins can be produced by expression in any suitable prokaryotic or eukaryotic host system. Examples of eukaryotic cells include, without limitation, mammalian cells, such as CHO, COS, HEK 293, BHK, SK-Hep, and HepG2; insect cells, for example SF9 cells, SF21 cells, S2 cells, and High Five cells; and yeast cells, for example *Saccharomyces* or *Schizosaccharomyces* cells. In one embodiment, the ADAMTS proteins can be expressed in bacterial cells, yeast cells, insect cells, avian cells, mammalian cells, and the like. For example, in a human cell line, a hamster cell line, or a murine cell line. In one particular embodiment, the cell line is a CHO, BHK, or HEK cell line. In a preferred embodiment, the cell line is a CHO cell line. In a specific embodiment, CHO clones capable of stably expressing rA13 are prepared by co-transfecting a CHO cell with coding sequences for rA13 and a dihydrofolate reductase (*e.g.*, a murine dhfr gene) and selecting for growth in the presence of increasing levels of methotrexate.

[0222] In one embodiment, the cells may be any mammalian cell that can be cultured, preferably in a manufacturing process (*i.e.*, at least 10 liter, preferrably at least 100 liters), to produce a desired ADAMTS protein such as ADAMTS13. Examples include the monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, *J. Gen Virol.*, 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR, such as

the DUKX-B11 subclone (CHO, Uriaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980)); mouse Sertoli cells (TM4, Mather, *Biol. Reprod*, 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, *Annals N. Y. Acad. Sci.*, 383:44-68 (1982)); MRC 5 cells; FS4 cells; and the human hepatoma line (Hep G2). Preferably, the cell line is a rodent cell line, especially a hamster cell line such as CHO or BHK.

[0223] A wide variety of vectors can be used for the expression of an ADAMTS protein (*e.g.*, ADAMTS13) and can be selected from eukaryotic and prokaryotic expression vectors. In certain embodiments, a plasmid vector is contemplated for use in expressing an ADAMTS protein (*e.g.*, ADAMTS13). In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector can carry a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. The plasmid will comprise a nucleotide sequence encoding an ADAMTS protein (*e.g.*, ADAMTS13) operable linked to one or more control sequences, for example, a promoter.

[0224] A preferred method of preparing stable CHO cell clones expressing a recombinant ADAMTS protein is as follows. A DHFR deficient CHO cell line DUKX-B11 is transfected with a DHFR expression vector to allow for expression of the relevant recombinant protein. An exemplary method is described by Plaimauer *et al.* (Blood. 2002 Nov 15;100(10):3626-32. Epub 2002 Jul 12), the content of which is hereby incorporated by reference in its entirety for all purposes. Selection is carried out by growth in Hypoxanthine/Thymidine (HT) free media and amplification of the relevant region coding for expression of the recombinant ADAMTS protein and DHFR gene is achieved by propagation of the cells in increasing concentrations of methotrexate. Where appropriate, CHO cell lines may be adapted for growth in serum and/or protein free medium, essentially as described in US 6,100,061 (Reiter *et al.*, Immuno Aktiengesellschaft).

[0225] In another preferred embodiment, stable HEK293 cells are prepared by transfecting with a construct containing a hygromycin selectable marker and selecting transformants by antibiotic resistance.

[0226] The ability of certain viruses to infect cells or enter cells via receptor-mediated endocytosis, and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign nucleic acids into cells (e.g., mammalian cells). Accordingly, in certain embodiments, a viral vector is used to introduce a nucleotide sequence encoding an ADAMTS protein (*e.g.*, ADAMTS13) into a host cell for expression. The viral vector will comprise a nucleotide sequence encoding an ADAMTS protein (*e.g.*, ADAMTS13) operable linked to one or more control sequences, for example, a promoter. Alternatively, the viral vector may not contain a control sequence and will instead rely on a control sequence within the host cell to drive expression of the ADAMTS protein. Non-limiting examples of virus vectors that may be used to deliver a nucleic acid include Adenoviral vectors, AAV vectors, and Retroviral vectors.

[0227] In one embodiment, an Adenovirus expression vector include those constructs containing adenovirus sequences sufficient to support packaging of the construct and to ultimately express an ADAMTS construct that has been cloned therein. Adenoviral vectors allow for the introduction of foreign sequences up to 7 kb (Grunhaus et al., Seminar in Virology, 200(2):535-546, 1992)).

[0228] In another embodiment, an adeno-associated virus (AAV) can be used to introduce a nucleotide sequence encoding an ADAMTS protein (*e.g.*, ADAMTS13) into a host cell for expression. AAV systems have been described previously and are generally well known in the art (Kelleher and Vos, *Biotechniques*, 17(6):1110-7, 1994; Cotten et al., *Proc Natl Acad Sci USA*, 89(13):6094-6098, 1992; Curiel, *Nat Immun*, 13(2-3):141-64, 1994; Muzyczka, *Curr Top Microbiol Immunol*, 158:97-129, 1992). Details concerning the generation and use of rAAV vectors are described, for example, in U.S. Patent Nos. 5,139,941 and 4,797,368, each incorporated herein by reference in their entireties for all purposes.

[0229] In one embodiment, a retroviral expression vector can be used to introduce a nucleotide sequence encoding an ADAMTS protein (*e.g.*, ADAMTS13) into a host cell for expression. These systems have been described previously and are generally well known in the art (Mann *et*

al., *Cell*, 33:153-159, 1983; Nicolas and Rubinstein, In: Vectors: A survey of molecular cloning vectors and their uses, Rodriguez and Denhardt, eds., Stoneham: Butterworth, pp. 494-513, 1988; Temin, In: Gene Transfer, Kucherlapati (ed.), New York: Plenum Press, pp. 149-188, 1986). In a specific embodiment, the retroviral vector is a lentiviral vector (*see*, for example, Naldini *et al.*, *Science*, 272(5259):263-267, 1996; Zufferey *et al.*, *Nat Biotechnol*, 15(9):871-875, 1997; Blomer *et al.*, *J Virol.*, 71(9):6641-6649, 1997; U.S. Pat. Nos. 6,013,516 and 5,994,136).

[0230] Non-limiting examples of vectors for prokaryotic expression include plasmids such as pRSET, pET, pBAD, etc., wherein the promoters used in prokaryotic expression vectors include lac, trc, trp, recA, araBAD, etc. Examples of vectors for eukaryotic expression include: (i) for expression in yeast, vectors such as pAO, pPIC, pYES, pMET, using promoters such as AOX1, GAP, GAL1, AUG1, etc; (ii) for expression in insect cells, vectors such as pMT, pAc5, pIB, pMIB, pBAC, etc., using promoters such as PH, p10, MT, Ac5, OpIE2, gp64, polh, etc., and (iii) for expression in mammalian cells, vectors such as pSVL, pCMV, pRc/RSV, pcDNA3, pBPV, etc., and vectors derived form viral systems such as vaccinia virus, adeno-associated viruses, herpes viruses, retroviruses, etc., using promoters such as CMV, SV40, EF-1, UbC, RSV, ADV, BPV, and β-actin. An exemplary vector for expressing rA13 is described by Plaimauer *et al.* (Blood. 2002 Nov 15;100(10):3626-32. Epub 2002 Jul 12), the content of which is hereby incorporated by reference in its entirety for all purposes.

[0231] In certain embodiments, the cell-culture methods of the invention may comprise the use of a microcarrier. The present invention provides, among other aspect, methods of large-scale ADAMTS protein expression. In some embodiments, the cell-cultures of the embodiments can be performed in large bioreactors under conditions suitable for providing high volume-specific culture surface areas to achieve high cell densities and protein expression. One means for providing such growth conditions is to use microcarriers for cell-culture in stirred tank bioreactors. In another embodiment, these growth requirements are met via the use of a suspension cell culture.

V. Specific embodiments

A. Recombinant Von Willebrand Factor (rVWF)

[0232] Recombinant vWF can be expressed in mammalian cells, but the specific activity of the vWF can vary widely depending on the cell culture conditions and has not been shown to be comparable or equal to that of vWF isolated from blood plasma. The present invention is based in-part on the surprising result that cell culture media having at least 2.4 μg/L of copper provides an advantageous effect of promoting expression of high molecular weight vWF having a high specific activity. In particular, the high molecular weight, recombinant vWF of the present invention can include a highly multimeric form comprising about 14 to about 22 dimers and a specific Ristocetin activity of at least about 30 mU/μg. The cell culture processes according to the present invention also allow for maintaining low NH_4^+ levels (*e.g.*, less than 10 mM) during the upstream process in cell culture systems, thereby reducing deleterious effects to post-translational modifications. It is believed that the present invention provides for the first time cell culture conditions comprising a medium having a suitable copper concentration in combination with appropriate levels of ammonium in the supernatant to express a highly multimeric vWF with a high specific activity.

[0233] In one aspect, the present invention relates to cell culture conditions for producing recombinant, high molecular weight vWF with a high specific activity. The cell culture conditions of the present invention can include, for example, a cell culture medium with an increased copper concentration and/or cell culture supernatant with a low ammonium (NH_4^+) concentration. The present invention also provides methods for cultivating cells in cell culture conditions to express a high molecular weight vWF with a high specific activity.

[0234] In one aspect, the present invention provides a cell culture solution for producing high molecular weight, recombinant vWF protein, the cell culture solution comprising: a cell culture medium comprising a copper concentration of at least about 2.4 μg/L; a cell culture supernatant comprising an ammonium concentration of less than 10 mM; and a plurality of cells expressing highly multimeric vWF protein, wherein the vWF protein comprises a specific Ristocetin activity of at least about 30 mU/μg.

[0235] In one specific embodiment of the cell cultures described above, the cell culture solution comprises a medium supplement comprising copper.

[0236] In one specific embodiment of the cell cultures described above, the medium supplement comprises a hydrolysate, optionally a soy hydrolysate.

[0237] In one specific embodiment of the cell cultures described above, the medium supplement comprises a copper salt, a copper chelate, or a combination thereof.

[0238] In one specific embodiment of the cell cultures described above, the copper salt is selected from the group consisting of copper sulfate, copper acetate, copper carbonate, copper chloride, copper hydroxide, copper nitrate, and copper oxide.

[0239] In one specific embodiment of the cell cultures described above, the copper concentration is at least about 4 μg/L.

[0240] In one specific embodiment of the cell cultures described above, the copper concentration is from about 2.4 μg/L to about 20 μg/L.

[0241] In one specific embodiment of the cell cultures described above, the vWF protein comprises about 14 to about 22 dimers.

[0242] In one aspect, the present invention provides a method of producing a high molecular weight, recombinant vWF protein, the method comprising the steps of: a) providing a culture of cells comprising a nucleic acid encoding recombinant vWF protein; b) expressing vWF protein in the cells under cell culture conditions comprising a cell culture medium comprising a copper concentration of at least about 2.4 μg/L and a cell culture supernatant comprising an ammonium concentration less than about 10 mM, wherein the vWF protein is highly multimeric vWF protein and comprises a specific Ristocetin activity of at least about 30 mU/μg.

[0243] In one specific embodiment of the methods described above, the cells are mammalian cells.

[0244] In one specific embodiment of the methods described above, the cells are from a continuous cell line.

[0245] In one specific embodiment of the methods described above, the cells are CHO cells.

[0246] In one specific embodiment of the methods described above, the copper concentration is at least about 4 μg/L.

[0247] In one specific embodiment of the methods described above, the copper concentration is from about 2.4 μg/L to about 20 μg/L.

[0248] In one specific embodiment of the methods described above, the recombinant vWF protein has a specific Ristocetin Cofactor activity of at least about 50 mU/μg.

[0249] In one specific embodiment of the methods described above, the recombinant vWF protein has a specific Ristocetin Cofactor activity from about 30 mU/μg to about 100 mU/μg.

[0250] In one specific embodiment of the methods described above, the recombinant vWF protein comprises about 14 to about 22 dimers.

[0251] In one aspect, the present invention provides a high molecular weight, recombinant vWF protein produced by a process, the process comprising the steps of: a) providing a culture of cells comprising a nucleic acid encoding recombinant vWF protein; and b) expressing vWF protein in the cells under cell culture conditions comprising a cell culture medium comprising a copper concentration of at least 2.4 μg/L and a cell culture supernatant comprising an ammonium concentration less than 10 mM, wherein the vWF protein is highly multimeric vWF protein and comprises a specific Ristocetin activity of at least about 30 mU/μg.

[0252] In one specific embodiment of the rVWF compositions described above, the recombinant vWF protein has a specific Ristocetin Cofactor activity of at least about 50 mU/μg.

[0253] In one specific embodiment of the rVWF compositions described above, the recombinant vWF protein has a specific Ristocetin Cofactor activity from about 30 mU/μg to about 100 mU/μg.

[0254] In one specific embodiment of the rVWF compositions described above, the recombinant vWF protein comprises about 14 to about 22 dimers.

[0255] In one aspect, the present invention provides a cell culture solution for producing high molecular weight, recombinant vWF protein, the cell culture solution comprising: a cell culture medium comprising a copper concentration of at least about 2.4 μg/L; a cell culture supernatant comprising an ammonium concentration of less than 10 mM; and a plurality of cells expressing highly multimeric vWF protein, wherein the vWF protein comprises about 14 to about 22 dimers and a specific Ristocetin activity of at least about 30 mU/μg.

[0256] In one aspect, the present invention relates to cell culture conditions for producing recombinant high molecular weight vWF that is in highly multimeric form with a high specific

activity. The cell culture conditions of the present invention can include, for example, a cell culture medium with an increased copper concentration and cell culture supernatant with a low ammonium (NH_4^+) concentration. The present invention also provides methods for cultivating cells in cell culture conditions to express a high molecular weight vWF with a high specific activity.

[0257] In one aspect, the present invention includes a cell culture solution for producing high molecular weight, recombinant vWF, comprising a cell culture medium comprising a copper concentration of at least about 2.4 μg/L; a cell culture supernatant comprising an ammonium concentration of less than 10 mM; and a plurality of cells expressing highly multimeric vWF comprising about 14 to about 22 dimers and a specific Ristocetin activity of at least about 30 mU/μg. In one embodiment of the cell culture solutions described above, at least 10% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In a specific embodiment, at least 15% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 20% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 25% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 30% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In certain embodiments, the copper concentration can be at least about 4 μg/L or the copper concentration can range from about 2.4 μg/L to about 20 μg/L. In some embodiments, the cell culture media comprise a medium supplement comprising copper. In certain embodiments, the medium supplement can comprise a hydrolysate or a copper salt, copper chelate, or a combination thereof. In some embodiments, the copper salt can include copper sulfate, copper acetate, copper carbonate, copper chloride, copper hydroxide, copper nitrate, or copper oxide. In certain embodiments, the cells that can be from a continuous cell line and can include mammalian cells, such as CHO cells. In some embodiments, the recombinant vWF has a specific Ristocetin Cofactor activity of at least about 50 mU/μg, or the specific Ristocetin Cofactor activity can range from about 30 mU/μg to about 100 mU/μg.

[0258] In another aspect, the present invention includes a method of producing a high molecular weight, recombinant vWF, comprising a) providing a culture of cells; b) introducing a nucleic

acid sequence coding for vWF; c) selecting the cells carrying the nucleic acid sequence; and, d) expressing vWF in the cells under cell culture conditions comprising a cell culture medium comprising a copper concentration of at least about 2.4 µg/L and a cell culture supernatant comprising an ammonium concentration less than about 10 mM, wherein the vWF is highly multimeric vWF comprising about 14 to about 22 dimers and a specific Ristocetin activity of at least about 30 mU/µg. In one embodiment of the cell culture supernatant described above, at least 10% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In a specific embodiment, at least 15% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 20% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 25% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 30% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In some embodiments, the cells that can be from a continuous cell line and can include mammalian cells, such as CHO cells. In certain embodiments, the copper concentration can be at least about 4 µg/L or the copper concentration can range from about 2.4 µg/L to about 20 µg/L. In some embodiments, the cell culture media comprise a medium supplement comprising copper. In certain embodiments, the medium supplement can comprise a hydrolysate or a copper salt, copper chelate, or a combination thereof. In some embodiments, the copper salt can include copper sulfate, copper acetate, copper carbonate, copper chloride, copper hydroxide, copper nitrate, or copper oxide. In some embodiments, the recombinant vWF has a specific Ristocetin Cofactor activity of at least about 50 mU/µg, or the specific Ristocetin Cofactor activity can range from about 30 mU/µg to about 100 mU/µg.

[0259] In yet another aspect, the present invention includes a high molecular weight, recombinant vWF produced by a process, comprising the steps of: a) providing a culture of cells; b) introducing a nucleic acid sequence coding for vWF; c) selecting the cells carrying the nucleic acid sequence; and, d) expressing vWF in the cells under cell culture conditions comprising a cell culture medium comprising a copper concentration of at least 2.4 µg/L and a cell culture supernatant comprising an ammonium concentration less than 10 mM, wherein the vWF is highly multimeric vWF comprising about 14 to about 22 dimers and a specific Ristocetin activity of at least about 30 mU/µg. In one embodiment of the cell culture supernatant described above,

at least 10% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In a specific embodiment, at least 15% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 20% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 25% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 30% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In some embodiments, the cells that can be from a continuous cell line and can include mammalian cells, such as CHO cells. In certain embodiments, the copper concentration can be at least about 4 μg/L or the copper concentration can range from about 2.4 μg/L to about 20 μg/L. In some embodiments, the cell culture media comprise a medium supplement comprising copper. In certain embodiments, the medium supplement can comprise a hydrolysate or a copper salt, copper chelate, or a combination thereof. In some embodiments, the copper salt can include copper sulfate, copper acetate, copper carbonate, copper chloride, copper hydroxide, copper nitrate, or copper oxide. In some embodiments, the recombinant vWF has a specific Ristocetin Cofactor activity of at least about 50 mU/μg, or the specific Ristocetin Cofactor activity can range from about 30 mU/μg to about 100 mU/μg.

[0260] In one aspect, the present invention provides a composition comprising recombinant Von Willebrand Factor (rVWF) having a specific ristocetin cofactor activity of at least 30 mU/μg. In a preferred embodiment, the composition has a specific ristocetin cofactor activity of at least 40 mU/μg. In a more preferred embodiment, the composition has a specific ristocetin cofactor activity of at least 50 mU/μg. In a more preferred embodiment, the composition has a specific ristocetin cofactor activity of at least 60 mU/μg. In a more preferred embodiment, the composition has a specific ristocetin cofactor activity of at least 70 mU/μg. In yet a more preferred embodiment, the composition has a specific ristocetin cofactor activity of at least 80 mU/μg.

[0261] In one embodiment of the compositions described above, at least 10% of the rVWF in the composition is present in a high molecular weight VWF multimer of more than 10 dimers. In a specific embodiment, at least 15% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 20% of the rVWF is

present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 25% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers. In another specific embodiment, at least 30% of the rVWF is present in a high molecular weight VWF multimer of more than 10 dimers.

[0262] In one embodiment of the compositions described above, the composition comprises a culture supernatant. In one specific embodiment the culture supernatant is a mammalian cell culture supernatant. In a more specific embodiment, the mammalian cell culture supernatant is a CHO cell supernatant.

[0263] In one embodiment of the compositions described above, the rVWF is expressed in a cell culture comprising at least 2.4 μg/L copper. In a specific embodiment, the cell culture comprises at least 4 μg/L copper. In a more specific embodiment, the culture comprises between 2.4 μg/L and 20 μg/L copper. In one embodiment, the copper is provided as a copper salt, a copper chelate, or a combination thereof. In a specific embodiment, the copper salt is selected from the group consisting of copper sulfate, copper acetate, copper carbonate, copper chloride, copper hydroxide, copper nitrate, and copper oxide.

[0264] In one embodiment of the compositions described above, the cell culture is a batch culture.

[0265] In one embodiment of the compositions described above, the cell culture is a continuous culture. In a specific embodiment, the continuous culture is performed in chemostatic mode. In another specific embodiment, the continuous culture is performed in perfusion mode.

[0266] In one embodiment of the compositions described above, the level of NH4+ in the culture is maintained at a concentration below 4 mM.

[0267] In one embodiment of the compositions described above, the cell density of the culture is maintained at less than $2.5x10^6$ cells per mL.

[0268] In one embodiment of the compositions described above, the cell density of the culture is maintained at less than $2.0x10^6$ cells per mL.

[0269] In one embodiment of the compositions described above, the culture is maintained at less than $1.5x10^6$ cells per mL.

[0270] In one embodiment of the compositions described above, the rVWF is co-expressed with recombinant Factor VIII (rFVIII). In a specific embodiment, a majority of the co-expressed rFVIII has been removed. In a more specific embodiment, the ratio of rVWF to rFVIII in the composition is at least 10:1.

[0271] In one embodiment of the compositions described above, the composition is formulated for pharmaceutical administration. In a specific embodiment, the composition is formulated for intravenous, subcutaneous, or intramuscular administration.

[0272] In one embodiment of the compositions described above, the composition is lyophilized.

[0273] In yet another aspect, the present invention includes a high molecular weight, recombinant vWF produced by a process, comprising the steps of: a) providing a culture of cells; b) introducing a nucleic acid sequence coding for vWF; c) selecting the cells carrying the nucleic acid sequence; and, d) expressing vWF in the cells under cell culture conditions comprising a cell culture medium comprising a copper concentration of at least 2.4 μg/L and a cell culture supernatant comprising an ammonium concentration less than 10 mM, wherein the vWF is highly multimeric vWF comprising about 14 to about 22 dimers and a specific Ristocetin activity of at least about 30 mU/μg. It is understood that all of the embodiments and concentrations described in the "Cell Culture Media" and "Methods of Producing Recombinant vWF" sections above can apply here.

[0274] The recombinant vWF of the present invention can include a high molecular weight recombinant vWF protein having a high specific activity. In one embodiment, the vWF of the present invention is a highly multimeric form of vWF. In some embodiments, the highly multimeric form of vWF includes at least up to about 14 dimers and in other embodiments at least up to about 22 dimers. In yet other embodiments, the highly multimeric form of vWF can range from about 10 to about 20 dimers, or from about 15 to about 25 dimers, or from about 20 to about 40 dimers. In certain embodiments, the recombinant vWF is comparable to plasmatic vWF.

[0275] As described herein, the present invention provides the surprising result that increased copper concentration in cell culture media can produce high molecular weight vWF with high specific activity. Cell culture media comprising a copper concentration, *e.g.*, greater than about

2.4 μg/L can increase the yield of recombinant multimeric vWF, as compared to media without copper. In certain embodiments, the percentage of multimeric vWF (*i.e.*, rVWF comprising at least 2 dimers) can be greater than about 50%, or greater than about 75%, or greater than about 90%. The multimeric distribution of the vWF can be analyzed using standard techniques such as, e.g., in Agarose electrophoresis under non-reducing conditions.

[0276] As provided herein, the recombinant vWF produced by the methods of the present invention can have a high specific activity, e.g., a high specific Ristocetin Cofactor activity. In one embodiment, the recombinant vWF produced by the methods of the present invention can include a specific Ristocetin Cofactor activity of at least 30 mU/μg and in another embodiment at least 50 mU/μg. In other embodiments, the specific Ristocetin Cofactor activity can range from about 30 mU/μg to about 100 mU/μg or from about 50 mU/μg to about 100 mU/μg.

B. Recombinant ADAMTS13 (rA13)

[0277] The ADAMTS proteins (*i.e.*, ADAMTS-1 to ADAMTS-20) are a family of secreted zinc metalloproteinases that share a common modular domain organization (for review, *see*, Flannery C.R., *Front Biosci.* 2006 Jan 1;11:544-69). All of the ADAMTS protein share a common core domain architecture, consisting of a signal peptide, followed by a prodomain, a zinc-dependent metalloproteinase catalytic domain, a disintegrin-like domain, a thrombospondin type I repeat, a cysteine-rich domain, and a spacer domain (Apte S.S., J Biol Chem. 2009 Nov 13;284(46):31493-7). Additionally, all but ADAMTS-4 contain at least one more thrombospondin type I repeat domain, and many of the ADAMTS protein contain one or more additional ancillary domains. Notably, it has been reported that all ADAMTS protein appear to contain at least one calcium binding site and at least one zinc binding site located within the metalloproteinase catalytic domain (Andreini *et al.*, *J. Proteome Res.*, 2005, 4 (3), pp 881–888).

[0278] Biological roles for ADAMTS proteins have been reported for various diseases and conditions, including, Antiangiogenesis, Renal interstitial fibrosis, Bone remodeling, Ovarian folliculogenesis, Atherosclerosis, Urogenital development, and Tumor growth/remodeling (ADAMTS-1); Ehler–Danlos syndrome type 7C and Bovine dermatopraxis (ADAMTS-2); Arthritis, Atherosclerosis, and Tendinopathy (ADAMTS-4); Arthritis and Glioblastoma (ADAMTS-5); Arthritis (ADAMTS-7); Antiangiogenesis, Brain malignancy, Arthritis, and

Atherosclerosis (ADAMTS-8); Arthritis (ADAMTS-9, -12); Thrombotic thrombocytopenic purpura (ADAMTS-13); and Antithrombosis/stroke (ADAMTS18) (for review, *see*, Lin and Liu, *Open Access Rheumatology Research and Reviews* 2009:1 121–131).

[0279] Recombinant ADAMTS13 (A13) has been expressed before in mammalian cells, however the specific activity varies widely dependent on the cell culture conditions. It has been found that many commercially available culture mediums are not sufficient for expression of rA13 with high specific activities, expressed as the ratio of activity, measured by FRETS-VWF73 assay, to antigen content, as determined by ELISA. In one aspect, the methods provided herein are based on several advantageous findings that allow for cell-culture expression of rA13 having increased levels of total and specific activity.

[0280] Accordingly, due to the shared structure-function relationship between the ADAMTS family of secreted metalloproteinases, the methods provided by the present invention allow for the expression of all ADAMTS proteins in cell culture and recovery from the cell medium.

[0281] In one aspect, the present invention provides a composition comprising recombinant ADAMTS13 (rA13) having a specific FRETS-VWF activity of at least 1600 mU/μg.

[0282] In one embodiment of the compositions described above, the rA13 has a specific FRETS-VWF activity of at least 800 mU/μg.

[0283] In one embodiment of the compositions described above, the composition comprises a culture supernatant. In a specific embodiment, the culture supernatant is a mammalian cell culture supernatant. In a more specific embodiment, the mammalian cell culture supernatant is a CHO cell supernatant.

[0284] In one embodiment of the compositions described above,the rA13 is expressed in a cell culture comprising at least 1 μg/L copper. In a specific embodiment, the cell culture comprises at least 2 μg/L copper. In a more specific embodiment, the culture comprises between 2 μg/L and 20 μg/L copper.

[0285] In one embodiment of the compositions described above, the copper is provided as a copper salt, a copper chelate, or a combination thereof. In a specific embodiment, the copper salt

is selected from the group consisting of copper sulfate, copper acetate, copper carbonate, copper chloride, copper hydroxide, copper nitrate, and copper oxide.

[0286] In one embodiment of the compositions described above, the cell culture is a batch culture.

[0287] In one embodiment of the compositions described above, the cell culture is a continuous culture. In a specific embodiment, the continuous culture is performed in chemostatic mode. In another specific embodiment, the continuous culture is performed in perfusion mode.

[0288] In one embodiment of the compositions described above, the level of $NH4^+$ in the culture is maintained at a concentration below 5 mM.

[0289] In one embodiment of the compositions described above, the level of $NH4^+$ in the culture is maintained at a concentration below 4 mM.

[0290] In one embodiment of the compositions described above, the cell density of the culture is maintained at less than $4.0x10^6$ cells per mL. In a specific embodiment, the cell density of the culture is maintained at less than $3.0x10^6$ cells per mL. In a specific embodiment, the cell density of the culture is maintained at less than $2.0x10^6$ cells per mL. In a more specific embodiment, the cell density of the culture is maintained at less than $1.5x10^6$ cells per mL.

[0291] In one embodiment of the compositions described above, the composition is formulated for pharmaceutical administration. In a specific embodiment, the composition is formulated for intravenous, subcutaneous, or intramuscular administration.

[0292] In one embodiment of the compositions described above, the composition is lyophilyzed.

VI. Formulations

[0293] In one aspect, the formulations comprising the recombinant therapeutic proteins rVWF or rA13 of the present invention are 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)].

[0294] Methods of preparing pharmaceutical formulations can include one or more of the following steps: adding a stabilizing agent as described herein to said mixture prior to lyophilizing, adding at least one agent selected from a bulking agent, an osmolarity regulating agent, and a surfactant, each of which as described herein, to said mixture prior to lyophilization. A lyophilized formulation is, in one aspect, at least comprised of one or more of a buffer, a bulking agent, and a stabilizer. In this aspect, the utility of a surfactant is evaluated and selected in cases where aggregation during the lyophilization step or during reconstitution becomes an issue. An appropriate buffering agent is included to maintain the formulation within stable zones of pH during lyophilization.

[0295] The standard reconstitution practice for lyophilized material is to add back a volume of pure water or sterile water for injection (WFI) (typically equivalent to the volume removed during lyophilization), although dilute solutions of antibacterial agents are sometimes used in the production of pharmaceuticals for parenteral administration [Chen, Drug Development and Industrial Pharmacy, 18:1311-1354 (1992)]. Accordingly, methods are provided for preparation of reconstituted recombinant VWF compositions comprising the step of adding a diluent to a lyophilized recombinant VWF composition of the invention.

[0296] The lyophilized material may be reconstituted as an aqueous solution. 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, an aqueous suspension that contains the active compound in admixture with excipients suitable for the manufacture of aqueous suspensions). In various aspects, such excipients are suspending agents, for example and without limitation, sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents are a naturally-occurring phosphatide, for example and without limitation, lecithin, or condensation products of an alkylene oxide with fatty acids, for example and without limitation, polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example and without limitation, 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 and without limitation,

polyethylene sorbitan monooleate. In various aspects, the aqueous suspensions also contain one or more preservatives, for example and without limitation, ethyl, or n-propyl, p-hydroxybenzoate.

[0297] 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.

[0298] The pharmaceutical formulations are administered intravenously, 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.

[0299] Single or multiple administrations of the compositions are carried out with the dose levels and pattern being selected by the treating physician. For the prevention or treatment of disease, the appropriate dosage depends on the type of disease to be treated, 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.

[0300] In one aspect, formulations of the invention are administered by an initial bolus followed by a continuous infusion to maintain therapeutic circulating levels of drug product. As another example, the inventive compound is 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 depends on the pharmacokinetic parameters of the agents and the route of administration.

The optimal pharmaceutical formulation is 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 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 is 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 is 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. 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. As studies are conducted, further information will emerge regarding the appropriate dosage levels and duration of treatment for various diseases and conditions.

VII. Methods of treatment

[0301] The present invention further contemplates methods of treating a patient in need of rVWF or rA13 produced according to the methods described herein. Such methods of treatment can include administration of pharmaceutical formulations comprising the recombinant rA13 or the high molecular weight, recombinant vWF of the present invention.

[0302] In another aspect, the present invention provides methods for therapeutic or prophylactic treatments comprising the administration of an rVWF or rA13 composition provided herein. Generally, for therapeutic applications, formulations are administered to a subject with a disease or condition associated with ADAMTS13 or VWF dysfunction or otherwise in need thereof, in a "therapeutically effective dose." Formulations and amounts effective for these uses will depend upon the severity of the disease or condition and the general state of the patient's health. Single or multiple administrations of the formulations may be administered depending on the dosage and frequency as required and tolerated by the patient.

[0303] In one embodiment, the present invention provides methods of treating or preventing a disease or condition associated with an ADAMTS13 or VWF dysfunction. In a further embodiment, the pharmaceutical formulations comprising recombinant vWF can be administered to treat diseases related to vWF, such as von Willebrand's disease or hemophilia. In another embodiment, the invention provides methods of treating or preventing a disease or condition associated with the formation and/or presence of one or more thrombus, comprising the administration of a rA13 composition provided herein. In another embodiment, the invention provides methods of disintegrating one or more thrombus in a subject in need thereof. In yet other embodiments, the invention provides methods of treating or preventing an infarction in subject in need thereof. Generally, the methods provided by the invention comprise administering an rADAMTS13 composition as provided herein to a subject in need thereof.

[0304] Non-limiting examples of disorders associated with the formation and/or the presence of one or more thrombus are hereditary thrombotic thrombocytopenic purpura (TTP), acquired TTP, arterial thrombosis, acute myocardial infarction (AMI), stroke, sepsis, and disseminated intravascular coagulation (DIC).

[0305] Non-limiting examples of disorders associated with an infarction, include without limitation, myocardial infarction (heart attack), pulmonary embolism, cerebrovascular events such as stroke, peripheral artery occlusive disease (such as gangrene), antiphospholipid syndrome, sepsis, giant-cell arteritis (GCA), hernia, and volvulus.

VIII. Examples

[0306] The present invention will now be further illustrated in the following examples, without being limited thereto.

Example 1

[0307] Continuous cell culture experiments were performed using cultures of a recombinant CHO cell line expressing vWF. The basal medium was DMEM/F12, which contained about 0.3 μg/L of Cu^{2+}. The medium was supplemented with soy hydrolysate and copper sulfate

($CuSO_4 \cdot 5H_2O$) to bring the final copper concentration in the medium to greater than at least 2.4 µg/L.

[0308] Recombinant CHO cells expressing vWF were cultivated by continuous cell cultures so that the ammonium levels (NH_4^+) were kept at a concentration less than about 10 mM. It was found that production systems providing a continuous supply of medium (e.g., perfusion or chemostat cultures) were preferable because conventional batch or fed batch techniques produced high NH_4^+ concentrations at the end of the culture. At the end of the culture, the highly multimeric vWF was isolated and the specific Ristocetin Cofactor activity of the vWF was measured.

Example 2

[0309] Recombinant Factor VIII (rFVIII) and Von Willebrand Factor (rVWF) were co-expressed in batch cultures of GD8/6 cells to determine the effect of the composition of the culture medium on VWF expression and activity. Briefly, GD8/6 cells were batch cultivated in BAV-SP medium composed of a modified DMEM/F12 basal powder (**Table 5**) and additional supplements also containing 4 g/L of soy hydrolysate, *see* **Table 6**, with and without additional copper supplementation. To test the effect of low copper concentraions on rVWF expression and activity, basal BAV-SP media was used. The basal media contained 0.3 µg/L copper and was supplemented with soy hydrolysate, which contributed an additional 0.7 µg/L copper, as determined experimentally, providing a final copper concentration of 1.0 µg/L. As a comparision, the BAV-SP media used for the batch cultures was further supplemented with an additional 3.3 µg/L of Cu^{2+}, providing a final copper concentration of 4.3 µg/L, to determine the effect high copper concentrations on VWF expression and activity.

Table 5. Composition of BAV-SP Culture Media

BAV-SP-Medium	
Components	mg/L
Amino Acids	
L-Alanine	4.45
L-Arginine HCl	147.50
L-Asparagine-H_2O	30.21
L-Aspartic Acid	6.65
L-Cystéine HCl-H_2O	32.55
L-Cystine 2HCl	57.35
L-Glutamic Acid	7.35

Glycine	18.75
L-Histidine-H_2O HCl	31.48
Hydroxy-L-Proline	
L-Isoleucine	54.47
L-Leucine	59.05
L-Lysine HCl	91.25
L-Methionine	17.24
L-Phenylalanine	35.48
L-Proline	52.24
L-Serine	26.25
L-Threonine	53.45
L-Tryptophan	29.01
L-Tyrosine 2Na 2H2O	55.79
L-Valine	52.85
Vitamins	
Ascorbic Acid	3.499
Biotin	0.0035
Choline Chloride	8.980
D-Ca-Pantothenate	2.240
Folic Acid	2.650
I-Inositol	12.600
Nicotinamide	2.020
Pyridoxine HCl	2.031
Riboflavin	0.219
Thiamine HCl	2.170
Vitamin B12	0.680
Anorganic Salts	
Calcium Chloride (CaCl2)	116.600
Copper Sulfate ($CuSO_4$-$5H_2O$)	0.0013
Ferric Nitrate ($Fe(NO_3)3$-$9H_2O$)	0.050
Ferrous Sulfate ($FeSO_4$-$7H_2O$)	0.417
Magnesium Chloride (MgCl2)	28.640
Magnesium Sulfate (MgSO4)	48.840
Potassium Chloride (KCl)	311.800
Sodium Chloride (NaCl)	6995.500
Sodium phosphate (Na2HPO4)	71.020
Sodium phosphate (NaH2PO4)	62.500
Zinc Sulfate Heptahydrate (ZnSO4-7H2O)	0.432
Sodium selenite	0.0131
Others	
D-Glucose	3151
Linoleic Acid	0.042
Lipoic Acid	0.105
Putrescine 2HCl	0.081
Thymidine	0.365
Hypoxantine Na	2.390
Sodium Pyruvate	55.000

Table 6. Composition of BAV-SP Supplement

Componets for final formulation	
L-Glutamine	600.00
Ethanolamine	1.530
Synperonic F68	250.000

Sodium Bicarbonate (NaHCO3)	2000.000
Soy peptone	4000.00
Total formulation	18596.8

[0310] GD8/6 cells expressing rVWF were grown in either low copper media (Table **7**) or high copper media (Table **8**) for 7 days. After 2 days the cultures were subcultured to perform a batch culture for a period of 5 days. Samples of the culture supernatant were tested daily for rVWF content (vWF ELISA), total (Ristocetin) and specific (Specific Activity) via a ristocetin cofactor assay. Various culture parameters, including cell count, cell viability, and ammonium concentration were also monitored daily (except for batch days 3 and 4).

[0311] Unexpectedly, cell cultures grown under high copper concentrations produced supernatants containing significantly higher total and specific rVWF activity (compare results in Table **7** and Table **8**). For example, at batch day 4, the cell culture grown under high copper concentration contained 1.52 IU rVWF activity/mL, as compared to 0.2 IU rVWF activity/mL for the low copper culture. This is despite the fact that the low copper cell culture produced nearly twice the amount of rVWF as the high copper cell culture. Furthermore, the specific activity of the supernatant obtained from the high copper culture was more than 13 times greater than that of the low copper culture supernatant (831 mU/10µg rVWF v. 62 mU/10µg rVWF).

[0312] As seen in Table **7** and Table **8**, the total and specific ristocetin cofactor activity of the high copper culture was twice that of the low copper culture at batch day 1. Furthermore, in contrast to the subsequent increases in activity observed in the high copper culture, no increase in the amount of ristocetin cofactor activity was seen after batch day 1 for the low copper cell culture. Consistent with this result, agarose gel electrophoresis analysis of the rVWF multimer state revealed that a low concentration of high molecular weight rVWF, relative to the concentration of low molecular weight rVWF species, was present in the supernatant of the low copper cell culture at batch day 1, and that the relative concentration further decreased over time (Figure 1A and 1B). In contrast, supplementation of the culture medium with Cu^{2+} resulted in a consistent formation of ristocetin cofactor (RiCoF) active antigen through the fourth day. Consistently, no loss in the amount of stable high molecular weight rVWF multimers occurred through day 4 of the batch culture (Figure 1A and 1B). The densitometric results of the agarose electrophoresis gel shown in Figure 1B revealed that under low copper conditions the culture

was only able to produce a rVWF population in which more than 10% (*i.e.*, 16.3%) of the rVWF was present in molecules having more than 10 dimers for one day of the batch culture (i.e. "day3" sample in Lane 2 of the agarose gel of Figure 1A), and notably, this population fell to only 4% at day 5 of the batch culture ("day 7" sample in Lane 6). In contrast under high copper conditions the relative amount of vWF multimers with more than 10 dimers is consistently around 30% through day 4 of the batch culture ("day 3" to "day 6" in Lanes 7-10; 28% to 31.4%).

[0313] Notably, beginning at batch day 5 of the high copper batch culture, when NH_4^+ levels exceeded 100 mg/L (greater than about 5.0 mM), the expression of additional antigen (18.3 to 35.4 µg/L; compare batch days 4 and 5 of Table **8**) did not result in a concomitant increase in the RiCoF activity present in the supernatant. Consistent with this result, the level of high molecular weight rVWF multimers at batch day 5 (day 7 of the culture) decreased relative to the concentration of low molecular weight rVWF multimers. Also only on batch day 5 ("day 7" sample of the culture in Lane 11) the relative amount of the vWF with more than 10 dimers is reduced to 21.4%.

[0314] Taken together, the data provided above demonstrate that the supplementation of copper concentrations in cell cultures expressing rVWF dramatically increases the total and specific rVWF ristocetin cofactor activity, as well the stable production of high molecular weight rVWF multimers. Furthermore, the data show a correlation between the presence of high NH_4^+ concentrations in the cell culture and the loss of rVWF ristocetin cofactor activity and high molecular weight rVWF multimer production.

Table 7. Expression of rVWF in mammalian cell culture performed in batch mode using BAV-SP media with a low copper concentration (1.0 µg/L).

batch day	Cell Count [10E6 cells/mL]	NH_4^+ [mg/l]	Viability [%]	vWF ELISA [µg/ml]	Ristocetin [IU/mL]	Specific Actifity mU/10µg
0	0.42	21	98.8	n.d.	n.d.	n.d.
1	0.78	43	n.d.	6.3	0.23	365
2	1.24	64	99.1	12.8	0.23	180
3	1.86	n.d.	n.d.	22.7	0.21	93
4	2.49	n.d.	n.d.	32.2	0.20	62
5	3.11	106	98.3	44.4	0.20	45

Table 8. Expression of rVWF in mammalian cell culture performed in batch mode using BAV-SP media with a high copper concentration (4.3 µg/L).

batch day	Cell Count [10E6 cells/mL]	NH_4^+ [mg/l]	Viability [%]	vWF ELISA [µg/ml]	Ristocetin [IU/mL]	Specific Actifity mU/10µg
0	0.40	21	99.3	n.d.	n.d.	n.d.
1	0.71	42	n.d.	4.8	0.40	833
2	1.23	63	99.5	8.7	0.62	713
3	1.85	n.d.	n.d.	15.0	1.07	713
4	2.54	n.d.	n.d.	18.3	1.52	831
5	3.32	109	98.9	35.4	1.55	438

Example 3

[0315] Recombinant Factor VIII (rFVIII) and Von Willebrand Factor (rVWF) were co-expressed in continuous cultures of GD8/6 cells operated under chemostatic conditions to determine the effect of the composition of the culture medium on VWF expression and activity. Briefly, GD8/6 cells were cultivated in BAV-SP medium containing 4 g/L of soy hydrolysate with and without copper supplementation as described in Example 2. To test the effect of low copper concentraions on rVWF expression and activity, basal BAV-SP media was used. The basal media contained 0.3 µg/L copper and was supplemented with soy hydrolysate, which contributed an additional 0.7 µg/L copper, providing a final copper concentration of 1.0 µg/L. As a comparision, the BAV-SP media used for the batch cultures was further supplemented with an additional 3.3 µg/L of Cu^{2+}, providing a final copper concentration of 4.3 µg/L, to determine the effect high copper concentrations on VWF expression and activity. Cultures grown in the presence of high and low copper concentrations were cultivated under both high ($2.8x10^6$ cells/mL) and low (appr. 1.4x10E06 cells/mL) cell densities.

[0316] As before, samples of the culture supernatant were tested for rVWF content (vWF ELISA), total (Ristocetin) and specific (Specific Activity) activity via a ristocetin cofactor assay. Various culture parameters, including cell count, cell viability, and ammonium concentration were also monitored. Data were generated from the steady state phase of weeks 2 and 3 of the chemostat cultures (Table **9** to Table **13**).

Table 9. Mean data for rVWF expression in chemostatic cell culture during weeks 2 and 3.

Cell Count	Copper Concentration	Cell Count [10E6 cells/mL]	NH4+ [mM]	Viability [%]	vWF ELISA [µg/ml]	Ristocetin [IU/mL]	specific actifity [mU/10µg]
high	low	**2.88**	**3.88**	**97.74**	**44.56**	**0.10**	**21.62**
high	high	**2.79**	**4.04**	**98.25**	**38.38**	**0.19**	**53.11**
low	low	**1.55**	**3.33**	**98.63**	**18.96**	**0.10**	**50.16**
low	high	**1.43**	**3.17**	**98.59**	**11.76**	**0.70**	**598.76**

Table 10. rVWF expression in chemostatic cell culture under high cell count, low copper conditions during weeks 2 and 3.

Day	Cell Count [10E6 cells/mL]	NH4+ [mM]	Viability [%]	vWF ELISA [µg/ml]	Ristocetin [IU/mL]	specific actifity [mU/10µg]
8	2.54	3.7	98.20	39.8	0.095	23.86934673
9	3.02	4.2	97.40			
10	2.97	3.9	97.90	41.3	0.095	23.00242131
11	2.78					
13	2.91	3.8	97.60	41.7	0.095	22.78177458
14	2.90	3.8	97.40			
15	3.05	3.9	97.70	44.7	0.095	21.25279642
16	2.99	3.8	98.30			
17	2.76	3.9	97.40	55.3	0.095	17.17902351
	2.88	**3.88**	**97.74**	**44.56**	**0.10**	**21.62**

Table 11. rVWF expression in chemostatic cell culture under high cell count, high copper conditions during weeks 2 and 3.

Day	Cell Count [10E6 cells/mL]	NH4+ [mM]	Viability [%]	vWF ELISA [µg/ml]	Ristocetin [IU/mL]	specific actifity [mU/10µg]
8	2.52	3.9	98.30	31.8	0.35	110.0628931
9	2.92	4.3	98.50			
10	2.80	4.2	98.60	37.4	0.32	85.56149733
11	2.57					
13	2.81	3.9	98.50	37.6	0.095	25.26595745
14	2.92	3.9	97.80			
15	2.77	3.9	97.80	43.0	0.095	22.09302326
16	2.72	4.0	98.30			
17	3.04	4.1	98.20	42.1	0.095	22.56532067
	2.79	**4.04**	**98.25**	**38.38**	**0.19**	**53.11**

Table 12. rVWF expression in chemostatic cell culture under low cell count, low copper conditions during weeks 2 and 3.

Day	Cell Count [10E6 cells/mL]	NH4+ [mM]	Viability [%]	vWF ELISA [µg/ml]	Ristocetin [IU/mL]	specific actifity [mU/10µg]
8	1.61	3.3	99.10	19.3	0.095	49.22279793

9	1.65	3.3	98.00			
10	1.49	3.3	98.90	18.6	0.095	51.07526882
11	1.58					
13	1.58	3.4	98.10	18.1	0.095	52.48618785
14	1.56	3.3	99.30			
15	1.52	3.3	98.50	18.9	0.095	50.26455026
16	1.50	3.3	98.40			
17	1.50	3.4	98.70	19.9	0.095	47.73869347
	1.55	**3.33**	**98.63**	**18.96**	**0.10**	**50.16**

Table 13. rVWF expression in chemostatic cell culture under low cell count, high copper conditions during weeks 2 and 3.

Day	Cell Count [10E6 cells/mL]	NH4+ [mM]	Viability [%]	vWF ELISA [µg/ml]	Ristocetin [IU/mL]	specific actifity [mU/10µg]
8	1.46	3.2	98.80	11.6	0.73	629.3103448
9	1.45	3.2	98.20			
10	1.37	3.1	98.90	11.0	0.7	636.3636364
11	1.43					
13	1.33	3.2	98.10	11.1	0.68	612.6126126
14	1.39	3.2	97.20			
15	1.51	3.2	99.70	12.4	0.69	556.4516129
16	1.49	3.2	98.60			
17	1.43	3.2	99.20	12.7	0.71	559.0551181
	1.43	**3.17**	**98.59**	**11.76**	**0.70**	**598.76**

[0317] As shown in Figure 2A, supernatant harvested from continuous rVWF cell culture grown at low cell densities and high copper concentrations contained high specific activity (average of 600 mU / 10 µg), while supernatants harvested from rVWF cell cultures grown at high cell densities in the presence of high or low copper concentrations and rVWF cell cultures grown at low cell density in the presence of low copper concentration contained low specific activities (less than 100 mU / 10 µg). Consistent with the results observed for batch cultures, Figure 2B shows that continuous mammalian cell cultures expressing rVWF with high specific activities have lower NH_4^+ concentrations than do cultures producing rVWF with low specific activities. This data further strengthen the correlation between NH_4^+ concentration in the cell culture and the specific activity of rVWF produced by the culture. Notably, the combination of high copper concentration and low ammonium concentration in the cell culture allowed for the production significantly improved rVWF activity.

[0318] Consistent with this result, agarose gel electrophoresis analysis of the rVWF multimer state of chemostat cultures (Figure 6) revealed that only supernatants from cultures operated at

high copper and low cell density and thus low ammonium resulted in a consistent expression high multuimeric vWF (about 23% to 27% on CST day 8, 17 and 24 in Lanes 4, 8, and 12, respectively). All other conditions do not reach consistently high amounts of more than 10% of vWF with more than 10 dimers over an extended cultivation period.

Example 4

[0319] To determine the effect of culture media copper concentration on the expression and specific activity of rA13, mammalian cell cultures expression rA13 were grown for 4 weeks under chemostatic continuous culture conditions in ADAMTS13 medium comprising a modified DMEM/F12 basal media BESP845 and additional supplements (Table **14**) containing copper concentrations ranging from 0.66 µg/L (without additional copper supplementation) and with additional copper supplementation to 4 µg/L. As shown in Table **15**, increasing copper concentration in the cell culture media resulted in a significant increase in volumetric (P) and specific (q) productivity expressed as the total rA13 activity produced per liter culture and day and the total rA13 activity produced per cell and day, respectively.

Table 14. Composition of ADAMTS13 medium.

DMEM/F12 BESP845	
Amino Acids	**mg/L**
L-Alanine	13.3500
L-Arginine HCl	147.5000
L-Asparagine-H_2O	45.1600
L-Aspartic Acid	19.9500
L-Cystéine HCl-H_2O	32.5500
L-Cystine 2HCl	102.3500
L-Glutamic Acid	22.0500
Glycine	26.2500
L-Histidine-H_2O HCl	51.4800
L-Isoleucine	74.4700
L-Leucine	119.0500
L-Lysine HCl	146.2500
L-Methionine	100.0000
L-Phenylalanine	60.4800
L-Proline	63.7400
L-Serine	36.7500
L-Threonine	53.4500
L-Tryptophan	29.0100
L-Tyrosine 2Na 2H2O	75.7900
L-Valine	82.8500
salts	**mg/L**
Calcium Chloride (CaCl2)	116.6000
Copper Sulfate ($CuSO_4$-$5H_2O$)	0.0026

Ferric Nitrate ($Fe(NO_3)3-9H_2O$)	0.0500
Ferrous Sulfate ($FeSO_4-7H_2O$)	1.0170
Magnesium Chloride (MgCl2)	28.6400
Magnesium Sulfate (MgSO4)	48.8400
Potassium Chloride (KCl)	311.8000
Sodium Chloride (NaCl)	5495.5000
Na2HPO4 Anhydrous	213.0200
NaH2PO4 Anhydrous	54.3500
Zinc Sulfate Heptahydrate (ZnSO4-27H2O)	0.4320
Sodium selenite.anhydrous	0.0087
Vitamins	**mg/L**
Ascorbic Acid	3.4990
Biotin	0.2035
Choline Chloride	26.9800
D-Ca-Pantothenate	6.2400
Folic Acid	6.6500
I-Inositol	36.6000
Nicotinamide	7.0200
Pyridoxine HCl	6.0310
Riboflavin	0.6590
Thiamine HCl	6.5100
Vitamin B12	2.6800
miscellaneous	**mg/L**
D-Glucose	5000.0000
Linoleic Acid	0.0420
Lipoic Acid	1.0050
Putrescine 2HCl	3.6810
Thymidine	0.3650
Hypoxantine Na	2.3900
Sodium Pyruvate	55.0000
DMEM/F12 BESP845: Total	12738.3
ADAMTS-13 Medium Supplements	**mg/L**
L-Glutamin	1300.0000
Pluronic F68	1000.0000
Etanolamin	1.5300
ZnSo4*7 H2O	1.0000
Na-Hydrogencarbonat	1500.0000
Supplements Total	**3802.5**
Total amount of Ingridients	**16540.8**

[0320] To determine if increased copper concentrations affected the integrity of the expressed rA13, supernatant harvested from rA13 cell cultures grown in media containing 0.66 µg/L, 1 µg/L, and 4 µg/L copper was examined by SDS-PAGE analysis. As seen in Figure 3A (silver stain) and Figure 3B (anti-A13 western blot), no obvious change in product quality by gel electrophoresis was observed. Specifically, no increase in the level of truncated 170 kD or other low MW rA13 variants and no other additional or increased HCP bands resulted from rA13 expression in the presence of increased copper concentrations.

[0321] To estimate an optimal concentration of copper on the activity of rA13, an extrapolation of data from Table **15** of P Frets versus copper concentration (Figure 4) shows that optimum effect is likely reached at about 2 µg/L, with a conservative estimation of a negative effect above about 4 µg/L.

Table 15. Expression of rA13 in mammalian cell culture performed in chemostatic continuous culture mode using media containing variable copper concentrations.

10 L Scale R&D Experiment Mean of 4 CST weeks	**ZZ - NC**	**P Frets**	**q Frets**	**Spec. Activity**
	[10E6 cells/mL]	**[U/(L*d)]**	**[U/(10E9 cells*d)]**	**U/mg**
0.66 µg/L Cu^{2+}	1.35	1322	1028	1759
1 µg/L Cu^{2+}	1.64	1962	1247	1690
4 µg/L Cu^{2+}	2.26	2960	1338	1768

[0322] Based on the results obtained above, an additional experiment was performed, which compared the expression of rA13 in cell culture media containing 0.66 µg/L copper with 2.0 µg/L copper. As shown in Table **16**, supplementation of the basal cell media with copper, to a final concentration of 2.0 µg/L copper, resulted in a significant increase in volumetric (P) and specific (q) productivity expressed as the total rA13 activity produced per liter culture and day and the total rA13 activity produced per cell and day, respectively, over a period of 8 weeks. The specific data for each week of rA13 production in the two cultures is shown in Figure 5. From these data, it is clear that copper supplementation has a measurable beneficial effect on cell metabolism, specific growth rate and rA13 productivity.

Table 16. Expression of rA13 in mammalian cell culture performed in chemostatic continuous culture mode using media containing variable copper concentrations.

10 L Scale Mean of 8 CST weeks	**ZZ - NC**	**P Frets**	**q Frets**	**Spec. Activity**
	[10E6 cells/mL]	**[U/(L*d)]**	**[U/(10E9 cells*d)]**	**U/mg**

0.66 µg/L Cu^{2+}	1.29	1049	821	1862
2 µg/L Cu^{2+}	2.17	2470	1146	1749

[0323] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

WHAT IS CLAIMED IS:

1. A method for producing a recombinant Von Willebrand Factor (rVWF) composition, the method comprising the steps of:

(a) providing a basal cell culture media;

(b) supplementing the basal cell culture media with copper to provide a final copper concentration of at least 2.4 µg/L;

(c) providing one or more cells comprising a nucleic acid encoding a rVWF protein;

(d) culturing the one or more cells in the copper supplemented cell culture media such that rVWF is expressed and excreted from the cells into a culture supernatant; and

(e) recovering at least a portion of the culture supernatant,

wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 30 mU/µg rVWF.

2. The method of claim 1, further comprising the step of supplementing the basal cell culture media with a hydrolysate prior to culturing the one or more cells.

3. The method of claim 2, wherein the hydrolysate is a plant hydrolysate.

4. The method of claim 3, wherein the hydrolysate is a soy hydrolysate.

5. The method according to any one of claims 1 to 4, wherein the basal cell culture media is an animal protein free culture media.

6. The method according to any one of claims 1 to 4, wherein the basal cell culture media is a protein free culture media.

7. The method according to any one of claims 1 to 6, wherein the basal cell culture media is a chemically defined culture media.

8. The method according to any one of claims 1 to 7, wherein the final copper concentration of the copper supplemented basal cell culture media is at least 4 µg/L copper.

9. The method according to any one of claims 1 to 8, wherein the final copper concentration of the copper supplemented basal cell culture media is between 2.4 µg/L and 20 µg/L copper.

10. The method according to any one of claims 1 to 9, wherein the copper supplementing the basal cell culture media is provided as a copper salt, a copper chelate, or a combination thereof.

11. The method of claim 10, wherein the copper salt is selected from the group consisting of copper sulfate, copper acetate, copper carbonate, copper chloride, copper hydroxide, copper nitrate, and copper oxide.

12. The method according to any one of claims 1 to 11, wherein the one or more cells are mammalian cells.

13. The method of claim 12, wherein the mammalian cells are CHO cells.

14. The method according to any one of claims 1 to 13, wherein culturing the one or more cells comprises batch cultivation of the cells.

15. The method according to any one of claims 1 to 13, wherein culturing the one or more cells comprises continuous cultivation of the cells.

16. The method of claim 15, wherein the continuous cultivation of cells is performed in chemostatic mode.

17. The method of claim 15, wherein the continuous cultivation of cells is performed in perfusion mode.

18. The method according to any one of claims 1 to 17, wherein the one or more cells is cultured in at least 100 L of the supplemented basal cell culture media.

19. The method according to any one of claims 1 to 18, wherein the cell density is maintained at less than $2.5x10^6$ cells per mL during the step of culturing the one or more cells.

20. The method of claim 19, wherein the cell density is maintained at less than $2.0x10^6$ cells per mL during the step of culturing the one or more cells.

21. The method of claim 19, wherein the cell density is maintained at less than $1.5x10^6$ cells per mL during the step of culturing the one or more cells.

22. The method according to any one of claims 1 to 21, wherein the step of recovering at least a portion of the culture supernatant comprises filtration or centrifugation to remove cells from the portion of culture supernatant.

23. The method according to any one of claims 1 to 22, wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 40 mU/µg rVWF.

24. The method of claim 23, wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU/µg rVWF.

25. The method of claim 23, wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 60 mU/µg rVWF.

26. The method of claim 23, wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU/µg rVWF.

27. The method of claim 23, wherein the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 80 mU/µg rVWF.

28. The method according to any one of claims 1 to 27, wherein at least 10% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers.

29. The method according to any one of claims 1 to 27, wherein at least 20% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers.

30. The method according to any one of claims 1 to 27, wherein at least 30% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers.

31. The method according to any one of claims 1 to 30, wherein the supernatant contains high molecular weight VWF multimers of 14 to 22 dimers.

32. The method according to any one of claims 1 to 31, wherein the NH_4^+ content of the cell culture supernatant is maintained at a concentration below 10 mM.

33. The method of claim 32, wherein the NH_4^+ content of the cell culture supernatant is maintained at a concentration below 4 mM.

34. The method according to any one of claims 1 to 33, wherein rVWF is co-expressed with recombinant Factor VIII (rFVIII).

35. The method of claim 34, further comprising a step of purifying rVWF away from at least 50% of the rFVIII present in the recovered supernatant.

36. The method of claim 35, wherein the ratio of rVWF to rFVIII after the purification step is at least 10:1.

37. The method according to any one of claims 1 to 36, wherein the method further comprises a rVWF enrichment step.

38. A cell culture supernatant comprising recombinant Von Willebrand Factor (rVWF), wherein the supernatant is prepared by a method according to any one of the preceding claims.

39. A cell culture supernatant comprising recombinant Von Willebrand Factor (rVWF), wherein at least 20% of the rVWF in the supernatant is present in a high molecular weight VWF multimer of more than 10 dimers.

40. A cell culture supernatant comprising recombinant Von Willebrand Factor (rVWF), wherein the supernatant contains at least 0.5 IU ristocetin cofactor activity per mL.

41. A recombinant Von Willebrand Factor (rVWF) composition prepared by a method according to any one of claims 1 to 37.

42. The composition of claim 41, wherein the composition further comprises recombinant Factor VIII (rFVIII).

43. The composition of claim 42, wherein the ratio of rVWF to rFVIII is at least 10:1.

44. The composition of claim 42 or 43, wherein the composition is formulated for pharmaceutical administration.

45. The composition according to any one of claims 42 to 44, wherein the composition is formulated for intravenous administration.