LYOPHILIZED FACTOR IX FORMULATIONS

BACKGROUND

[0001] The present invention relates generally to the field of therapeutics for hemostatic disorders.

[0002] Hemophilia B (also known as Christmas disease) is one of the most common inherited bleeding disorders in the world. It results in decreased *in vivo* and *in vitro* blood clotting activity and requires extensive medical monitoring throughout the life of the affected individual. In the absence of intervention, the afflicted individual will suffer from spontaneous bleeding in the joints, which produces severe pain and debilitating immobility; bleeding into muscles results in the accumulation of blood in those tissues; spontaneous bleeding in the throat and neck can cause asphyxiation if not immediately treated; renal bleeding; and severe bleeding following surgery, minor accidental injuries, or dental extractions also are prevalent.

[0003] Normal *in vivo* blood coagulation at minimum requires the serine proteases Factors II (prothrombin), VII, IX, X and XI (soluble plasma proteins); cofactors including the transmembrane protein tissue factor and the plasma proteins Factors V and VIII; fibrinogen, the transglutaminase Factor XIII, phospholipid (including activated platelets), and calcium. Additional proteins including kallikrein, high molecular weight kininogen, and Factor XII are required for some *in vitro* clotting tests, and can play a role *in vivo* under pathologic conditions.

[0004] In hemophilia, blood clotting is disturbed by a lack of certain plasma blood clotting factors. Hemophilia B is caused by a deficiency in Factor IX that can result from either the decreased synthesis of the Factor IX protein or a defective molecule with reduced activity. The treatment of hemophilia occurs by replacement of the missing clotting factor by exogenous factor concentrates highly enriched in Factor IX. However, generating such a concentrate from blood is fraught with technical difficulties, as is described below.

[0005] Purification of Factor IX from plasma (plasma derived Factor IX; pdFIX) almost exclusively yields active Factor IX. However, such purification of Factor IX from plasma is very difficult because Factor IX is only present in low concentration in plasma (5 µg/mL. Andersson, Thrombosis Research 7: 451 459 (1975). Further, purification from

blood requires the removal or inactivation of infectious agents such as HIV and HCV. In addition, pdFIX has a short half-life and therefore requires frequent dosing. Recombinant factor IX (rFIX) is also available, but suffers from the same short half-life and need for frequent dosing (*e.g.,* 2-3 times per week for prophylaxis) as pdFIX. rFIX also has a lower incremental recovery (K value) compared to pdFIX, which necessitates the use of higher doses of rFIX than those for pdFIX.

[0006] Reduced mortality, prevention of joint damage and improved quality of life have been important achievements due to the development of plasma-derived and recombinant Factor IX. Prolonged protection from bleeding would represent another key advancement in the treatment of hemophilia B subjects. However, to date, no products that allow for prolonged protection have been developed. Therefore, there remains a need for improved methods of treating hemophilia due to Factor IX deficiency that are more tolerable and more effective than current therapies.

[0007] In particular, there remains a need for improved lyophilized FIX formulations with higher drug product strength, longer shelf life, reduced lyophilization process time, and shorter reconstitution time.

BRIEF SUMMARY OF THE INVENTION

[0008] The present invention is directed to a pre-lyophilization formulation comprising: (a) a Factor IX (FIX) polypeptide having FIX coagulation activity; (b) a buffering agent; (c) a stabilizing agent; (d) a bulking agent; and (e) a surfactant, wherein the formulation has a fill volume of less than about 5 mL, less than about 4mL, or less than about 3 mL and wherein each of (a) – (e) are at an amount per vial (mg/vial) sufficient to allow (1) improved stability of the FIX polypeptide when lyophilized; (2) reduced reconstitution time when lyophilized; (3) reduced splashing onto a stopper comprising the formulation; (4) reduced lyophilization cycle time; (5) increased shelf-life of a lyophilate prepared from the pre-lyophilization formulation at room temperature; or (6) any combinations thereof, compared to a reference pre-lyophilization formulation, wherein the reference formulation comprises (a) – (e) at the amount per vial identical to the pre-lyophilization formulation, but has at least a 5mL fill volume. In a particular embodiment, the fill volume of the formulation is about 2.65mL.

[0009] In some embodiments, the pre-lyophilization formulation comprises at least 100 IU/vial of the FIX polypeptide. In some embodiments, the pre-lyophilization formulation comprises about 200 IU/vial to about 10,000 IU/vial of the FIX polypeptide.

[0010] In some embodiments, the FIX polypeptide comprises wild-type FIX. In some embodiments, the FIX polypeptide further comprises a heterologous moiety fused to wild-type FIX. In one embodiment, the heterologous moiety is a moiety extending half-life of FIX. In another embodiment, the heterologous moiety comprises a polypeptide or a non-polypeptide moiety. In one embodiment, the moiety extending half-life of FIX comprises an FcRn binding partner or an Fc region. In one embodiment, the FIX polypeptide is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identical to SEQ ID NO: 2.

[0011] In some embodiments, the fill volume is about 4 mL, about 3.5 mL, about 3.0 mL, about 2.9 mL, about 2.8 mL, about 2.7 mL, about 2.65 mL, about 2.6mL, about 2.5 mL, about 2.4 mL, about 2.3 mL, about 2.2 mL, about 2.1 mL, or about 2.0 mL.

[0012] In some embodiments, the reduced reconstitution time is less than 1.5 minute, less than 1 minute, less than 50 seconds, less than 40 seconds, less than 30 seconds, less than 20 seconds, or less than 10 seconds.

[0013] In some embodiments, the buffering agent is L-histidine. In one embodiment, the buffering agent is at a concentration (mg/mL) between about 3 mg/mL and about 15 mg/mL. In another embodiment, the buffering agent is at a concentration between about 8 mg and about 39 mg per vial.

[0014] In some embodiments, the stabilizing agent is sucrose. In one embodiment, the stabilizing agent is at a concentration (mg/mL) between 10 mg/mL and about 50 mg/mL. In another embodiment, the stabilizing agent is at a concentration between about 27 mg and about 132 mg per vial.

[0015] In some embodiments, the bulking agent is mannitol. In one embodiment, the bulking agent is at a concentration (mg/mL) between 20 mg/mL and about 100 mg/mL. In another embodiment, the bulking agent is at a concentration between about 53 mg per vial and about 265 mg per vial.

[0016] In some embodiments, the surfactant is polysorbate 20. In one embodiment, the surfactant is at a concentration (mg/mL) between 0.01 mg/mL and about 5 mg/mL. In

another embodiment, the surfactant is at a concentration between about 0.03 mg and about 13 mg per vial.

[0017] In one aspect, the invention is directed to pre-lyophilization formulation comprising: (a) about 80 to about 2,750 IU/mL of rFIXFc; (b) about 7.76 mg/mL of L-histidine; (c) about 47.6 mg/mL of mannitol; (d) about 23.8 mg/mL of sucrose; and, (e) about 0.2 mg/mL of polysorbate-20.

[0018] The present invention is further directed to a lyophilate powder comprising a FIX polypeptide, a buffering agent, a stabilizing agent, a bulking agent, a surfactant, or any combinations thereof.

[0019] In some embodiment, the residual moisture level of the lyophilate powder is below 1%.

[0020] In one embodiment, the lyophilate powder comprises: (a) a FIX polypeptide at an amount between about 2 mg per vial and about 150 mg per vial; (b) a buffering agent at an amount between 10 mg per vial and about 30 mg per vial; (c) a bulking agent at an amount between 70 mg vial and about 200 mg per vial; (d) a stabilizing agent at an amount between 30 mg per vial and 100 mg per vial; and (e) a surfactant at an amount between 0.05 mg per vial and about 5 mg per vial.

[0021] In another embodiment, the lyophilate powder comprises: (a) the lyophilized FIX polypeptide at an amount between about 2.2 mg per vial and about 125 mg per vial; (b) the buffering agent at an amount between about 12.5 mg per vial and 25 mg per vial; (c) the stabilizing agent at an amount between about 32.5 mg per vial and 80 mg per vial; (d) the bulking agent at an amount between about 75 mg per vial and 150 mg per vial; and (e) the surfactant at an amount between about 0.1 mg/mL and about 2 mg/mL.

[0022] In another embodiment, the lyophilate powder comprises: (a) about 2.2 to about 125 mg/vial of the FIX polypeptide; (b) about 20.6 mg/vial of L-histidine; (c) about 126.1 mg/vial of mannitol; (d) about 63.1 mg/vial of sucrose; and, (e) about 0.53 mg/vial of polysorbate-20;

[0023] The present invention is also directed to a reconstituted formulation comprising the lyophilate powder described herein reconstituted by a reconstitution buffer.

[0024] In one embodiment, the reconstituted formulation comprises: (a) the FIX polypeptide at a concentration between about 0.9 mg/mL and about 50 mg/mL; (b) the buffering agent at a concentration between 1.5 mg/mL and about 7.5 mg/mL; (c) the

bulking agent at a concentration between 10 mg/mL and about 50 mg/mL; (d) the stabilizing agent at a concentration between 5 mg/mL and 25 mg/mL per vial; and (e) the surfactant at a concentration between 0.005 mg/mL and about 2.5 mg/mL.

[0025] In another embodiment, the reconstituted formulation comprises: (a) the FIX polypeptide at a concentration between about 0.9 mg/mL and about 50 mg/mL; (b) the buffering agent at a concentration of about 3.88 mg/mL; (c) the bulking agent at a concentration of about 23.8 mg/mL; (d) the stabilizing agent at a concentration of about 11.9 mg/mL; (e) the surfactant at a concentration of about 0.1 mg/mL; and (f) the reconstitution buffer.

[0026] In another embodiment, the reconstituted formulation comprises: (a) the FIX polypeptide at a concentration between about 80 IU/mL and about 2,750 IU/mL; (b) the buffering agent at a concentration of about 25 mM; (c) the bulking agent at a concentration of about 131 mM; (d) the stabilizing agent at a concentration of about 35 mM; (e) the surfactant at a concentration of 0.01 % (w/v); and (f) the reconstitution buffer.

[0027] The present invention further pertains to a method of administering a FIX polypeptide to a hemophilia B patient in need thereof, or a method of preventing, treating, ameliorating, or managing hemophilia B in a patient in need thereof, comprising administering to the patient the reconstituted formulations described herein.

[0028] The present invention is also directed to a method of producing a lyophilate powder comprising a FIX polypeptide comprising lyophilizing the pre-lyophilization formulations described herein.

[0029] In one aspect, the present invention is directed to a method of lyophilizing a FIX polypeptide comprising: (a) a "freezing step" comprising freezing a pre-lyophilization formulation comprising the FIX polypeptide and an aqueous solvent; (b) a "vacuum step" comprising reducing the pressure of the frozen pre-lyophilization formulation by an amount effective to remove the aqueous solvent from the frozen pre-lyophilization formulation; and, (c) a single "drying step" comprising increasing the temperature of the frozen pre-lyophilization formulation above the collapse temperature, thereby producing a lyophilate powder. In some embodiments, the pre-lyophilization formulation is aseptically filtered and aseptically filled into a vial prior to step (a).

[0030] In another aspect, the present invention is directed to methods of producing a lyophilate powder comprising a FIX polypeptide, comprising: (a) a "freezing step" comprising freezing a pre-lyophilization formulation comprising a FIX polypeptide by ramping down the temperature for about 2 hours to a freezing temperature of about -55°C, and holding the freezing temperature for about 2 hours; (a') an "annealing step" comprising ramping up for about 1.5 hours the temperature of the frozen pre-lyophilization formulation of step (a) to an annealing temperature of about -6°C, holding the annealing temperature for about 3 hours, and ramping down the temperature for about 1.5 hours to about -55°C; (b) a "vacuum step" comprising holding the frozen pre-lyophilization formulation of step (a') at about -55°C for two hours at atmospheric pressure and ramping down the pressure for about 2 hours to about 0.33 mbar; and, (c) a single "drying step" comprising ramping up for 3 hours the temperature of the frozen pre-lyophilization formulation of step (b) to about 40°C, while holding the pressure at about 0.33 mbar, and holding the temperature of the frozen pre-lyophilization formulation at about 40°C for about 25 hours, while holding the pressure at about 0.33 mbar, thereby producing the lyophilate powder.

[0031] In a futher aspect, the lyophilate powder has one or more characteristics selected from the group consisting of: (1) improved stability of the FIX polypeptide when lyophilized; (2) reduced reconstitution time when lyophilized; (3) reduced splashing onto a stopper comprising the formulation; (4) reduced lyophilization cycle time; (5) increased shelf-life of a lyophilate prepared from the pre-lyophilization formulation at room temperature; or (6) any combinations thereof,

[0032] In one aspect, the disclosure provides a method of stabilizing a lyophilate powder comprising a FIX polypeptide, comprising lyophilizing a pre-lyophilization formulation according to the methods described herein, wherein the lyophilate powder is stabilized as measured by Size Exclusion Chromatography (SEC) with respect to a lyophilate powder prepared by using a lyophilization method comprising more than one drying step.

[0033] In another aspect, the disclosure provides a method of increasing the shelf-life of a lyophilate powder comprising a FIX polypeptide, comprising lyophilizing a pre-lyophilization formulation according to the methods described herein, wherein the shelf-life of the lyophilate powder is increased as measured by SEC and/or FIX clotting activity

assay with respect to the shelf-life of a lyophilate powder prepared by using a lyophilization method comprising more than one drying step.

[0034] This disclosure also provides a method to decrease the reconstitution time of a lyophilate powder comprising a FIX polypeptide, comprising lyophilizing a pre-lyophilization formulation according to the methods described herein, wherein the reconstitution time of the lyophilate powder is decreased with respect to the reconstitution time of a lyophilate powder prepared by using a lyophilization method comprising more than one drying step.

[0035] This disclosure further provides a method to reduce lyophilization process time of producing a lyophilate powder comprising a FIX polypeptide, comprising lyophilizing a pre-lyophilization formulation according to the methods described herein, wherein the lyophilization process time of the pre-lyophilization formulation is reduced with respect to the lyophilization process time of producing a lyophilate powder using a lyophilization method comprising more than one drying step.

BRIEF DESCRIPTION OF THE FIGURES

[0036] Figure 1 shows the vial loading pattern for each lyophilization cycle. The numbers 1, 2, and 3 indicate the thermocouple locations.

[0037] Figure 2 shows the prediction profiler from the DOE analysis of residual moisture as a function of the lyophilization parameters – temperature, vacuum, and time.

[0038] Figure 3 shows the prediction profiler from the DOE analysis of product temperature during sublimation as a function of the lyophilization parameters – temperature and vacuum.

[0039] Figure 4 shows the prediction profiler from the DOE analysis of vial mass flow during sublimation as a function of the lyophilization parameters – temperature and vacuum.

[0040] Figure 5 shows the lyophilization data from DOE Run 8 in Example 2, which is similar to the proposed rFIXFc-2G lyophilization cycle (shelf temperature of 40 °C, a chamber vacuum of 250 mTorr (0.33 mBar) and a drying time of 25 hours).

DETAILED DESCRIPTION

[0041] This disclosure provides, among other things, pre-lyophilization formulations, reconstituted formulations, and lyophilate powder compositions comprising a Factor IX (FIX) polypeptide. The disclosure also provides lyophilization methods for producing lyophilate powder comprising a FIX polypeptide. Also provided are methods of stabilizing a lyophilate powder comprising a FIX polypeptide, method to increase the shelf-life of a lyophilate powder comprising a FIX polypeptide, method to decrease the reconstitute time of a lyophilate powder comprising a FIX polypeptide, and method to reduce lyophilization process time of a pre-lyophilization formulation comprising a FIX polypeptide. In addition, the disclosure provides methods of preventing, treating, ameliorating, or managing hemophilia B in a patient in need of by administering a reconstituted formulation comprising a FIX polypeptide.

Definitions

[0042] Throughout this disclosure, the term "a" or "an" entity refers to one or more of that entity; for example, "a polynucleotide," is understood to represent one or more polynucleotides. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

[0043] Furthermore, "and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. Thus, the term "and/or" as used in a phrase such as "A and/or B" herein is intended to include "A and B," "A or B," "A" (alone), and "B" (alone). Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following aspects: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

[0044] It is understood that wherever aspects are described herein with the language "comprising," otherwise analogous aspects described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[0045] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure is related. For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular

Biology, 3rd ed., 1999, Academic Press; and the Oxford Dictionary Of Biochemistry And Molecular Biology, Revised, 2000, Oxford University Press, provide one of skill with a general dictionary of many of the terms used in this disclosure.

[0046] Units, prefixes, and symbols are denoted in their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, amino acid sequences are written left to right in amino to carboxy orientation. The headings provided herein are not limitations of the various aspects of the disclosure, which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification in its entirety.

[0047] The term "about" is used herein to mean approximately, roughly, around, or in the regions of, and the value will depend on how limitations of the measuring system. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" can modify a numerical value above and below the stated value by a variance of, *e.g.,* 10 percent or 20 percent, up or down (higher or lower). Unless otherwise stated, the meaning of "about" should be assumed to be within an acceptable error range for the particular value for the formulation or composition.

[0048] The term "polypeptide," "peptide" and "protein" are used interchangeably and refer to a polymeric compound comprised of covalently linked amino acid residues.

[0049] The term "polynucleotide" and "nucleic acid" are used interchangeably and refer to a polymeric compound comprised of covalently linked nucleotide residues. Polynucleotides can be DNA, cDNA, RNA, single stranded, or double stranded, vectors, plasmids, phage, or viruses.

[0050] The term "administering," as used herein, means to, *e.g.,* prescribe or give a pharmaceutical composition comprising an FIX polypeptide to a subject. Examples of routes of administration include, but are not limited to, intravenous, *e.g.,* intravenous injection and intravenous infusion, *e.g.,* via central venous access. Additional routes of administration include subcutaneous, intramuscular, oral, nasal, and pulmonary administration. A pharmaceutical composition comprising an FIX polypeptide can comprise one or more excipients, as described herein. Advantages of the methods, compositions, and pharmaceutical kits provided herein include: improved regimen

compliance; reduced break through bleeds; increased protection of joints from bleeds; prevention of joint damage; reduced morbidity; reduced mortality; prolonged protection from bleeding; decreased thrombotic events; and improved quality of life. Administering includes parenteral administration. In some embodiments, the parenteral administration is intravenous or subcutaneous administration.

[0051] The term "treatment" or "treating" as used herein means amelioration or reduction of one or more symptoms of bleeding diseases or disorders including, but not limited to, hemophilia B. In one embodiment, "treatment of" or "treating" a bleeding disease or disorder includes prevention of one or more symptoms of a bleeding disease or disorder. In a bleeding disease or disorder caused by a FIX deficiency (*e.g.,* a low baseline FIX activity), the term "treatment" or "treating" can mean FIX replacement therapy. By administering a FIXFc polypeptide to a subject, the subject can achieve and/or maintain a plasma trough level of a FIX activity at about 1 IU/dl or above 1 IU/dl. In other embodiments, "treatment" or "treating" means reduction of the frequency of one or more symptoms of bleeding diseases or disorders, *e.g.,* spontaneous or uncontrollable bleeding episodes. "Treatment," however, need not be a cure.

[0052] "Patient" as used herein includes an individual who is known to have at least one incidence of uncontrolled bleeding episodes, who has been diagnosed with a disease or disorder associated with uncontrolled bleeding episodes, *e.g.,* a bleeding disease or disorder, *e.g.,* hemophilia B, who are susceptible to uncontrolled bleeding episodes, *e.g.,* hemophilia, or any combinations thereof. Patients can also include an individual who is in danger of one or more uncontrollable bleeding episodes prior to a certain activity, *e.g.,* a surgery, a sport activity, or any strenuous activities. The patient can have a baseline FIX activity less than 1%, less than 0.5%, less than 2%, less than 2.5%, less than 3%, or less than 4%. Patients also include pediatric humans. Pediatric patients are birth to 20 years, preferably birth to 18 years, birth to 16 years, birth to 15 years, birth to 12 years, birth to 11 years, birth to 6 years, birth to 5 years, birth to 2 years, and 2 to 11 years of age.

[0053] "Baseline," as used herein, is the lowest measured plasma Factor IX level in a subject prior to administering a dose. The Factor IX plasma levels can be measured at two time points prior to dosing: at a screening visit and immediately prior to dosing. Alternatively, (a) the baseline in subjects whose pretreatment FIX activity is <1%, who have no detectable FIX antigen, and have nonsense genotypes can be defined as 0%, (b)

the baseline for subjects with pretreatment FIX activity <1% and who have detectable FIX antigen can be set at 0.5%, (c) the baseline for subjects whose pretreatment FIX activity is between 1 – 2% is Cmin (the lowest activity throughout the PK study), and (d) the baseline for subjects whose pretreatment FIX activity is ≥2% can be set at 2%. Activity above the baseline pre-dosing can be considered residue drug from prior treatment, and can be decayed to baseline and subtracted from the PK data following rFIXFBP dosing.

[0054] "Trough," as used herein, is the lowest plasma Factor IX activity level reached after administering a dose of chimeric polypeptide of the invention or another Factor IX molecule and before the next dose is administered, if any. Trough is used interchangeably herein with "threshold." Baseline Factor IX levels are subtracted from measured Factor IX levels to calculate the trough level.

[0055] As used herein, the term "half-life" refers to a biological half-life of a particular polypeptide in vivo. Half-life can be represented by the time required for half the quantity administered to a subject to be cleared from the circulation and/or other tissues in the animal.

[0056] The terms "long-acting" and "long-lasting" are used interchangeably herein. In one embodiment, the term "long-acting" or "long-lasting" indicates that a FIX activity as a result of administration of the rFIXFBP polypeptide is longer than the FIX activity of a wild-type FIX (*e.g.,* BENEFIX® or plasma-derived FIX ("pdFIX")). The "longer" FIX activity can be measured by any known methods in the art, *e.g.,* aPTT assay, chromogenic assay, ROTEM, TGA, and etc. In one embodiment, the "longer" FIX activity can be shown by the $T_{1/2beta}$ (activity). In another embodiment, the "longer" FIX activity can be inferred by the level of FIX antigen present in plasma, *e.g.,* by the $T_{1/2beta}$ (antigen).

[0057] The terms "lyophilate," "lyophilate powder," "lyophilized product," or "product cake," as used herein, denote a formulation which is manufactured by freeze-drying methods. The solvent (*e.g.* water) is removed by freezing following sublimation under vacuum and desorption of residual water at elevated temperature. In the pharmaceutical field, the lyophilate is present as a powder or a physical stable cake. The lyophilate is characterized by a fast dissolution after addition of a reconstitution medium.

[0058] The term "pre-lyophilization formulation" or "lyophilization feedstock" as used herein denotes a liquid formulation before the solvent (*e.g.,* water) is removed by a

freeze-drying method. The "fill volume" of a pre-lyophilization formulation is the total volume of the liquid formulation prior to lyophilization.

[0059] "$T_{1/2\beta,}$" or "$T_{1/2\ beta}$" or "Beta HL," as used herein, is half-life associated with elimination phase, $t_{1/2\beta}$=(ln2)/elimination rate constant associated with the terminal phase. The $T_{1/2\ beta}$ can be measured by FIX activity or by FIX antigen level in plasma. The $T_{1/2\ beta}$ based on activity is shown as $T_{1/2\ beta}$ (activity), and the $T_{1/2\ beta}$ based on the FIX antigen level can be shown as $T_{1/2\ beta}$ (antigen). Both $T_{1/2\ beta}$ (activity) and $T_{1/2\ beta}$ (antigen) can be shown as ranges or a geometric mean.

[0060] The term "reconstituted formulation" or "post-reconstitution composition" as used herein denotes a formulation which is lyophilized and re-dissolved by addition of a diluent. The diluent can contain, without limitation, water for injection (WFI), bacteriostatic water for injection (BWFI), sodium chloride solutions (*e.g.,* 0.9% (w/v) NaCl), glucose solutions (*e.g.,* 5% glucose), surfactant containing solutions (*e.g.,* 0.01% polysorbate 20 or polysorbate 80), a pH-buffered solution (*e.g.* phosphate-buffered solutions) and combinations thereof.

The lyophilization process in general

[0061] Lyophilization, or freeze-drying, is a process widely used in the pharmaceutical industry for the preservation of biological and pharmaceutical materials. The lyophilization process, also known as the lyophilization cycle, is traditionally divided into three distinct stages: freezing, primary drying, and secondary drying. "Lyophilizing," as used herein, refers to the entire process of lyophilization, including both the freezing steps and the drying steps.

[0062] In lyophilization, water present in a material is converted to ice during a freezing step and then removed from the material by direct sublimation under low-pressure conditions during a primary drying step. During freezing, however, not all of the water is transformed to ice. Some portion of the water is trapped in a matrix of solids containing, for example, formulation components and/or the active ingredient. The excess bound water within the matrix can be reduced to a desired level of residual moisture during a secondary drying step. All lyophilization steps, freezing, primary drying and secondary drying, are determinative of the final product properties. The primary drying is typically the longest step in a lyophilization process, therefore, optimization of this portion of the process has significant economic effect.

[0063] In certain aspects of the invention, the lyophilization process only comprises the primary drying step.

[0064] In certain aspects of the invention, the lyophilization process also comprises a separate "vacuum step" between the freezing step and the primary drying step.

[0065] In other aspects of the invention, the lyophilization process further comprises an "annealing step" between the freezing step and the primary drying step.

[0066] The term "annealing step" as used herein refers to a step in the lyophilization process of a polypeptide preparation undergoing lyophilization, prior to the drying step of the preparation, in which the temperature of the preparation is raised from a lower temperature to a higher temperature and then cooled again after a period of time.

[0067] Cycle and formulation optimization has traditionally been performed to assure that the product temperature during primary drying would never exceed the collapse temperature. The term "collapse temperature" as used herein refers to the product temperature during freeze-drying above which product cake begins to lose its original structure. Above the collapse temperature, product could experience slow sporadic bubbling, swelling, foaming, cavitation, fenestration, gross collapse, retraction and beading that may have consequences on the appearance of the product. As a result, Collapse may result in poor product stability, long drying times, uneven drying and loss of texture. *See*, *e.g.,* US 2010/0041870.

[0068] Lyophilized product in accordance with the present invention can be assessed based on product quality analysis, reconstitution time, quality of reconstitution, high molecular weight, moisture, glass transition temperature (T_g), and biological or biochemical activity. Typically, product quality analysis includes product degradation rate analysis using methods including, but not limited to, size exclusion chromatography (SEC), cation exchange-HPLC (CEX-HPLC), X-ray diffraction (XRD), modulated differential scanning calorimetry (mDSC), reversed phase HPLC (RP-HPLC), multi-angle light scattering detector (MALS), fluorescence, ultraviolet absorption, nephelometry, capillary electrophoresis (CE), SDS-PAGE, and combinations thereof. In some embodiments, evaluation of lyophilized product in accordance with the present invention includes a step of evaluating cake appearance. Additionally, lyophilized product may be assessed based on biological or biochemical activities of the product, typically, after reconstitution.

Lyophilized Factor IX Formulations

[0069] This disclosure provides pre-lyophilization, lyophilized, and post-reconstitution formulations, or pharmaceutical compositions, comprising a FIX polypeptide.

[0070] In certain aspects of the invention, the formulations disclosed herein comprise a FIX polypeptide, a buffering agent, a stabilizing agent, a bulking agent, and a surfactant, or any combinations thereof. The formulation can also contain any other agents that are useful for pharmaceutical formulation.

Factor IX (FIX) polypeptide

[0071] The FIX polypeptide or FIX protein useful for the formulation is a functional Factor FIX protein in its normal role in coagulation, unless otherwise specified. Thus, the FIX polypeptide includes variant polypeptides that are functional and the polynucleotides that encode such functional variant polypeptides. In one embodiment, the FIX polypeptides are the human, bovine, porcine, canine, feline, and murine FIX polypeptides. The full length polypeptide and polynucleotide sequences of FIX are known, as are many functional variants, *e.g.,* fragments, mutants and modified versions. FIX polypeptides include full-length FIX, full-length FIX minus Met at the N-terminus, full-length FIX minus the signal sequence, mature FIX (minus the signal sequence and propeptide), and mature FIX with an additional Met at the N-terminus. FIX can be made by recombinant means ("recombinant Factor IX" or "rFIX"), i.e., it is not naturally occurring or derived from plasma.

[0072] A great many functional FIX variants are known. International publication number WO 02/040544 A3, which is herein incorporated by reference in its entirety, discloses mutants that exhibit increased resistance to inhibition by heparin at page 4, lines 9-30 and page 15, lines 6-31. International publication number WO 03/020764 A2, which is herein incorporated by reference in its entirety, discloses FIX mutants with reduced T cell immunogenicity in Tables 2 and 3 (on pages 14-24), and at page 12, lines 1-27. International publication number WO 2007/149406 A2, which is herein incorporated by reference in its entirety, discloses functional mutant FIX molecules that exhibit increased protein stability, increased in vivo and in vitro half-life, and increased resistance to proteases at page 4, line 1 to page 19, line 11. WO 2007/149406 A2 also discloses chimeric and other variant FIX molecules at page 19, line 12 to page 20, line 9.

International publication number WO 08/118507 A2, which is herein incorporated by reference in its entirety, discloses FIX mutants that exhibit increased clotting activity at page 5, line 14 to page 6, line 5. International publication number WO 09/051717 A2, which is herein incorporated by reference in its entirety, discloses FIX mutants having an increased number of N-linked and/or O-linked glycosylation sites, which results in an increased half-life and/or recovery at page 9, line 11 to page 20, line 2. International publication number WO 09/137254 A2, which is herein incorporated by reference in its entirety, also discloses Factor IX mutants with increased numbers of glycosylation sites at page 2, paragraph [006] to page 5, paragraph [011] and page 16, paragraph [044] to page 24, paragraph [057]. International publication number WO 09/130198 A2, which is herein incorporated by reference in its entirety, discloses functional mutant FIX molecules that have an increased number of glycosylation sites, which result in an increased half-life, at page 4, line 26 to page 12, line 6. International publication number WO 09/140015 A2, which is herein incorporated by reference in its entirety, discloses functional FIX mutants that an increased number of Cys residues, which can be used for polymer (*e.g.,* PEG) conjugation, at page 11, paragraph [0043] to page 13, paragraph [0053]. The FIX polypeptides described in International Application No. PCT/US2011/043569 filed July 11, 2011 and published as WO 2012/006624 on January 12, 2012 are also incorporated herein by reference in its entirety.

[0073] In certain embodiments, the FIX polypeptide comprises wild-type FIX. In some embodiments, the FIX polypeptide further comprises a heterologous moiety fused to wild-type FIX. In certain embodiments, the heterologous moiety is a moiety extending half-life of FIX. In certain embodiments, the heterologous moiety comprises a polypeptide or a non-polypeptide moiety.

[0074] In other embodiments, the FIX polypeptide is a long-acting FIX polypeptide. A long-acting FIX polypeptide can comprise a FIX portion and a non FIX portion, *e.g.,* a heterologous moiety that is capable of extending *in vivo* or *in vitro* half-life of the FIX polypeptide. Exemplary non- FIX portions include, *e.g.,* Fc, albumin, a PAS sequence, transferrin, CTP (28 amino acid C-terminal peptide (CTP) of human chorionic gonadotropin (hCG) with its 4 O-glycans), polyethylene glycol (PEG), hydroxyethyl starch (HES), albumin binding polypeptide, albumin-binding small molecules, or any combination thereof. Exemplary long-acting FIX polypeptides of the invention include,

e.g., Factor IX-Fc polypeptides, Factor IX-albumin polypeptides, Factor IX-PAS polypeptides, Factor IX -transferrin polypeptides, Factor IX -CTP polypeptides, Factor IX -PEG polypeptides, Factor IX -HES polypeptides, Factor IX -albumin binding polypeptide polypeptides, or Factor IX -albumin-binding small molecule polypeptides.

[0075] In one embodiment, the FIX polypeptide is rFIXFc, a recombinant fusion protein comprised of human coagulation Factor IX (FIX) and an Fc domain of a human antibody (IgG1 isotype). *See, e.g.,* PCT Application No. PCT/US2011/043569, filed July 11, 2011 and published as WO 2012/006624, which is incorporated herein by reference in its entirety. The rFIXFc polypeptide is a heterodimeric protein with a FIXFc single chain (FIXFc-sc) and an Fc single chain (Fc-sc) bound together through two disulfide bonds in the hinge region of Fc. rFIXFc requires two protein subunits, FIXFc-sc (642 amino acids, SEQ ID NO:2) and Fc-sc (227 amino acids, SEQ ID NO:4), to assemble within a transfected cell line to form the final protein product, rFIXFc. The polynucleotide sequences encoding FIXFc-sc and Fc-sc are presented as SEQ ID NO:1 and SEQ ID NO:3, respectively.

[0076] In certain embodiments, the Factor IX portion of rFIXFc has a primary amino acid sequence that is identical to the Thr148 allelic form of plasma derived Factor IX and has structural and functional characteristics similar to endogenous Factor IX. The Fc domain of rFIXFc contains the hinge, CH2 and CH3 regions of IgG1. The assembled heterodimer mature form of rFIXFc contains 869 amino acids with a molecular weight of approximately 98 kilodaltons. In some embodiments, the rFIXFc polypeptide comprises an amino acid sequence at least 90%, 95%, or 100% identical to amino acids 1 to 642 of SEQ ID NO:2.

[0077] In one embodiment, the second portion fused to FIX is an FcRn binding partner. In another embodiment, an FcRn binding partner fused to FIX is an Fc fragment. An FcRn binding partner is any molecule that can be specifically bound by the FcRn receptor with consequent active transport by the FcRn receptor of the FcRn binding partner. Thus, the term Fc includes any variants of IgG Fc that are functional. The region of the Fc portion of IgG that binds to the FcRn receptor has been described based on X-ray crystallography (Burmeister *et al.*, *Nature 372*:379 (1994), incorporated herein by reference in its entirety). The major contact area of the Fc with the FcRn is near the junction of the CH2 and CH3 domains. Fc-FcRn contacts are all within a single Ig heavy

chain. The FcRn binding partners include, *e.g.,* whole IgG, the Fc fragment of IgG, and other fragments of IgG that include the complete binding region of FcRn. The major contact sites include amino acid residues 248, 250-257, 272, 285, 288, 290-291, 308-311, and 314 of the CH2 domain and amino acid residues 385-387, 428, and 433-436 of the CH3 domain. References made to amino acid numbering of immunoglobulins or immunoglobulin fragments, or regions, are all based on Kabat *et al.* 1991, Sequences of Proteins of Immunological Interest, U. S. Department of Public Health, Bethesda; MD, incorporated herein by reference in its entirety. (The FcRn receptor has been isolated from several mammalian species including humans. The sequences of the human FcRn, rat FcRn, and mouse FcRn are known (Story et al., *J. Exp. Med. 180*: 2377 (1994), incorporated herein by reference in its entirety.) An Fc can comprise the CH2 and CH3 domains of an immunoglobulin with or without the hinge region of the immunoglobulin. Exemplary Fc variants are provided in WO 2004/101740 and WO 2006/074199, incorporated herein by reference in its entirety.

[0078] Fc (or Fc portion of a chimeric polypeptide) can contain one or more mutations, and combinations of mutations.

[0079] Fc (or Fc portion of a chimeric polypeptide) can contain mutations conferring increased half-life such as M252Y, S254T, T256E, and combinations thereof, as disclosed in Oganesyan *et al., Mol. Immunol. 46*:1750 (2009), which is incorporated herein by reference in its entirety; H433K, N434F, and combinations thereof, as disclosed in Vaccaro *et al., Nat. Biotechnol. 23*:1283 (2005), which is incorporated herein by reference in its entirety; the mutants disclosed at pages 1-2, paragraph [0012], and Examples 9 and 10 of US 2009/0264627 A1, which is incorporated herein by reference in its entirety; and the mutants disclosed at page 2, paragraphs [0014] to [0021] of US 20090163699 A1, which is incorporated herein by reference in its entirety.

[0080] Fc (or Fc portion of a chimeric polypeptide) can also include, *e.g.,* the following mutations: The Fc region of IgG can be modified according to well recognized procedures such as site directed mutagenesis and the like to yield modified IgG or Fc fragments or portions thereof that will be bound by FcRn. Such modifications include, *e.g.,* modifications remote from the FcRn contact sites as well as modifications within the contact sites that preserve or even enhance binding to the FcRn. For example the following single amino acid residues in human IgG1 Fc (Fcy1) can be substituted without

significant loss of Fc binding affinity for FcRn: P238A, S239A, K246A, K248A, D249A, M252A, T256A, E258A, T260A, D265A, S267A, H268A, E269A, D270A, E272A, L274A, N276A, Y278A, D280A, V282A, E283A, H285A, N286A, T289A, K290A, R292A, E293A, E294A, Q295A, Y296F, N297A, S298A, Y300F, R301A, V303A, V305A, T307A, L309A, Q311A, D312A, N315A, K317A, E318A, K320A, K322A, S324A, K326A, A327Q, P329A, A330Q, A330S, P331A, P331S, E333A, K334A, T335A, S337A, K338A, K340A, Q342A, R344A, E345A, Q347A, R355A, E356A, M358A, T359A, K360A, N361A, Q362A, Y373A, S375A D376A, A378Q, E380A, E382A, S383A, N384A, Q386A, E388A, N389A, N390A, Y391F, K392A, L398A, S400A, D401A, D413A, K414A, R416A, Q418A, Q419A, N421A, V422A, S424A, E430A, N434A, T437A, Q438A, K439A, S440A, S444A, and K447A, where for example P238A represents wildtype proline substituted by alanine at position number 238. In addition to alanine other amino acids can be substituted for the wildtype amino acids at the positions specified above. Mutations can be introduced singly into Fc giving rise to more than one hundred FcRn binding partners distinct from native Fc. Additionally, combinations of two, three, or more of these individual mutations can be introduced together, giving rise to hundreds more FcRn binding partners. Certain of these mutations can confer new functionality upon the FcRn binding partner. For example, one embodiment incorporates N297A, removing a highly conserved N-glycosylation site. The effect of this mutation is to reduce immunogenicity, thereby enhancing circulating half-life of the FcRn binding partner, and to render the FcRn binding partner incapable of binding to FcγRI, FcγRIIA, FcγRIIB, and FcγRIIIA, without compromising affinity for FcRn (Routledge et al. 1995, Transplantation 60:847, which is incorporated herein by reference in its entirety; Friend et al. 1999, Transplantation 68:1632, which is incorporated herein by reference in its entirety; Shields et al. 1995, J. Biol. Chem. 276:6591, which is incorporated herein by reference in its entirety). Additionally, at least three human Fc gamma receptors appear to recognize a binding site on IgG within the lower hinge region, generally amino acids 234-237. Therefore, another example of new functionality and potential decreased immunogenicity can arise from mutations of this region, as for example by replacing amino acids 233-236 of human IgG1 "ELLG" to the corresponding sequence from IgG2 "PVA" (with one amino acid deletion). It has been shown that FcγRI, FcγRII, and FcγRIII which mediate various effector functions will not

bind to IgG1 when such mutations have been introduced (Ward and Ghetie, *Therapeutic Immunology 2*:77 (1995), which is incorporated herein by reference in its entirety; and Armour *et al.*, *Eur. J. Immunol. 29*:2613 (1999), which is incorporated herein by reference in its entirety). As a further example of new functionality arising from mutations described above affinity for FcRn can be increased beyond that of wild type in some instances. This increased affinity can reflect an increased "on" rate, a decreased "off" rate or both an increased "on" rate and a decreased "off" rate. Mutations believed to impart an increased affinity for FcRn include, *e.g.,* T256A, T307A, E380A, and N434A (Shields *et al.*, *J. Biol. Chem. 276*:6591 (2001), which is incorporated herein by reference in its entirety).

[0081] The Fc (or Fc portion of a chimeric polypeptide) can be at least about 60%, about 70%, about 80%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or about 100% identical to the Fc amino acid sequence shown in Table 14 (*e.g.,* amino acids 21 to 247 of SEQ ID NO: 4). The Fc (or Fc portion of a chimeric polypeptide) can be identical to the Fc amino acid sequence shown in Table 14 (*e.g.,* amino acids 21 to 247 of SEQ ID NO: 4).

[0082] As discussed above, exemplary long-acting polypeptides also include FIX fused to one or more albumin polypeptides, albumin binding polypeptides, or albumin-binding small molecules. In one embodiment, the albumin is human albumin. The albumin or albumin binding protein can be fused to either the N-terminal end of FIX or to the C-terminal end of FIX or inserted between two amino acids in FIX. Examples of albumin, *e.g.,* fragments thereof, that can be used in the present invention are known. *e.g.,* U.S. Patent No. 7,592,010; U.S. Patent No. 6,686,179; and Schulte, *Thrombosis Res. 124* Suppl. 2:S6-S8 (2009), each of which is incorporated herein by reference in its entirety.

[0083] The albumin binding polypeptides can compromise, without limitation, bacterial albumin-binding domains, albumin-binding peptides, or albumin-binding antibody fragments that can bind to albumin. Domain 3 from streptococcal protein G, as disclosed by Kraulis *et al.*, *FEBS Lett.* 378:190-194 (1996) and Linhult *et al.*, *Protein Sci.* 11:206-213 (2002) is an example of a bacterial albumin-binding domain. Examples of albumin-binding peptides include a series of peptides having the core sequence DICLPRWGCLW (SEQ ID NO: 5). See, *e.g.,* Dennis *et al.*, *J. Biol. Chem.* 2002, 277: 35035-35043 (2002). Examples of albumin-binding antibody fragments are disclosed in Muller and

Kontermann, *Curr. Opin. Mol. Ther.* 9:319-326 (2007); Rooverset *et al.*, *Cancer Immunol. Immunother.* 56:303-317 (2007), and Holt *et al.*, *Prot. Eng. Design Sci.*, 21:283-288 (2008), which are incorporated herein by reference in their entireties.

[0084] In certain aspects, a recombinant FIX polypeptide of the invention comprises at least one attachment site for a non-polypeptide small molecule, variant, or derivative that can bind to albumin thereof. An example of such albumin binding moieties is 2-(3-maleimidopropanamido)-6-(4-(4-iodophenyl)butanamido)hexanoate ("Albu" tag) as disclosed by Trusselet *et al.*, *Bioconjugate Chem.* 20:2286-2292 (2009).

[0085] As discussed above, exemplary long-acting polypeptides also include FIX fused to at least one C-terminal peptide (CTP) of the β subunit of human chorionic gonadotropin or fragment, variant, or derivative thereof. The CTP can be fused to FIX either the N-terminal end of FIX or to the C-terminal end of FIX. One or more CTP peptides fused to or inserted into a recombinant protein is known to increase the *in vivo* half-life of that protein. *See, e.g.,* U.S. Patent No. 5,712,122, incorporated by reference herein in its entirety. Exemplary CTP peptides include DPRFQDSSSSKAPPPSLPSPSRLPGPSDTPIL (SEQ ID NO: 6) or SSSSKAPPPSLPSPSRLPGPSDTPILPQ. (SEQ ID NO: 7). *See, e.g.,* U.S. Patent Application Publication No. US 2009/0087411 A1, incorporated by reference.

[0086] As discussed above, exemplary long-acting polypeptides also include FIX fused to at least one PAS sequence or fragment, variant, or derivative thereof. The PAS sequence can be fused to either the N-terminal end of FIX or to the C-terminal end of FIX. A PAS peptide or PAS sequence, as used herein, means an amino acid sequence comprising mainly alanine and serine residues or comprising mainly alanine, serine, and proline residues, the amino acid sequence forming random coil conformation under physiological conditions. Accordingly, the PAS sequence is a building block, an amino acid polymer, or a sequence cassette comprising, consisting essentially of, or consisting of alanine, serine, and proline which can be used as a part of the heterologous moiety in the chimeric protein. An amino acid polymer also can form random coil conformation when residues other than alanine, serine, and proline are added as a minor constituent in the PAS sequence. By "minor constituent" is meant that that amino acids other than alanine, serine, and proline can be added in the PAS sequence to a certain degree, *e.g.,* up to about 12%, *i.e.*, about 12 of 100 amino acids of the PAS sequence, up to about 10%, up to about

9%, up to about 8%, about 6%, about 5%, about 4%, about 3%, *i.e.* about 2%, or about 1%, of the amino acids. The amino acids different from alanine, serine and proline can be selected from the group consisting of Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr, and Val. Under physiological conditions, a PAS peptide forms a random coil conformation and thereby can mediate an increased *in vivo* and/or *in vitro* stability to a recombinant protein of the invention, and has procoagulant activity.

[0087] Non-limiting examples of the PAS peptides include ASPAAPAPASPAAPAPSAPA (SEQ ID NO: 8), AAPASPAPAAPSAPAPAAPS (SEQ ID NO: 9), APSSPSPSAPSSPSPASPSS (SEQ ID NO: 10), APSSPSPSAPSSPSPASPS (SEQ ID NO: 11), SSPSAPSPSSPASPSPSSPA (SEQ ID NO: 12), AASPAAPSAPPAAASPAAPSAPPA (SEQ ID NO: 13), ASAAAPAAASAAASAPSAAA (SEQ ID NO: 14) or any variants, derivatives, fragments, or combinations thereof. Additional examples of PAS sequences are known from, *e.g.,* US Pat. Publ. No. 2010/0292130 A1, PCT Appl. Publ. No. WO 2008/155134 A1, and European issued patent EP2173890.

[0088] As discussed above, exemplary long-acting polypeptides also include FIX fused to at least one transferrin peptide or fragment, variant, or derivative thereof. At least one transferrin peptide can be fused to either the N-terminal end of FIX or to the C-terminal end of FIX or inserted between two amino acids in FIX. Any transferrin can be fused to or inserted into a recombinant FIX protein of the invention. As an example, wild-type human Tf (Tf) is a 679 amino acid protein, of approximately 75 KDa (not accounting for glycosylation), with two main domains, N (about 330 amino acids) and C (about 340 amino acids), which appear to originate from a gene duplication. *See* GenBank accession numbers NM001063, XM002793, M12530, XM039845, XM 039847 and S95936 (www.ncbi.nlm.nih.gov), all of which are herein incorporated by reference in their entirety.

[0089] Transferrin transports iron through transferrin receptor (TfR)-mediated endocytosis. After the iron is released into an endosomal compartment and Tf-TfR complex is recycled to cell surface, the Tf is released back extracellular space for next cycle of iron transporting. Tf possesses a long half-life that is in excess of 14-17 days (Li *et al., Trends Pharmacol. Sci.* 23:206-209 (2002)).Transferrin fusion proteins have been studied for half-life extension, targeted deliver for cancer therapies, oral delivery and

sustained activation of proinsulin (Brandsma *et al.*, *Biotechnol. Adv.*, 29: 230-238 (2011); Bai *et al.*, *Proc. Natl. Acad. Sci. USA* 102:7292–7296 (2005); Kim *et al.*, *J. Pharmacol. Exp. Ther.*, 334:682-692 (2010); Wang *et al.*, *J. Controlled Release* 155:386-392 (2011)).

[0090] As discussed above, exemplary long-acting polypeptides also include FIX fused to at least one polyethylene glycol (PEG) moieties.

[0091] PEGylated FIX can refer to a conjugate formed between FIX and at least one polyethylene glycol (PEG) molecule. PEG is commercially available in a large variety of molecular weights and average molecular weight ranges. Typical examples of PEG average molecular weight ranges include, but are not limited to, about 200, about 300, about 400, about 600, about 1000, about 1300-1600, about 1450, about 2000, about 3000, about 3000-3750, about 3350, about 3000-7000, about 3500-4500, about 5000-7000, about 7000-9000, about 8000, about 10000, about 8500-11500, about 16000-24000, about 35000, about 40000, about 60000, and about 80000 daltons. These average molecular weights are provided merely as examples and are not meant to be limiting in any way.

[0092] A recombinant long-acting FIX protein of the invention can be PEGylated to include mono- or poly-(*e.g.*, 2-4) PEG moieties. PEGylation can be carried out by any of the PEGylation reactions known in the art. Methods for preparing a PEGylated protein product will generally include (i) reacting a polypeptide with polyethylene glycol (such as a reactive ester or aldehyde derivative of PEG) under conditions whereby the peptide of the invention becomes attached to one or more PEG groups; and (ii) obtaining the reaction product(s). In general, the optimal reaction conditions for the reactions will be determined case by case based on known parameters and the desired result.

[0093] There are a number of PEG attachment methods available to those skilled in the art, for example Malik F *et al.*, *Exp. Hematol.* 20:1028-35 (1992); Francis, *Focus on Growth Factors* 3(2):4-10 (1992); European Pat. Pub. Nos. EP0401384, EP0154316, and EP0401384; and International Pat. Appl. Pub. Nos. WO92/16221 and WO95/34326. As a non-limiting example, FIX variants can contain cysteine substitutions in one or more insertion sites in FIX, and the cysteines can be further conjugated to PEG polymer. See Mei *et al.*, *Blood* 116:270-279 (2010) and U.S. Patent No. 7,632,921, which are incorporated herein by reference in their entireties.

[0094] As discussed above, exemplary long-acting polypeptides also include FIX fused to at least one hydroxyethyl starch (HES) polymer. HES is a derivative of naturally

occurring amylopectin and is degraded by alpha-amylase in the body. HES exhibits advantageous biological properties and is used as a blood volume replacement agent and in hemodilution therapy in the clinics. See, *e.g.,* Sommermeyer *et al.*, *Krankenhauspharmazie* 8:271-278 (1987); and Weidler *et al.*, *Arzneim.-Forschung/Drug Res.* 41: 494-498 (1991).

[0095] HES is mainly characterized by the molecular weight distribution and the degree of substitution. HES has a mean molecular weight (weight mean) of from 1 to 300 kD, from 2 to 200kD, from 3 to 100 kD, or from 4 to 70kD. Hydroxyethyl starch can further exhibit a molar degree of substitution of from 0.1 to 3, from 0.1 to 2, from 0.1 to 0.9, or from 0.1 to 0.8, and a ratio between C2:C6 substitution in the range of from 2 to 20 with respect to the hydroxyethyl groups. HES with a mean molecular weight of about 130 kD is VOLUVEN® from Fresenius. VOLUVEN ® is an artificial colloid, employed, *e.g.,* for volume replacement used in the therapeutic indication for therapy and prophylaxis of hypovolaemia. There are a number of HES attachment methods available to those skilled in the art, *e.g.,* the same PEG attachment methods described above.

[0096] Factor IX coagulant activity is expresses as International Unit(s) (IU). One IU of Factor IX activity corresponds approximately to the quantity of Factor IX in one milliliter of normal human plasma. Several assays are available for measuring Factor IX activity, including the one stage clotting assay (activated partial thromboplastin time; aPTT), thrombin generation time (TGA) and rotational thromboelastometry (ROTEM®).

Buffering Agent

[0097] Buffering agents useful for the present invention can be a weak acid or base used to maintain the acidity (pH) of a solution near a chosen value after the addition of another acid or base. Suitable buffering agents can maximize the stability of the pharmaceutical formulations by maintaining pH control of the formulation. Suitable buffering agents can also ensure physiological compatibility or optimize solubility. Rheology, viscosity and other properties can also dependent on the pH of the formulation. Common buffering agents include, but are not limited to, histidine, citrate, succinate, acetate and phosphate. In some embodiments, a buffering agent comprises L-histidine or mixtures of L-histidine with L-histidine hydrochloride with isotonicity agents and potentially pH adjustment with an acid or a base known in the art. In certain embodiments, the buffering agent is L-

histidine. In certain embodiments, the pH of the formulation is maintained between about 6 and about 8, or between about 6.5 and about 7.5.

Stabilizing Agent

[0098] Stabilizing agents are added to a pharmaceutical product in order to stabilize that product. Such agents can stabilize proteins in a number of different ways. Common stabilizing agents include, but are not limited to, amino acids such as glycine, alanine, lysine, arginine, or threonine, carbohydrates such as glucose, sucrose, trehalose, raffinose, or maltose, polyols such as glycerol, mannitol, sorbitol, cyclodextrins or destrans of any kind and molecular weight, or PEG. In one aspect of the invention, the stabilizing agent is chosen in order to maximize the stability of FIX polypeptide in lyophilized preparations. In certain embodiments, the stabilizing agent is sucrose.

Bulking Agent

[0099] Bulking agents can be added to a pharmaceutical product in order to add volume and mass to the product, thereby facilitating precise metering and handling thereof. Common bulking agents include, but are not limited to, lactose, sucrose, glucose, mannitol, sorbitol, calcium carbonate, or magnesium stearate. In certain embodiments, the bulking agent is mannitol.

Surfactant

[0100] Surfactants are amphipathic substances with lyophilic and lyophobic groups. A surfactant can be anionic, cationic, zwitterionic, or nonionic. Examples of nonionic surfactants include, but are not limited to, alkyl ethoxylate, nonylphenol ethoxylate, amine ethoxylate, polyethylene oxide, polypropylene oxide, fatty alcohols such as cetyl alcohol or oleyl alcohol, cocamide MEA, cocamide DEA, polysorbates, or dodecyl dimethylamine oxide. In certain embodiments, the surfactant is polysorbate 20 or polysorbate 80.

Pre-Lyophilization Formulation

[0101] In one aspect, the disclosure provides a pre-lyophilization formulation comprising:

(a) a Factor IX (FIX) polypeptide having FIX coagulation activity;

(b) a buffering agent;

(c) a stabilizing agent;

(d) a bulking agent; and

(e) a surfactant,

wherein the formulation has a fill volume of less than about 5 mL, less than about 4mL, or less than about 3 mL and wherein each of (a) – (e) are at an amount per vial (mg/vial) sufficient to allow

(1) improved stability of the FIX polypeptide when lyophilized;

(2) reduced reconstitution time when lyophilized;

(3) reduced splashing onto a stopper comprising the formulation;

(4) reduced lyophilization cycle time;

(5) increased shelf-life of a lyophilate prepared from the pre-lyophilization formulation at room temperature; or

(6) any combinations thereof,

compared to a reference pre-lyophilization formulation,

and wherein the reference formulation comprises (a) – (e) at the amount per vial identical to the pre-lyophilization formulation, but has at least 5mL fill volume. In some embodiments, the reference formulation has a 5.3mL fill volume or a 5mL fill volume.

[0102] In other embodiments, the pre-lyophilization formulation allows at least two, at least three, at least four, or at least five properties selected from (1) improved stability of the FIX polypeptide when lyophilized; (2)reduced reconstitution time when lyophilized; (3) reduced splashing onto a stopper comprising the formulation; (4) reduced lyophilization cycle time; and n(5) increased shelf-life of a lyophilate prepared from the pre-lyophilization formulation at room temperature. In certain embodiments, the pre-lyophilization formulation allows (1) improved stability of the FIX polypeptide when lyophilized. In certain embodiments, the pre-lyophilization formulation allows (2) reduced reconstitution time when lyophilized. In certain embodiments, the pre-lyophilization formulation allows (3) reduced splashing onto a stopper comprising the formulation. In certain embodiments, the pre-lyophilization formulation allows (4) reduced lyophilization cycle time. In certain embodiments, the pre-lyophilization formulation allows (5) increased shelf-life of a lyophilate prepared from the pre-

lyophilization formulation at room temperature. In certain embodiments, the pre-lyophilization formulation allows (6) any combinations of properties described herein.

[0103] In certain embodiments, the pre-lyophilization formulation comprises at least about 100 IU/vial of the FIX polypeptide. In certain embodiments, the pre-lyophilization formulation comprises at least about 200 IU/vial to about 10,000 IU/vial of the FIX polypeptide, about 200IU/vial to about 6,000 IU/vial, or about 500IU/vial to about 5,000IU/vial. In certain embodiments, the pre-lyophilization formulation comprises about 220 IU/vial, about 250 IU/vial, about 300 IU/vial, about 400 IU/vial, about 500 IU/vial, about 600 IU/vial, about 700 IU/vial, about 800 IU/vial, about 900 IU/vial, about 1,000 IU/vial, about 1,100 IU/vial, about 1,200 IU/vial, about 1,300 IU/vial, about 1,400 IU/vial, about 1,500 IU/vial, about 2,000 IU/vial, about 2,500 IU/vial, about 3,000 IU/vial, about 4,000 IU/vial, about 5,000 IU/vial, about 5,500 IU/vial, about 6,000 IU/vial, about 6,500 IU/vial, about 7,000 IU/vial, about 7,500 IU/vial, about 8,000 IU/vial, about 8,500 IU/vial, about 9,000 IU/vial, about 9,500 IU/vial or about 10,000 IU/vial of the FIX polypeptide.

[0104] In some embodiments, a higher concentration of the pre-lyophilization formulation is achieved by reducing the fill volume. In certain embodiments, the pre-lyophilization formulation has the fill volume of about 4.0 mL, about 3.5 mL, about 3.0 mL, about 2.9 mL, about 2.8 mL, about 2.7 mL, about 2.65 mL, about 2.6mL, about 2.5 mL, about 2.4 mL, about 2.3 mL, about 2.2 mL, about 2.1 mL, or about 2.0 mL. In some embodiments, the fill volume of the pre-lyophilization formulation is about 2.65 mL. In some embodiments, the fill volume of the pre-lyophilization formulation is less than about 5 mL.

[0105] In some embodiments, the FIX polypeptide can be further concentrated by an additional purification step, *e.g.*, a second ultra filtration step.

[0106] In certain embodiments, the reduced reconstitution time is less than 1.5 minute, less than 1 minute, less than 50 seconds, less than 40 seconds, less than 30 seconds, less than 20 seconds, or less than 10 seconds. In specific embodiments, the reduced reconstitution time is less than 30 seconds.

[0107] In certain embodiments, the reduced lyophilization cycle time of the pre-lyophilization formulation is about 4 days or less, about 3 days or less, about 2 days or less, or about a day or less.

[0108] In certain embodiments, the concentration of the buffering agent in the pre-lyophilization formulation is between about 3 mg/mL and about 15 mg/mL, between 4mg/mL and between 12 mg/mL, between about 5 mg/mL and about 10 mg/mL, or between about 5.82 mg/mL and about 9.7 mg/mL. In one embodiment, the buffering agent is at a concentration of between about 3.88 mg/mL and about 9.7 mg/mL. In one embodiment, the buffering agent is at a concentration of about 7.76 mg/mL. In some embodiments, the pre-lyophilization formulation contains L-histidine at a concentration of about 7.76 mg/mL.

[0109] In certain embodiments, the concentration of the stabilizing agent in the pre-lyophilization formulation is between 10 mg/mL and about 50 mg/mL, between about 13 mg/mL and about 40 mg/mL, between about 15 mg/mL and about 35 mg/mL, or between about 17.85 mg/mL and about 29.95 mg/mL. In one embodiment, the buffering agent is at a concentration of about 23.8 mg/mL. In some embodiments, the pre-lyophilization formulation contains sucrose at a concentration of 23.8mg/mL.

[0110] In certain embodiments, the concentration of the bulking agent in the pre-lyophilization formulation is between about 20 mg/mL and about 100 mg/mL, between about 30 mg/mL and about 70 mg/mL, between about 30 mg/mL and about 60 mg/mL, or between about 35.7 mg/mL and about 59.5 mg/mL. In one embodiment, the bulking agent is at a concentration of about 47.6 mg/mL. In some embodiments, the pre-lyophilization formulation contains mannitol at a concentration of about 47.6 mg/mL.

[0111] In certain embodiments, the concentration of the surfactant in the pre-lyophilization formulation is between about 0.01 mg/mL and about 5 mg/mL, between about 0.1 mg/mL and about 4 mg/mL, between about 0.1 mg/mL and about 3 mg/mL, between about 0.01 mg/mL and about 2 mg/mL, or between about 0.05 mg/mL and about 1 mg/mL. In one embodiment, the surfactant is at a concentration of about 0.2 mg/mL. In some embodiments, the pre-lyophilization formulation contains polysorbate 20 or polysorbate 80 at a concentration of about 0.2 mg/mL.

[0112] In certain embodiments, the concentration of the FIX polypeptide in the pre-lyophilization formulation is between about 80 IU/mL and about 2,750 IU/mL. In some embodiments, the concentration of the FIX polypeptide in the pre-lyophilization formulation is at least about 100 IU/mL, at least about 200 IU/mL, at least about 300 IU/mL, at least about 400 IU/mL, at least about 500 IU/mL, at least about 600 IU/mL, at

least about 700 IU/mL, at least about 800 IU/mL, at least about 900 IU/mL, at least about 1000 IU/mL, at least about 1500 IU/mL, at least about 2000 IU/mL, or at least about 2500 IU/mL.

[0113] In one aspect, the disclosure further provides a pre-lyophilization formulation comprising:

(a) about 80 to about 2,750 IU/mL of rFIXFc;

(b) about 7.76 mg/mL of L-histidine.

(c) about 47.6 mg/mL of mannitol;

(d) about 23.8 mg/mL of sucrose; and,

(e) about 0.2 mg/mL of polysorbate-20.

[0114] In certain embodiments, the fill volume of such pre-lyophilization formulation is about 3 mL, about 2.9 mL, about 2.8 mL, about 2.7 mL, about 2.65 mL, about 2.6 mL, about 2.5 mL, about 2.4 mL, about 2.3 mL, about 2.2 mL, about 2.1 mL, or about 2.0 mL. In one embodiment, the fill volume of such pre-lyophilization formulation is about 2.65 mL.

[0115] In addition, this disclosure provides a lyophilate powder which is lyophilized from any of the above pre-lyophilization formulations. In some embodiments, the pre-lyophilization formulation is any formulation described herein.

Lyophilate Power

[0116] The disclosure also provides a lyophilate powder comprising a FIX polypeptide, a buffering agent, a stabilizing agent, a bulking agent, a surfactant, or any combinations thereof.

[0117] In certain embodiments, the lyophilate powder comprises between about 8 mg and about 39 mg per vial, between about 9mg and about 35 mg per vial, between about 10 mg and about 30 mg per vial, between about 12 mg and about 25 mg per vial, between about 15 mg and about 23 mg per vial of the buffering agent (*e.g.,* L-histidine). In one embodiment, the lyophilate powder comprises about 25 mg per vial, about 24 mg per vial, about 23 mg per vial, about 22 mg per vial, about 21 mg per vial, about 20 mg per vial, about 19 mg per vial, about 18 mg per vial, about 17 mg per vial, about 16 mg per vial, about 15 mg per vial of the buffering agent. In another embodiment, the lyophilate powder comprises about 20.6 mg per vial of the buffering agent. In some embodiments, the buffering agent is L-histidine.

[0118] In certain embodiments, the lyophilate powder comprises between about 27 mg and about 132 mg per vial, between about 30 mg and about 120 mg per vial, between about 40 mg and about 110 mg per vial, between about 50 mg and about 100 mg per vial, between about 60 mg and about 90 mg per vial of the stabilizing agent. In one embodiment, the lyophilate powder comprises about 68 mg per vial, about 67 mg per vial about 66 mg per vial about 65 mg per vial about 64 mg per vial about 63 mg per vial about 62 mg per vial about 61 mg per vial about 60 mg per vial about 59 mg per vial of the stabilizing agent. In another embodiment, the lyophilate power comprises about 63.1 mg per vial of the stabilizing agent. In some embodiments, the stabilizing agent is sucrose.

[0119] In certain embodiments, the lyophilate powder comprises between about 50 mg and about 265 mg per vial, between about 53 mg and about 265 mg per vial, between about 50 mg and about 250 mg per vial, between about 53 mg and about 265 mg per vial, between about 80 mg and about 200 mg per vial, between about 100 mg and about 150 mg per vial, or between about 110 mg and about 140 mg per vial of the bulking agent. In one embodiment, the lyophilate powder comprises about 131 mg per vial, about 130 mg per vial, about 129 mg per vial, about 128 mg per vial, about 127 mg per vial, about 126 mg per vial, about 125 mg per vial, about 124 mg per vial, about 123 mg per vial, or about 122 mg per vial of the bulking agent. In another embodiment, the lyophilate powder comprises about 126.1 mg per vial of the bulking agent. In some embodiments, the bulking agent is mannitol.

[0120] In certain embodiments, the lyophilate powder comprises between about 0.03 mg and about 13 mg per vial, between about 0.05 mg and about 10 mg per vial between about 0.07 mg and about 8 mg per vial between about 0.1 mg and about 2 mg per vial of the surfactant. In one embodiment, the lyophilate powder comprises about 1 mg per vial, about 0.9 mg per vial, about 0.8 mg per vial, about 0.7 mg per vial, about 0.6 mg per vial, about 0.5 mg per vial, about 0.4 mg per vial, about 0.3 mg per vial, about 0.2 mg per vial, or about 0.1 mg per vial of the surfactant. In another embodiment, the lyophilate power comprises about 0.5 mg per vial of the surfactant. In one embodiment, the lyophilate powder comprises about 0.53 mg per vial of the surfactant. In some embodiments, the surfactant is polysorbate 20 or polysorbate 80.

[0121] In certain embodiments, the lyophilate powder comprises:

(a) a FIX polypeptide at an amount between about 2 mg per vial and about 150 mg per vial;

(b) a buffering agent at an amount between about 10 mg per vial and about 30 mg per vial;

(c) a bulking agent at an amount between about 70 mg vial and about 200 mg per vial.

(d) a stabilizing agent at an amount between about 30 mg per vial and 100 mg per vial; and

(e) a surfactant at an amount between about 0.05 mg per vial and about 5 mg per vial.

[0122] In some embodiments, the lyophilate powder comprises:

(a) a FIX polypeptide at an amount between about 2.2 mg per vial and about 125 mg per vial;

(b) a buffering agent at an amount between about 8 mg per vial and about 39 mg per vial;

(c) a bulking agent at an amount between about 53 mg vial and about 265 mg per vial.

(d) a stabilizing agent at an amount between about 27 mg per vial and 132 mg per vial; and

(e) a surfactant at an amount between about 0.03 mg per vial and about 13 mg per vial.

[0123] In certain embodiments, the lyophilate powder comprises:

(a) the lyophilized FIX polypeptide at an amount between about 2.2 mg per vial and about 125 mg per vial;

(b) the buffering agent at an amount between about 12.5 mg per vial and 25 mg per vial;

(c) the stabilizing agent at an amount between about 32.5 mg per vial and 80 mg per vial;

(d) the bulking agent at an amount between about 75 mg per vial and 150 mg per vial; and

(e) the surfactant at an amount between about 0.1 mg/mL and about 2 mg/mL.

[0124] In one embodiment, the lyophilate powder comprises:

(a) about 2.2 to about 125 mg/vial of the FIX polypeptide;

(b) about 20.6 mg/vial of L-histidine;

(c) about 126.1 mg/vial of mannitol;

(d) about 63.1 mg/vial of sucrose; and

(e) about 0.53 mg/vial of polysorbate 20.

Reconstituted Formulation

[0125] Furthermore, this disclosure provides a reconstituted formulation comprising any of the above lyophilate powder reconstituted by a reconstitution buffer.

[0126] In certain embodiments, the reconstitution buffer is a NaCl solution. In some embodiments, the reconstitution buffer is 5 mL.

[0127] In certain embodiments, the reconstituted formulation comprises:

(a) the FIX polypeptide at a concentration between about 0.9 mg/mL and about 50 mg/mL;

(b) the buffering agent at a concentration between 2 mg/mL and about 5 mg/mL;

(c) the bulking agent at a concentration between 20 mg/mL and about 30 mg/mL;

(d) the stabilizing agent at a concentration between 8 mg/mL and 15 mg/mL per vial; and

(e) the surfactant at a concentration between 0.05 mg/mL and about 0.4 mg/mL.

[0128] In certain embodiments, the reconstituted formulation comprises:

(a) the FIX polypeptide at a concentration between about 0.9 mg/mL and about 50 mg/mL;

(b) the buffering agent at a concentration of about 3.88 mg/mL;

(c) the bulking agent at a concentration of about 23.8 mg/mL;

(d) the stabilizing agent at a concentration of about 11.9 mg/mL;

(e) the surfactant at a concentration of about 0.1 mg/mL; and

(f) the reconstitution buffer comprising about 3.25 mg/mL NaCl.

[0129] In certain embodiments, the reconstituted formulation comprises:

(a) the FIX polypeptide at a concentration between about 80 IU/mL and about 2,750 IU/mL;

(b) the buffering agent at a concentration of about 25 mM;

(c) the bulking agent at a concentration of about 131 mM;

(d) the stabilizing agent at a concentration of about 35 mM;

(e) the surfactant at a concentration of 0.01 % (w/v); and

(f) the reconstitution buffer.

[0130] Examples of the formulation compositions are further provided in Tables 2-4.

[0131] In one aspect, the disclosure further provides a vial comprising the pre-lyophilization formulations, the lyophilate powder, or the reconstituted formulations described herein.

[0132] In another aspect, the disclosure provides a kit comprising a first container comprising the lyophilate powder described herein and a second container comprising a reconstitution buffer at a volume sufficient to produce a reconstituted formulation, when combined with the lyophilate powder of the first container. In certain embodiments, the volume of the reconstitution buffer in the kit is about 5 mL. In some embodiments, the volume is about 5.3 mL. In certain embodiments, the reconstitution buffer of the kit comprises NaCl. In some embodiments, the kit is used to treat hemophilia B.

[0133] In yet another aspect, the disclosure provides a method of administering a FIX polypeptide to a hemophilia B patient in need thereof, comprising administering to the patient the reconstituted formulation described herein, wherein the administration prevents or reduces the frequency or severity of bleeding episodes in the patient.

[0134] The disclosure further provides a method of preventing, treating, ameliorating, or managing hemophilia B in a patient in need thereof by administering the reconstituted formulation described herein.

Methods of producing a lyophilate powder comprising a FIX polypeptide

[0135] This disclosure provides methods of producing a lyophilate powder comprising a FIX polypeptide. In one aspect, the disclosure provides lyophilization methods comprising lyophilizing the pre-lyophilization formulations described herein. In another aspect, the disclosure provides a lyophilization method comprising a single drying step.

[0136] In one aspect, this disclosure provides a method of lyophilizing a FIX polypeptide comprising:

(a) a "freezing step" comprising freezing a pre-lyophilization formulation comprising the FIX polypeptide and an aqueous solvent;

(b) a "vacuum step" comprising reducing the pressure of the frozen pre-lyophilization formulation by an amount effective to remove the aqueous solvent from the frozen pre-lyophilization formulation; and,

(c) a single "drying step" comprising increasing the temperature of the frozen pre-lyophilization formulation above the collapse temperature,

thereby producing a lyophilate powder. In another aspect, the lyophilization process time is reduced compared to a reference method, *e.g.,* lyophilization process having two or more drying steps.

[0137] In other aspects, the lyophilate power produced by the present method has one or more characteristics of the following: (1) improved stability of the FIX polypeptide; (2) reduced reconstitution time; (3) reduced splashing onto a stopper comprising the formulation; or (4) increased shelf-life of the lyophilate powder at room temperature.

[0138] In certain embodiments, the collapse temperature is -1.5 ^{o}C.

[0139] In certain embodiments, the pre-lyophilization formulation is frozen to a freezing temperature of about -65 to about -40^{o}C, about -65 to about -45 ^{o}C, about -65 to about -55^{o}C, about -60 to about -40 ^{o}C, about -60 to about -50^{o}C or about -60 to about -55 ^{o}C during the freezing step. In certain embodiments, the pre-lyophilization formulation is frozen to a freezing temperature of about -55^{o}C during the freezing step. In certain embodiments, the freezing temperature is ramped down from about 5^{o}C to about -55^{o}C during the freezing step.

[0140] In certain embodiments, the freezing temperature is held from about 30 minutes to about 5 hours, about 1 hour to about 5 hours, about 1.5 hours to about 5 hours, about 1.5 hours to about 4 hours, about 1.5 hours to about 3 hours, or about 1.5 hours to about 2.5 hours during the freezing step. In certain embodiments, the freezing temperature is held for about 2 hours during the freezing step.

[0141] In certain embodiments, the frozen pre-lyophilization formulation of step (a) is further subject to an "annealing step" (a') prior to the "vacuum step" (b). In certain embodiments, the temperature of the frozen pre-lyophilization formulation of step (a) is ramped up to an annealing temperature of about -15^{o}C to about -2^{o}C during the annealing step. In certain embodiments, the temperature of the frozen pre-lyophilization formulation of step (a) is ramped up to an annealing temperature of about -6^{o}C during the annealing step.

[0142] In certain embodiments, the annealing temperature is held for about 2 hours to about 4 hours during the annealing step. In certain embodiments, the annealing temperature is held for about 30 minutes to about 5 hours, about 1 hour to about 5 hours, about 2 hours to about 5 hours, about 2 hours to about 4 hours or about 2.5 hours to about 3.5 hours during the annealing step. In certain embodiments, the annealing temperature is held for about 3 hours during the annealing step.

[0143] In certain embodiments, the temperature of the frozen pre-lyophilization formulation is ramped down from the annealing temperature to a temperature of about -65°C to about -40°C during the annealing step. In certain embodiments, the temperature of the frozen pre-lyophilization formulation is ramped down from the annealing temperature to a temperature of -55°C during the annealing step.

[0144] In certain embodiments, the "vacuum step" comprises subjecting the frozen pre-lyophilization formulation to a vacuum between about 0.05 and about 1 mbar, between about 0.05 and about 0.50 mbar, between about 0.10 and about 0.50 mbar, between about 0.15 and about 0.50 mbar, between about 0.20 and about 0.50 mbar, or between about 0.25 and about 0.50 mbar. In certain embodiments, the vacuum in the "vacuum step" is about 0.33 mbar.

[0145] In certain embodiments, the vacuum is held in the "vacuum step" for about 5 hours, about 4 hours, about 3 hours, about 2 hours, or about 1 hour. In some embodiments, the vacuum is held in the "vacuum step" for about 2 hours.

[0146] In certain embodiments, the "drying step" comprises ramping up the temperature of the frozen pre-lyophilization formulation from about -55°C to a drying temperature of about 40°C. In certain embodiments, the drying temperature is at least about 30°C, at least about 32°C , at least about 34°C, at least about 35°C, at least about 36°C, at least about 38°C, at least about 39°C, at least about 40°C. In other embodiments, the drying temperature is about 35°C, about 40°C, about 32°C, or about 45 °C.

[0147] In certain embodiments, the drying step further comprises holding the drying temperature for about 10 hours to about 40 hours, about 10 hours to about 30 hours, or about 20 hours to about 30 hours. In certain embodiments, the drying temperature is held for about 25 hours.

[0148] In certain embodiments, the drying step is carried out at a pressure of about 0.05 mbar to about 1 mbar, between about 0.05 and about 0.50 mbar, between about 0.10 and

about 0.50 mbar, between about 0.15 and about 0.50 mbar, between about 0.20 and about 0.50 mbar, or about 0.20 mbar to about 0.45 mbar. In certain embodiments, the pressure is held at about 0.33 mbar during the drying step. The unit of mbar can be converted to Torr or any other units. For example, 1 mbar can be converted to 0.75006375541921 Torr.

[0149] In one aspect, the disclosure provides a method of producing a lyophilate powder comprising a FIX polypeptide, comprising:

(a) a "freezing step" comprising freezing a pre-lyophilization formulation comprising a FIX polypeptide by ramping down the temperature for about 2 hours to a freezing temperature of about -55°C, and holding the freezing temperature for about 2 hours;

(a') an "annealing step" comprising ramping up for about 1.5 hours the temperature of the frozen pre-lyophilization formulation of step (a) to an annealing temperature of about -6°C, holding the annealing temperature for about 3 hours, and ramping down the temperature for about 1.5 hours to about -55°C;

(b) a "vacuum step" comprising holding the frozen pre-lyophilization formulation of step (a') at about -55°C for two hours at atmospheric pressure and ramping down the pressure for about 2 hours to about 0.33 mbar; and,

(c) a single "drying step" comprising ramping up for 3 hours the temperature of the frozen pre-lyophilization formulation of step (b) to about 40°C, while holding the pressure at about 0.33 mbar, and holding the temperature of the frozen pre-lyophilization formulation at about 40°C for about 25 hours, while holding the pressure at about 0.33 mbar,

thereby producing the lyophilate powder. In some embodiments, the lyophilization method takes less cycle time.

[0150] In certain embodiments, the lyophilate powder produced by the methods described herein has the following characteristics:

(1) improved stability of the FIX polypeptide;

(2) reduced reconstitution time;

(3) reduced splashing onto a stopper comprising the formulation;

(4) increased shelf-life of the lyophilate powder at room temperature; or

(5) any combinations thereof,

[0151] In some embodiments, the lyophilization cycle period can be less than about 4.5 days, about 4 days, about 3.5 days, about 3 days, about 2.5 days, or about 2 days. In other embodiments, the lyophilization cycle period is about 3 days or less. In certain embodiments, the fill volume of the pre-lyophilization formulation used in the lyophilization method is less than about 5 mL. In certain embodiments, the fill volume is about 4 mL, about 3.5 mL, about 3.0 mL, about 2.9 mL, about 2.8 mL, about 2.7 mL, about 2.65 mL, about 2.6 mL, about 2.5 mL, about 2.4 mL, about 2.3 mL, about 2.2 mL, about 2.1 mL, or about 2.0 mL. In one embodiment, the fill volume is about 2.65 mL.

[0152] In certain embodiments, the reduced reconstitution time of the lyophilate powder produced by the lyophilization method is less than about 1.5 minutes, less than about 1 minute, less than about 50 seconds, less than about 40 seconds, less than about 30 seconds, less than about 20 seconds, or less than about 10 seconds. In certain embodiments, the reduced reconstitution time of the lyophilate powder produced by the lyophilization method is less than about 30 seconds.

[0153] In certain embodiments, the reduced lyophilization cycle time of the pre-lyophilization formulation used in the lyophilization method is about 4 days or less, about 3 days or less, about 2 days or less, or about a day or less.

[0154] In some embodiments, the lyophilate powder is produced from the pre-lyophilization formulation in about 90 hours or less, about 80 hours or less, about 70 hours or less, about 60 hours or less, about 50 hours or less, about 45 hours or less, about 40 hours or less, or about 30 hours or less. In certain embodiments, the lyophilate powder is produced from the pre-lyophilization formulation in about 45 hours or less.

[0155] In certain embodiments, the residual moisture in the lyophilate powder is less than about 1.0%, about 0.7%, about 0.6%, about 0.5%, about 0.4% or about 0.3%. In some embodiments, the residual moisture in the lyophilate powder is less than about 0.5%.

[0156] In one aspect, the disclosure provides a method of stabilizing a lyophilate powder comprising a FIX polypeptide, comprising lyophilizing a pre-lyophilization formulation according to the methods described herein, wherein the lyophilate powder is stabilized as measured by Size Exclusion Chromatography (SEC) with respect to a lyophilate powder prepared by using a lyophilization method comprising more than one drying step.

[0157] In another aspect, the disclosure provides a method of increasing the shelf-life of a lyophilate powder comprising a FIX polypeptide, comprising lyophilizing a pre-lyophilization formulation according to the methods described herein, wherein the shelf-life of the lyophilate powder is increased as measured by SEC and/or FIX clotting activity assay with respect to the shelf-life of a lyophilate powder prepared by using a lyophilization method comprising more than one drying step.

[0158] This disclosure also provides a method to decrease the reconstitution time of a lyophilate powder comprising a FIX polypeptide, comprising lyophilizing a pre-lyophilization formulation according to the methods described herein, wherein the reconstitution time of the lyophilate powder is decreased with respect to the reconstitution time of a lyophilate powder prepared by using a lyophilization method comprising more than one drying step.

[0159] This disclosure further provides a method to reduce lyophilization process time of producing a lyophilate powder comprising a FIX polypeptide, comprising lyophilizing a pre-lyophilization formulation according to the methods described herein, wherein the lyophilization process time of the pre-lyophilization formulation is reduced with respect to the lyophilization process time of producing a lyophilate powder using a lyophilization method comprising more than one drying step.

[0160] Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention. All patents and publications referred to herein are expressly incorporated by reference.

EXAMPLES

Example 1: Factor IX-Fc Drug Substance and Drug Product Compositions

rFIXFc Description

[0161] rFIXFc is a long-acting, fully recombinant fusion protein consisting of human coagulation Factor IX (FIX) covalently linked to the Fc domain of human immunoglobulin G1 (IgG1). The Factor IX portion of rFIXFc has a primary amino acid sequence that is identical to the Thr^{148} allelic form of plasma derived Factor IX and has structural and functional characteristics similar to endogenous Factor IX. The Fc domain

of rFIXFc contains the hinge, CH2 and CH3 regions of IgG1. rFIXFc contains 869 amino acids with a molecular weight of approximately 98 kilodaltons.

[0162] rFIXFc is produced by recombinant DNA technology in a human embryonic kidney (HEK) cell line and then purified.

[0163] The rFIXFc drug product formulation of the invention comprising rFIXFc can allow development of high concentration drug products, *e.g.,* 4000+ IU/vial drug product strengths. This requires a higher concentration of rFIXFc protein in the drug substance, as shown in the comparison Table 1 below. The rFIXFc drug product formulation of the invention can increase the shelf life of the 250 & 500 IU/vial drug product strengths to allow increased room temperature stability. Development data suggests that the 250 and 500 IU/vial drug product strengths are significantly more stable under accelerated conditions than the reference drug product.

[0164] The rFIXFc drug product formulation of the invention can also decrease the reconstitution time when lyophilized. Reference drug product reconstitution time varies between 1-2 minutes. Development data suggests that the LCM drug product reduces the reconstitution time to less than 30 seconds.

[0165] The rFIXFc drug product formulation of the invention can also allow reduction of the lyophilization process time. Currently the lyophilization cycle is ~4.5 days. With a lower vial fill volume this can likely be reduced to ~3 days or less for more economical manufacturing.

Drug Substance (DS)

[0166] The drug substance for the rFIXFc formulation of the invention will use the same formulation excipients as the reference drug substance. The higher concentration will be achieved using a second ultrafiltration step during drug substance manufacturing. *See* Table 1.

Table 1: Drug Substance Compositions

Drug Substance Compositions

Component	Reference Drug Product	Drug Product
rFactorIX-Fc	10-13 mg/mL	65-75 mg/mL
L-Histidine	3.88 mg/mL (25 mM)	3.88 mg/mL (25 mM)

Polysorbate 20	0.1 mg/mL (0.01 %)	0.1 mg/mL (0.1 %)

Drug Product (DP)

[0167] In order to accomplish the objectives stated above, a drug product was designed that doubled the concentrations of all components of the reference drug product (protein & excipients), while reducing the fill volume of the vial prior to lyophilization. This ensures that the dose of all components to the patient remains constant, while improving the drug product performance parameters mentioned above.

[0168] Tables 2-4 below detail the composition of the lyophilization feedstock, the contents of the solid product in the vial after lyophilization and the composition after reconstitution. It is important to note the wide variance of the rFIXFc protein concentrations. Due to the fact that each batch of Factor IX is slightly different in its activity in IU/mg, the feedstock is compounded using the assayed activity. This results in a range of protein concentrations, and this variance added to the different strengths of drug products gives the ranges stated below.

Table 2: Drug Product – Pre-lyophilization Formulation Compositions

Drug Product Lyophilization Feedstock Compositions

Component	Reference Drug Product 250-2000 IU/Vial	Reference Drug Product 3000 IU/Vial	Drug Product 250-5500 IU/Vial
rFactorIX-Fc	0.45-9.5 mg/mL 40-500 IU/mL	5.5-13.7 mg/mL 480-750 IU/mL	0.9-50 mg/mL 80-2750 IU/mL
L-Histidine	3.88 mg/mL (25 mM)	3.88 mg/mL (25 mM)	7.76 mg/mL (50 mM)
Mannitol	23.8 mg/mL (131 mM)	23.8 mg/mL (131 mM)	47.6 mg/mL (261 mM)
Sucrose	11.9 mg/mL (35 mM)	11.9 mg/mL (35 mM)	23.8 mg/mL (70 mM)
Polysorbate 20	0.1 mg/mL (0.01 %)	0.1 mg/mL (0.01 %)	0.2 mg/mL (0.2 %)
Feedstock Fill Volume	5.3 mL	7.4 mL	2.65 mL

Table 3: Drug Product – Lyophilate Compositions

Drug Product Lyophilized Vial Solid Compositions

Component	Reference Drug Product	Reference Drug Product	Drug Product 250-5500 IU/Vial*

	250-2000 IU/Vial	3000 IU/Vial	
rFactorIX-Fc	2.2-46 mg/vial	28-69 mg/vial	2.2-125 mg/vial
L-Histidine	19.4 mg/vial	27.2 mg/vial	19.4 mg/vial
Mannitol	119 mg/vial	167 mg/vial	119 mg/vial
Sucrose	59.5 mg/vial	83.3 mg/vial	59.5 mg/vial
Polysorbate 20	0.5 mg/vial	0.7 mg/vial	0.5 mg/vial

*The values are the nominal values that do not include the overfill. The values including the overfill are: 20.6 mg/vial (L-histidine), 126.01 mg/vial (mannitol), 63.1 mg/vial (sucrose), and 0.53 mg/vial (polysorbate).

Table 4: Drug Product – Reconstituted Formulation Compositions

Post-Reconstitution Drug Product Vial Compositions

Component	Reference Drug Product 250-2000 IU/Vial	Reference Drug Product 3000 IU/Vial	Drug Product 250-5500 IU/Vial
Diluent	5.2 mL of NaCl in Water for Injection		
rFactorIX-Fc	0.45-9.5 mg/mL 40-500 IU/mL	5.5-13.7 mg/mL 480-750 IU/mL	0.9-50.0 mg/mL 80-2750 IU/mL
L-Histidine	3.88 mg/mL (25 mM)	5.43 mg/mL (35 mM)	3.88 mg/mL (25 mM)
Mannitol	23.8 mg/mL (131 mM)	33.3 mg/mL (183 mM)	23.8 mg/mL (131 mM)
Sucrose	11.9 mg/mL (35 mM)	16.7 mg/mL (48 mM)	11.9 mg/mL (35 mM)
Polysorbate 20	0.1 mg/mL (0.01 %)	0.14 mg/mL (0.014 %)	0.1 mg/mL (0.01 %)
Reconstitution buffer	3.25 mg/mL	3.25 mg/mL	3.25 mg/mL
Reconstituted Volume	5.3 mL	5.3 mL	5.3 mL

Example 2: Development of Lyophilization Cycle Parameters for the Second Generation rFIXFc Drug Product

SUMMARY

[0169] The goal of this study was to evaluate ranges around the drying phase process parameters of the lyophilization cycle for the rFIXFc drug product of the invention.

[0170] This report summarizes a statistical design of experiments (DOE) study evaluating the lyophilization process parameters (Drying Shelf Temperature, Chamber Vacuum Level and Drying Time) and their effect on the product temperature during drying, the resulting residual moisture and drying rate of the drug product.

[0171] Preliminary lyophilization cycle design experiments on the Second Generation rFIXFc drug product demonstrated that distinct primary and secondary drying steps were not necessary due to the formulation's high collapse temperature of approximately -1.5 °C. A 12 experiment Design of Experiments (DOE) study was developed to evaluate the effect of the shelf temperature, vacuum level and drying time on the residual moisture levels and product temperatures during the lyophilization process on the placebo. An analysis of the data shows that in order to achieve a residual moisture level below 1%, a minimum shelf temperature of 30 °C during the drying phase is required. The analysis also demonstrated that drying times longer than 25 hours do not further decrease the residual moisture in the vials, and the chamber vacuum level has only a small effect on the residual moisture. The product temperatures during drying are significantly affected by the shelf temperature and chamber vacuum level, but the most aggressive drying conditions in the study (40 °C Shelf Temperature at 1000 mTorr Chamber Vacuum) resulted in a product temperature that was more than 10 °C colder than the collapse temperature. The vial mass flow is predominantly a function of the shelf temperature, and in order to maintain vacuum control, the commercial lyophilizer will need to be able to handle moisture flow rates of 0.7 g/hr/vial. A lyophilization cycle was proposed to achieve product with < 0.5% residual moisture using a shelf temperature of 40 °C, a chamber vacuum of 250 mTorr (0.33 mBar) and a drying time of 25 hours.

INTRODUCTION

[0172] The Second Generation FactorIX-Fc (rFIXFc-2G) Drug Product composition was designed to allow for improved protein stability during accelerated storage, improved reconstitution time, and reduced fill volume to reduce splashing onto the stoppers. This was accomplished by reducing the fill volume from 5.3 mL to 2.65 mL, and doubling the concentrations of protein and excipients in the formulation such that the reconstituted product is the same as the first generation composition. Another benefit to reducing the fill volume was a decrease in the amount of water that needs to be removed during the lyophilization process.

[0173] Since the new drug product design required revalidation of the drug product process, the lyophilization cycle was redeveloped. The collapse temperature of the placebo formulation was measured as approximately -1.5 °C, which would allow a shorter lyophilization cycle to be used than was designed for the reference rFIXFc lyophilization process. It was determined through initial experiments that no separate primary drying step was required, as the product did not undergo collapse even at shelf temperatures in excess of 40 °C. A cycle was designed using the freezing profile from the reference drug product combined with a direct step to the primary drying temperature after vacuum is applied. The placebo is a good surrogate for active rFIXFc-2G vials since the mannitol provides the crystalline structure for the cake making the appearance the same as the active vials. Amorphous sugar is also more difficult to dry than protein, so the resulting residual moisture is slightly higher, providing a worst-case value for the process. Removal of the protein from the vials also reduces the resistance to water vapor, giving a worst case estimate for the vial mass flow rate.

[0174] A statistical design of experiments (DOE) study was carried out to evaluate the lyophilization process parameters (Drying Shelf Temperature, Chamber Vacuum Level and Drying Time) and their effect on the product temperature during the drying process, the resulting residual moisture, and drying rate of the drug product.

MATERIALS AND METHODS

[0175] The goal of this study was to evaluate ranges around the drying phase process parameters of the lyophilization cycle for the second generation rFIXFc drug product. The drying phase parameters and ranges used to design the DOE study in JMP 9 are shown in Table 5. The resulting 12 experiment DOE plan showing the individual run parameter setpoints is shown in Table 6.

Table 5: The Drying Parameter Ranges Used in the DOE Study Design

Drying DOE Parameter	Minimum Value	Maximum Value
Shelf Temperature (°C)	0	40
Chamber Vacuum (mTorr)	100	1000
Drying Time (h)	15	35

Table 6: The Study Design of Experiments Template Output from JMP 9

Run	Temp (C)	Vacuum (mT)	Time (hr)
1	40	100	35
2	0	100	15
3	40	1000	25
4	20	100	28.2
5	20	500	25
6	0	100	25
7	20	550	25
8	40	250	15
9	20	1000	15
10	0	1000	35
11	10	500	25
12	40	500	25

[0176] For each lyophilization cycle in the study, eighty 10 mL Schott vials (P/N: 68000320) were filled with 2.75 mL of Second Generation rFIXFc Placebo as shown in Table 7, which provides a worst case fill volume for residual moisture evaluation. The filled vials were arranged on a single shelf with three thermocouples for each experiment as shown in Figure 1.

Table 7: Second Generation rFIXFc Drug Product Placebo Recipe

Component	FW	g/L	mM	% (w/v)
Histidine	155.15	7.76	50.00	0.776
PS-20	1227.54	0.2	0.1629	0.020
Mannitol	182.172	47.6	261.29	4.760
Sucrose	342.30	23.8	69.53	2.380
	ρ=	1.029	g/mL	

[0177] The lyophilization cycles used were variations of the cycle shown in Table 8. The Lyophilizer Shelf Drying Temperature, Drying Step Time and Drying Vacuum Level were varied based on the Design of Experiments table shown in Table 6. An SP Industries Lyostar II was used for each lyophilization cycle, and the vials were placed on the middle shelf.

Table 8: Lyophilization Cycle Parameters Showing the DOE Inputs of Temperature, Time and Vacuum

Phase	Step	Temp (°C)	Ramp (°C/min)	Soak (min)	Vacuum (mTorr)
Load	Load	25	-	As Needed	-
Freeze	Equilibrate	0	0.5	60	-
	Freeze	-55	0.5	120	-
	Anneal	-6	0.5	180	-
	Freeze	-55	0.5	120	-
Pulldown	Initial Vac	-55	-	-	DOE Vacuum
Drying	1°/2° Drying	DOE Temp	Direct Step	DOE Time	DOE Vacuum
Final	Final	5	5.0	As Needed	DOE Vacuum

[0178] After each lyophilization cycle, five vials were selected from corner and middle positions and measured for residual moisture using procedure TDMP-74, and averaged over the shelf. The product temperature during drying and the vial mass flow rate were measured using thermocouples and by Manometric Temperature Measurement in the Lyostar II software. These outputs were analyzed using JMP 9 software to evaluate the effect of the drying parameters on the second generation rFIXFc lyophilization process. A JMP stepwise analysis was performed to determine the significant variables, and these variables were then analyzed using a standard least squares effect screening algorithm which shows how the process outputs (residual moisture, product temperature and mass flow during drying) respond to the input variables.

RESULTS AND DISCUTION

[0179] The results of the twelve lyophilization experiments are shown in Table 9.

Table 9: The DOE Results of Residual Moisture, Vial Mass Flow and Product Temperature

Run	Temp (C)	Vacuum (mT)	Time (hr)	NB Ref	% Moisture	dm/dt (g/hr/vial)	Tp (MTM, °C)
1	40	100	35	16322-117	0.33	0.53	-26.5
2	0	100	15	16322-110	2.82	0.28	-32.2
3	40	1000	25	16322-090	0.57	0.45	-15.2
4	20	100	28.2	16322-139	1.28	0.41	-29.5
5	20	500	25	16322-098	1.58	0.57	-20.3
6	0	100	25	16322-151	2.18	0.27	-32.5
7	20	550	25	16322-146	1.49	0.56	-19.7
8	40	250	15	18266-004	0.59	0.70	-21.9
9	20	1000	15	16322-131	1.95	0.38	-15.6
10	0	1000	35	16322-124	2.56	0.17	-15.8
11	10	500	25	16322-104	2.01	0.35	-21.8
12	40	500	25	16322-083	0.49	0.56	-19.8

1. Analysis of Lyophilization Cycle Parameters on Residual Moisture

[0180] The rFIXFc-2G placebo was used as a worst-case surrogate for the drug product as it is generally more difficult to remove residual moisture from sugars during secondary drying than protein. The resulting prediction profiler showing the outcome of the DOE analysis is shown in Figure 2. Several observations are apparent: The shelf temperature has the most significant impact on the residual moisture in the drug product. This is expected based on the fact that secondary drying, which removes tightly bound water, is a diffusion and desorption controlled process. The model predicts with high confidence that shelf temperatures higher than 30 °C are required to achieve residual moisture levels lower than 1%. The vacuum level appears to have a small but measurable effect on the resulting residual moisture. The drying time appears to show a point of diminishing returns starting at 25 hours where the addition of further drying time does not continue to decrease the residual moisture level. This type of behavior is consistent with the kinetic approach to an equilibrium boundary determined by the shelf temperature, and the residual moisture approaches an asymptote where further drying is not possible. Based on this residual moisture DOE analysis the drying shelf temperature should be 30 °C or greater and the drying time should be fixed at 25 hours or less.

2. *Analysis of Lyophilization Cycle Parameters on Product Temperature During Sublimation*

[0181] The freeze drying collapse temperature of the rFIXFc-2G Drug Product placebo has been measured as approximately -1.5 °C. Practically, this means that the drug product will maintain an elegant cake structure so long as the product temperature is maintained below this collapse temperature as the bulk water is removed from the vial during lyophilization. The DOE analysis determined that the shelf temperature and chamber vacuum levels both had a significant effect on the product temperature as shown in Figure 3. The chamber vacuum had the largest effect, with higher pressures translating to higher product temperatures during sublimation. Even at 1000 mTorr (1.33 mBar) the highest measured product temperature was -15.2 °C, approximately 13 °C below the product collapse temperature. The shelf temperature also had a modest effect on the product temperature, but the results are less pronounced than the vacuum effect. This analysis shows that there is little risk of collapse even at a shelf temperature of 40 °C and a chamber vacuum level of 1000 mTorr, essentially eliminating the potential of collapse from any practical lyophilization cycle design space.

3. *Analysis of Lyophilization Cycle Parameters on Vial Mass Flow Rate During Sublimation*

[0182] The vial mass flow rate (dm/dt) is a measure of the rate at which water is being removed from the vials during the sublimation process. While faster drying is desirable to reduce the time required for the lyophilization cycle, too much moisture can overwhelm the condensers in manufacturing scale freeze driers and lead to a loss of vacuum control in the product chamber. The placebo represents the worst case vial mass flow conditions. Since there is no protein present in the formulation, the solids percentage in the cake is minimized and this results in a lower resistance to mass flow from the freeze dried cake. The shelf temperature has a significant effect on the vial mass flow as shown in Figure 4 with increasing temperature causing faster sublimation. The chamber vacuum level was included in the DOE analysis model, but the p value is 0.136 which is not significant with 95% confidence. The highest measured dm/dt in the study was 0.7 g/hr/vial.

4. Proposed Second Generation rFIXFc Lyophilization Cycle Based on the Placebo DOE Study

[0183] The data from the Placebo DOE study suggest it is feasible to design a lyophilization cycle to achieve a residual moisture target of < 0.5% while maintaining the product below its collapse temperature using a single drying step. The proposed lyophilization cycle is shown in Table 10, and the data from DOE Run 8 which is under similar conditions to the proposed rFIXFc-2G lyophilization cycle is shown in Figure 5. The residual moisture target of 0.5 % was selected as this is the average value of the first generation rFIXFc drug product strength series. This moisture level provides a cushion so that as the product absorbs moisture during accelerated stability, product quality attributes will not be affected.

[0184] The freezing and annealing portions of the lyophilization cycle were used as developed for the reference rFIXFc drug product lyophilization cycle, and the separate primary and secondary drying steps have been replaced with a single drying step at 40 °C shelf temperature and 250 mTorr vacuum for 25 hours.

Table 10: The Proposed Lyophilization Cycle for rFIXFc-2G

Phase	Step	Temp (°C)	Ramp (°C/min)	Soak (min)	Vacuum (mTorr)
Load	Load	25	-	As Needed	-
Freeze	Equilibrate	0	0.5	60	-
	Freeze	-55	0.5	120	-
	Anneal	-6	0.5	180	-
	Freeze	-55	0.5	120	-
Pulldown	Initial Vac	-55	-	-	250
Drying	1°/2° Drying	40	0.5	1500	250
Final	Final	5	5.0	As Needed	250

CONCLUSIONS

[0185] A 12 experiment DOE study evaluating the Second Generation rFIXFc Drug Product lyophilization process parameters on the residual moisture, product temperature and vial mass flow rate of the placebo was completed. An analysis of the data shows that in order to achieve a residual moisture level below 1%, a minimum shelf temperature of 30 °C during the drying phase is required. The analysis also demonstrated that drying times longer than 25 hours do not significantly decrease the residual moisture in the vials, and the vacuum level has only a small effect on the residual moisture. The product temperatures during drying are significantly affected by the shelf temperature and chamber vacuum level. The most aggressive conditions in the

study (40 °C Shelf Temperature at 1000 mTorr Chamber Vacuum) resulted in a product temperature that was more than 10 °C colder than the collapse temperature.

[0186] A lyophilization cycle was proposed to achieve product with < 0.5% residual moisture using a shelf temperature of 40 °C, a chamber vacuum of 250 mTorr (0.33 mBar) and a drying time of 25 hours.

[0187] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art, readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance.

[0188] The breadth and scope of the present invention should not be limited by any of the above-described exemplary embodiments, but should be defined only in accordance with the following claims and their equivalents. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein.

[0189] All documents, articles, publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

[0190] The present application claims priority to U.S. Provisional Application No. 61/969,801, filed March 24, 2014, which is incorporated herein by reference in its entirety.

TABLE OF SEQUENCES

Table 11: Polynucleotide Sequences of FIX

FIX-Fc Chain DNA Sequence (FIX signal peptide underlined, FIX sequence double underlined, Fc region in bold) (SEQ ID NO:1, which encodes SEQ ID NO:2)

pSYN-FIX-030 Nucleotide sequence (nt 1 to 7583):

FIX exon 1 (signal peptide, 1st amino acid propeptide): nt 690-777
FIX mini intron: nt 778-1076
FIX sequence : nt 1077-2371
Fc : nt 2372-3052

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 151 caataatgac gtatgttccc atagtaacgc caatagggac tttccattga
 201 cgtcaatggg tggagtattt acggtaaact gcccacttgg cagtacatca
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1251 ggaagcagta tgttgatgga gatcagtgtg agtccaatcc atgtttaaat

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6401 atagcaggca tgctggggat gcggtgggct ctatggcttc tgaggcggaa

6451 agaaccagtg gcggtaatac ggttatccac agaatcaggg gataacgcag

6501 gaaagaacat gtgagcaaaa ggccagcaaa aggccaggaa ccgtaaaaag

6551 gccgcgttgc tggcgttttt ccataggctc cgcccccctg acgagcatca

6601 caaaaatcga cgctcaagtc agaggtggcg aaacccgaca ggactataaa

6651 gataccaggc gtttcccccct agaagctccc tcgtgcgctc tcctgttccg

6701 accctgccgc ttaccggata cctgtccgcc tttctccctt cgggaagcgt

6751 ggcgctttct catagctcac gctgtaggta tctcagttcg gtgtaggtcg

6801 ttcgctccaa gctgggctgt gtgcacgaac cccccgttca gcccgaccgc

6851 tgcgccttat ccggtaacta tcgtcttgag tccaacccgg taagacacga

6901 cttatcgcca ctggcagcag ccactggtaa caggattagc agagcgaggt

6951 atgtaggcgg tgctacagag ttcttgaagt ggtggcctaa ctacggctac

7001 actagaagaa cagtatttgg tatctgcgct ctgctgaagc cagttacctt

7051 cggaaaaaga gttggtagct cttgatccgg caaacaaacc accgctggta

7101 gcggtggttt ttttgtttgc aagcagcaga ttacgcgcag aaaaaaagga

7151 tctcaagaag atcctttgat cttttctacg gggtctgacg ctcagtggaa

7201 cgaaaactca cgttaaggga ttttggtcat gacattaacc tataaaaata

7251 ggcgtatcac gaggcccttt cgtctcgcgc gtttcggtga tgacggtgaa

7301 aacctctgac acatgcagct cccggagacg gtcacagctt gtctgtaagc

7351 ggatgccggg agcagacaag cccgtcaggg cgcgtcagcg ggtgttggcg

7401 ggtgtcgggg ctggcttaac tatgcggcat cagagcagat tgtactgaga

7451 gtgcaccata tatgcggtgt gaaataccgc acagatgcgt aaggagaaaa

7501 taccgcatca ggcgccattc gccattcagg ctgcgcaact gttgggaagg

7551 gcgatcggtg cgggcctctt cgctattacg cca

Table 12: Polypeptide Sequences of FIX

FIX-Fc Monomer Hybrid: created by coexpressing FIX-Fc and Fc chains.

A. FIX-Fc chain (SEQ ID NO: 2)

The c-terminal lysine is not present in either subunit; this processing is often observed in recombinant proteins produced in mammalian cell culture, as well as with plasma derived proteins.

FIX-Fc-SC Subunit (the Fc part of FIX-Fc is in bold):

1 YNSGKLEEFV QGNLERECME EKCSFEEARE VFENTERTTE FWKQYVDGDQ

51 CESNPCLNGG SCKDDINSYE CWCPFGFEGK NCELDVTCNI KNGRCEQFCK

101 NSADNKVVCS CTEGYRLAEN QKSCEPAVPF PCGRVSVSQT SKLTRAETVF

151 PDVDYVNSTE AETILDNITQ STQSFNDFTR VVGGEDAKPG QFPWQVVLNG

201 KVDAFCGGSI VNEKWIVTAA HCVETGVKIT VVAGEHNIEE TEHTEQKRNV

251 IRIIPHHNYN AAINKYNHDI ALLELDEPLV LNSYVTPICI ADKEYTNIFL

301 KFGSGYVSGW GRVFHKGRSA LVLQYLRVPL VDRATCLRST KFTIYNNMFC

351 AGFHEGGRDS CQGDSGGPHV TEVEGTSFLT GIISWGEECA MKGKYGIYTK

401 VSRYVNWIKE KTKLT**DKTHT CPPCPAPELL GGPSVFLFPP KPKDTLMISR**

451 **TPEVTCVVVD VSHEDPEVKF NWYVDGVEVH NAKTKPREEQ YNSTYRVVSV**

501 **LTVLHQDWLN GKEYKCKVSN KALPAPIEKT ISKAKGQPRE PQVYTLPPSR**

551 **DELTKNQVSL TCLVKGFYPS DIAVEWESNG QPENNYKTTP PVLDSDGSFF**

601 **LYSKLTVDKS RWQQGNVFSC SVMHEALHNH YTQKSLSLSP GK**

Table 13: Polynucleotide Sequences of Fc

Fc DNA sequence (mouse Igκ signal peptide underlined) (SEQ ID NO:3, which encodes SEQ ID NO:4)

1 atggagacag acacactcct gctatgggta ctgctgctct gggttccagg

51 ttccactggt gacaaaactc acacatgccc accgtgccca gcacctgaac

101 tcctgggagg accgtcagtc ttcctcttcc ccccaaaacc caaggacacc

151 ctcatgatct cccggacccc tgaggtcaca tgcgtggtgg tggacgtgag

201 ccacgaagac cctgaggtca agttcaactg gtacgtggac ggcgtggagg

251 tgcataatgc caagacaaag ccgcgggagg agcagtacaa cagcacgtac

301 cgtgtggtca gcgtcctcac cgtcctgcac caggactggc tgaatggcaa

351 ggagtacaag tgcaaggtct ccaacaaagc cctcccagcc cccatcgaga

401 aaaccatctc caaagccaaa gggcagcccc gagaaccaca ggtgtacacc

451 ctgcccccat cccgcgatga gctgaccaag aaccaggtca gcctgacctg

501 cctggtcaaa ggcttctatc ccagcgacat cgccgtggag tgggagagca

551 atgggcagcc ggagaacaac tacaagacca cgcctcccgt gttggactcc

601 gacggctcct tcttcctcta cagcaagctc accgtggaca agagcaggtg

651 gcagcagggg aacgtcttct catgctccgt gatgcatgag gctctgcaca

701 accactacac gcagaagagc ctctccctgt ctccgggtaa a

Table 14: Polypeptide Sequences of Fc

Fc chain (SEQ ID NO:4)

```
  1 DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
 51 PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK
101 CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSRDELTK NQVSLTCLVK
151 GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG
201 NVFSCSVMHE ALHNHYTQKS LSLSPGK
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WHAT IS CLAIMED IS:

1. A pre-lyophilization formulation comprising:

(a) a Factor IX (FIX) polypeptide having FIX coagulation activity;

(b) a buffering agent;

(c) a stabilizing agent;

(d) a bulking agent; and

(e) a surfactant,

wherein the formulation has a fill volume of less than about 5 mL, less than about 4mL, or less than about 3 mL and wherein each of (a) – (e) are at an amount per vial (mg/vial) sufficient to allow

(1) improved stability of the FIX polypeptide when lyophilized;

(2) reduced reconstitution time when lyophilized;

(3) reduced splashing onto a stopper comprising the formulation;

(4) reduced lyophilization cycle time;

(5) increased shelf-life of a lyophilate prepared from the pre-lyophilization formulation at room temperature; or

(6) any combinations thereof,

compared to a reference pre-lyophilization formulation,

wherein the reference formulation comprises (a) – (e) at the amount per vial identical to the pre-lyophilization formulation, but has a 5mL fill volume.

2. The pre-lyophilization formulation of claim 1, wherein the fill volume is about 4 mL, about 3.5 mL, about 3.0 mL, about 2.9 mL, about 2.8 mL, about 2.7 mL, about 2.65 mL, about 2.6mL, about 2.5 mL, about 2.4 mL, about 2.3 mL, about 2.2 mL, about 2.1 mL, or about 2.0 mL.

3. The pre-lyophilization formulation of claim 1 or 2, wherein the fill volume is about 2.65 mL.

4. The pre-lyophilization formulation of any one of claims 1 to 3, wherein the reduced reconstitution time is less than 1.5 minute, less than 1 minute, less than 50 seconds, less than 40 seconds, less than 30 seconds, less than 20 seconds, or less than 10 seconds.

5. The pre-lyophilization formulation of any one of claims 1 to 4, wherein the reduced reconstitution time is less than 30 seconds.

6. The pre-lyophilization formulation of any one of claims 1 to 5, wherein the reduced lyophilization cycle time is about 4 days or less, about 3 days or less, about 2 days or less, or about a day or less.
7. The pre-lyophilization formulation of any one of claims 1 to 6, wherein the buffering agent is L-histidine.
8. The pre-lyophilization formulation of any one of claims 1 to 7, wherein the stabilizing agent is sucrose.
9. The pre-lyophilization formulation of any one of claims 1 to 8, wherein the bulking agent is mannitol.
10. The pre-lyophilization formulation of any one of claims 1 to 9, wherein the surfactant is polysorbate 20.
11. The pre-lyophilization formulation of any one of claims 1 to 10, wherein the formulation comprises at least 100 IU/vial of the FIX polypeptide.
12. The pre-lyophilization formulation of any one of claims 1 to 11, wherein the formulation comprises about 200 IU/vial to about 10,000 IU/vial of the FIX polypeptide.
13. The pre-lyophilization formulation of any one of claims 1 to 12, wherein the FIX polypeptide comprises about 220 IU/vial, about 250 IU/vial, about 300 IU/vial, about 400 IU/vial, about 500 IU/vial, about 600 IU/vial, about 700 IU/vial, about 800 IU/vial, about 900 IU/vial, about 1,000 IU/vial, about 1,100 IU/vial, about 1,200 IU/vial, about 1,300 IU/vial, about 1,400 IU/vial, about 1,500 IU/vial, about 2,000 IU/vial, about 2,500 IU/vial, about 3,000 IU/vial, about 4,000 IU/vial, about 5,000 IU/vial, about 5,500 IU/vial, about 6,000 IU/vial, about 6,500 IU/vial, about 7,000 IU/vial, about 7,500 IU/vial, about 8,000 IU/vial, about 8,500 IU/vial, about 9,000 IU/vial, about 9,500 IU/vial or about 10,000 IU/vial.
14. The pre-lyophilization formulation of any one of claims 1 to 13, wherein the buffering agent is at a concentration (mg/mL) between about 3 mg/mL and about 15 mg/mL.
15. The pre-lyophilization formulation of any one of claims 1 to 14, wherein the buffering agent is at a concentration (mg/mL) between about 3.88 mg/mL and about 9.7 mg/mL.
16. The pre-lyophilization formulation of any one of claims 1 to 15, wherein the buffering agent is at a concentration (mg/mL) of about 7.76 mg/mL.
17. The pre-lyophilization formulation of any one of claims 1 to 16, wherein the stabilizing agent is at a concentration (mg/mL) between 10 mg/mL and about 50 mg/mL.

18. The pre-lyophilization formulation of any one of claims 1 to 17, wherein the stabilizing agent is at a concentration (mg/mL) between about 17.85 mg/mL and about 29.95 mg/mL.
19. The pre-lyophilization formulation of any one of claims 1 to 18, wherein the stabilizing agent is at a concentration (mg/mL) of about 23.8 mg/mL.
20. The pre-lyophilization formulation of any one of claims 1 to 19, wherein the bulking agent is at a concentration (mg/mL) between 20 mg/mL and about 100 mg/mL.
21. The pre-lyophilization formulation of any one of claims 1 to 20, wherein the bulking agent is at a concentration (mg/mL) between 35.7 mg/mL and 59.5 mg/mL.
22. The pre-lyophilization formulation of any one of claims 1 to 21, wherein the bulking agent is at a concentration (mg/mL) of 47.6 mg/mL.
23. The pre-lyophilization formulation of any one of claims 1 to 22, wherein the surfactant is at a concentration (mg/mL) between 0.01 mg/mL and about 5 mg/mL.
24. The pre-lyophilization formulation of claim 23, wherein the surfactant is at a concentration (mg/mL) of 0.2 mg/mL.
25. The pre-lyophilization formulation of any one of claims 1 to 24, wherein the FIX polypeptide has a concentration (IU/mL) between about 80 IU/mL and about 2,750 IU/mL.
26. A pre-lyophilization formulation comprising:
    (a) about 80 to about 2,750 IU/mL of rFIXFc;
    (b) about 7.76 mg/mL of L-histidine;
    (c) about 47.6 mg/mL of mannitol;
    (d) about 23.8 mg/mL of sucrose; and,
    (e) about 0.2 mg/mL of polysorbate-20.
27. The pre-lyophilization formulation of claim 26, wherein the formulation has a fill volume per vial of about 3 mL, about 2.9 mL, about 2.8 mL, about 2.7 mL, about 2.65 mL, about 2.6 mL, about 2.5 mL, about 2.4 mL, about 2.3 mL, about 2.2 mL, about 2.1 mL, or about 2.0 mL.
28. The pre-lyophilization formulation of claim 27, wherein the formulation has a fill volume per vial of about 2.65 mL.
29. A lyophilate powder, which is lyophilized from the pre-lyophilization formulation of any one of claims 1 to 28.

30. The lyophilate powder of claim 29, which has a residual moisture level below 1%.

31. The lyophilate powder of claim 29 or 30, comprising a buffering agent, a stabilizing agent, a bulking agent, a surfactant, or any combinations thereof.

32. The lyophilate powder of claim 31, wherein the buffering agent is L-histidine.

33. The lyophilate powder of claim 31, wherein the stabilizing agent is sucrose.

34. The lyophilate powder of claim 31, wherein the surfactant is polysorbate 20.

35. The lyophilate powder of any one of claims 31 to 34, wherein the buffering agent is at a concentration between about 8 mg and about 39 mg per vial.

36. The lyophilate powder of claim 35, wherein the buffering agent is at a concentration of about 20.6 mg per vial.

37. The lyophilate powder of any one of claims 31 to 36, wherein the stabilizing agent is at a concentration between about 27 mg and about 132 mg per vial.

38. The lyophilate powder of claim 37, wherein the stabilizing agent is at a concentration of about 63.1 mg per vial.

39. The lyophilate powder of any one of claims 31 to 38, wherein the bulking agent is at a concentration between about 53 mg per vial and about 265 mg per vial.

40. The lyophilate powder of claim 39, wherein the bulking agent is at a concentration of about 126.1 mg per vial.

41. The lyophilate powder of any one of claims 31 to 40, wherein the surfactant is at a concentration between about 0.03mg and about 13 mg per vial.

42. The lyophilate powder of claim 41, wherein the surfactant is at a concentration of about 0.53 mg per vial.

43. A lyophilate powder comprising:

(a) a FIX polypeptide at an amount between about 2 mg per vial and about 150 mg per vial;

(b) a buffering agent at an amount between 10 mg per vial and about 30 mg per vial;

(c) a bulking agent at an amount between 70 mg vial and about 200 mg per vial;

(d) a stabilizing agent at an amount between 30 mg per vial and 100 mg per vial; and

(e) a surfactant at an amount between 0.05 mg per vial and about 5 mg per vial.

44. The lyophilate powder of claim 43, comprising:

(a) the lyophilized FIX polypeptide at an amount between about 2.2 mg per vial and about 125 mg per vial;

(b) the buffering agent at an amount between about 12.5 mg per vial and 25 mg per vial;

(c) the stabilizing agent at an amount between about 32.5 mg per vial and 80 mg per vial;

(d) the bulking agent at an amount between about 75 mg per vial and 150 mg per vial; and

(e) the surfactant at an amount between about 0.1 mg/mL and about 2 mg/mL.

45. The lyophilate powder of claim 44 comprising:

(a) about 2.2 to about 125 mg/vial of the FIX polypeptide;

(b) about 20.6 mg/vial of L-histidine.

(c) about 126.1 mg/vial of mannitol;

(d) about 63.1 mg/vial of sucrose; and,

(e) about 0.53 mg/vial of polysorbate-20;

46. A reconstituted formulation comprising the lyophilate power of any one of claims 29 to 45 reconstituted by a reconstitution buffer.

47. The reconstituted formulation of claim 46, which is used to treat hemophilia B.

48. The reconstitution formulation of claim 46 or 47, which is suitable for parenteral administration.

49. The reconstituted formulation of claim 48, wherein the parenteral administration is intravenous or subcutaneous administration.

50. The reconstituted formulation of any one of claims 47 to 49, comprising:

(a) the FIX polypeptide at a concentration between about 0.9 mg/mL and about 50 mg/mL;

(b) the buffering agent at a concentration between 1.5 mg/mL and about 7.5 mg/mL;

(c) the bulking agent at a concentration between 10 mg/mL and about 50 mg/mL;

(d) the stabilizing agent at a concentration between 5 mg/mL and 25 mg/mL per vial; and

(e) the surfactant at a concentration between 0.005 mg/mL and about 2.5 mg/mL.

51. The reconstituted formulation of claim 50, comprising:

(a) the FIX polypeptide at a concentration between about 0.9 mg/mL and about 50 mg/mL;

(b) the buffering agent at a concentration of about 3.88 mg/mL;

(c) the bulking agent at a concentration of about 23.8 mg/mL;

(d) the stabilizing agent at a concentration of about 11.9 mg/mL;

(e) the surfactant at a concentration of about 0.1 mg/mL; and

(f) the reconstitution buffer.

52. The reconstituted formulation of claim 49, comprising

(a) the FIX polypeptide at a concentration between about 80 IU/mL and about 2,750 IU/mL;

(b) the buffering agent at a concentration of about 25 mM;

(c) the bulking agent at a concentration of about 131 mM;

(d) the stabilizing agent at a concentration of about 35 mM;

(e) the surfactant at a concentration of 0.01 % (w/v); and

(f) the reconstitution buffer.

53. A vial comprising the pre-lyophilization formulation of any one of claims 1 to 28, the lyophilate power of any one of claims 29 to 45, or the reconstituted formulation of any one of claims 46 to 52.

54. The pre-lyophilization formulation of any one of claims 1 to 28, the lyophilate power of any one of claims 29 to 45, the reconstituted formulation of 46 to 52, or the vial of 53, wherein the FIX polypeptide comprises wild-type FIX.

55. The pre-lyophilization formulation of any one of claims 1 to 28, the lyophilate power of any one of claims 29 to 45, the reconstituted formulation of 46 to 52, or the vial of 53, wherein the FIX polypeptide further comprises a heterologous moiety fused to wild-type FIX.

56. The pre-lyophilization formulation of any one of claims 1 to 28, the lyophilate power of any one of claims 29 to 45, the reconstituted formulation of 46 to 52, or the vial of 53, wherein the heterologous moiety is a moiety extending half-life of FIX.

57. The pre-lyophilization formulation of any one of claims 1 to 28, the lyophilate power of any one of claims 29 to 45, the reconstituted formulation of 46 to 52, or the vial of 53, wherein the heterologous moiety comprises a polypeptide or a non-polypeptide moiety.

58. The pre-lyophilization formulation of any one of claims 1 to 28, the lyophilate power of any one of claims 29 to 45, the reconstituted formulation of 46 to 52, or the vial of 53, wherein the moiety extending half-life of FIX comprises an FcRn binding partner or an Fc region.

59. The pre-lyophilization formulation of any one of claims 1 to 28, the lyophilate power of any one of claims 29 to 45, the reconstituted formulation of 46 to 52, or the vial of 53, wherein the FIX polypeptide is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identical to SEQ ID NO: 2.

60. A kit comprising a first container comprising the lyophilate power of any one of claims 29 to 45 and a second container comprising a reconstitution buffer at a volume sufficient to produce, when combined with the lyophilization formulation of the first container.

61. The kit of claim 60, which is used to treat hemophilia B.

62. The kit of claim 60 or 61, wherein the reconstitution buffer comprises NaCl.

63. A method of administering a FIX polypeptide to a hemophilia B patient in need thereof, comprising administering to the patient the reconstituted formulation of any one of 46 to 52, wherein the administration prevents or reduces the frequency or severity of bleeding episodes in the patient.

64. A method of preventing, treating, ameliorating, or managing hemophilia B in a patient in need thereof by administering the reconstituted formulation of any one of claims 46 to 52.

65. A method of producing a lyophilate powder comprising a FIX polypeptide comprising lyophilizing the pre-lyophilization formulation of any one of claims 1 to 28.

66. A method of lyophilizing a FIX polypeptide comprising:

(a) a "freezing step" comprising freezing a pre-lyophilization formulation comprising the FIX polypeptide and an aqueous solvent;

(b) a "vacuum step" comprising reducing the pressure of the frozen pre-lyophilization formulation by an amount effective to remove the aqueous solvent from the frozen pre-lyophilization formulation; and,

(c) a single "drying step" comprising increasing the temperature of the frozen pre-lyophilization formulation above the collapse temperature,

thereby producing a lyophilate powder.

67. The method of claim 66, wherein the collapse temperature is about -1.5 ^{o}C.

68. The method of claim 66 or 67, wherein the pre-lyophilization formulation is frozen to a freezing temperature of about -65 to about -40°C during the freezing step.

69. The method of any one of claims 66 to 68, wherein the pre-lyophilization formulation is frozen to a freezing temperature of about -55°C during the freezing step.

70. The method of any one of claims 66 to 69, wherein the freezing temperature is ramped down from about 5°C to about -55°C during the freezing step.

71. The method of any one of claims 66 to 70, wherein the freezing temperature is held from about 30 minutes to about 5 hours during the freezing step.

72. The method of any one of claims 66 to 71, wherein the freezing temperature is held for about 2 hours during the freezing step.

73. The method of any one of claims 66 to 72, wherein the frozen pre-lyophilization formulation of step (a) is further subject to an "annealing step" (a') prior to the "vacuum step" (b).

74. The method of claim 73 wherein the temperature of the frozen pre-lyophilization formulation of step (a) is ramped up to an annealing temperature of about -15°C to about -2°C during the annealing step.

75. The method of claim 74, wherein the temperature of the frozen pre-lyophilization formulation of step (a) is ramped up to an annealing temperature of about -6°C during the annealing step.

76. The method of any one of claims 73 to 75, wherein the annealing temperature is held for about 30 minutes to about 5 hours during the annealing step.

77. The method of claim 76, wherein the annealing temperature is held for about 3 hours during the annealing step.

78. The method of any one of claims 73 to 77, wherein the temperature of the frozen pre-lyophilization formulation is ramped down from the annealing temperature to a temperature of about -65°C to about -40°C during the annealing step.

79. The method of claim 78, wherein the temperature of the frozen pre-lyophilization formulation is ramped down from the annealing temperature to a temperature of -55°C during the annealing step.

80. The method of any one of claims 66 to 79, wherein the "vacuum step" comprises subjecting the frozen pre-lyophilization formulation to a vacuum between about 0.05 and about 1 mbar.

81. The method of claim 80, wherein the vacuum in the "vacuum step" is about 0.33 mbar.

82. The method of any one of claims 66 to 81, wherein the vacuum is held in the "vacuum step" for about 2 hours.

83. The method of any one of claims 66 to 82, wherein the "drying step" comprises ramping up the temperature of the frozen pre-lyophilization formulation from about -55°C to a drying temperature of about 40°C.

84. The method of any one of claims 66 to 83, wherein the drying step further comprises holding the drying temperature for about 10 hours to about 40 hours.

85. The method of claim 84, wherein the drying temperature is held for about 25 hours.

86. The method of any one of claims 66 to 85, wherein the drying step is carried out at a pressure of about 0.05 mbar to about 1 mbar.

87. The method of claim 86, wherein the pressure is held at about 0.33 mbar during the drying step.

88. A method of producing a lyophilate powder comprising a FIX polypeptide, comprising:

(a) a "freezing step" comprising freezing a pre-lyophilization formulation comprising a FIX polypeptide by ramping down the temperature for about 2 hours to a freezing temperature of about -55°C, and holding the freezing temperature for about 2 hours;

(a') an "annealing step" comprising ramping up for about 1.5 hours the temperature of the frozen pre-lyophilization formulation of step (a) to an annealing temperature of about -6°C, holding the annealing temperature for about 3 hours, and ramping down the temperature for about 1.5 hours to about -55°C;

(b) a "vacuum step" comprising holding the frozen pre-lyophilization formulation of step (a') at about -55°C for two hours at atmospheric pressure and ramping down the pressure for about 2 hours to about 0.33 mbar; and,

(c) a single "drying step" comprising ramping up for 3 hours the temperature of the frozen pre-lyophilization formulation of step (b) to about 40°C, while holding the pressure at about 0.33 mbar, and holding the temperature of the frozen pre-lyophilization formulation at about 40°C for about 25 hours, while holding the pressure at about 0.33 mbar,

thereby producing the lyophilate powder.

89. The method of any one of claims 66 to 88, wherein the pre-lyophilization formulation is the formulation of any one of claims 1 to 28.

90. The method of any one of claims 65 to 89, wherein the lyophilate powder has one or more characteristics selected from the group consisting of:

(1) improved stability of the FIX polypeptide when lyophilized;

(2) reduced reconstitution time when lyophilized;

(3) reduced splashing onto a stopper comprising the formulation;

(4) reduced lyophilization cycle time;

(5) increased shelf-life of a lyophilate prepared from the pre-lyophilization formulation at room temperature; or

(6) any combinations thereof,

91. The method of any one of claims 65 to 90, wherein the pre-lyophilization formulation is in a fill volume of less than 5 mL.

92. The method of claim 91, wherein the fill volume of the pre-lyophilization formulation is about 4 mL, about 3.5 mL, about 3.0 mL, about 2.9 mL, about 2.8 mL, about 2.7 mL, about 2.65 mL, about 2.6 mL, about 2.5 mL, about 2.4 mL, about 2.3 mL, about 2.2 mL, about 2.1 mL, or about 2.0 mL.

93. The method of claim 91 or 92, wherein the fill volume of the pre-lyophilization formulation is about 2.65 mL.

94. The method of any one of claims 90 to 93, wherein the reduced reconstitution time is less than 1.5 minutes, less than 1 minute, less than 50 seconds, less than 40 seconds, less than 30 seconds, less than 20 seconds, or less than 10 seconds.

95. The method of any one of claims 90 to 94, wherein the reduced reconstitution time is less than 30 seconds.

96. The method of any one of claims 90 to 95, wherein the reduced lyophilization cycle time is about 4 days or less, about 3 days or less, about 2 days or less, or about a day or less.

97. The method of any one of claims 66 to 96, wherein the pre-lyophilization formulation is aseptically filtered and aseptically filled into a vial prior to step (a).

98. The method of any one of claims 65 to 97, wherein the lyophilate powder is produced from the pre-lyophilization formulation in about 45 hours or less.

99. The method of any one of claims 65 to 98, wherein the residual moisture in the lyophilate powder is less than 0.7%.

100. The method of any one of claims 65 to 99, wherein the residual moisture in the lyophilate powder is about 0.5%.

101. A method of stabilizing a lyophilate powder comprising a FIX polypeptide, comprising lyophilizing a pre-lyophilization formulation according to the method of any one of claims 65 to 100, wherein the lyophilate powder is stabilized as measured by Size Exclusion Chromatography (SEC) with respect to a lyophilate powder prepared by using a lyophilization method comprising more than one drying step.

102. A method of increasing the shelf-life of a lyophilate powder comprising a FIX polypeptide, comprising lyophilizing a pre-lyophilization formulation according to the method of any one of claims 65 to 100, wherein the shelf-life of the lyophilate powder is increased as measured by SEC and/or FIX clotting activity assay with respect to the shelf-life of a lyophilate powder prepared by using a lyophilization method comprising more than one drying step.

103. A method to decrease the reconstitution time of a lyophilate powder comprising a FIX polypeptide, comprising lyophilizing a pre-lyophilization formulation according to the method of any one of claims 65 to 100, wherein the reconstitution time of the lyophilate powder is decreased with respect to the reconstitution time of a lyophilate powder prepared by using a lyophilization method comprising more than one drying step.

104. A method to reduce lyophilization process time of producing a lyophilate powder comprising a FIX polypeptide, comprising lyophilizing a pre-lyophilization formulation according to the method of any one of claims 65 to 100, wherein the lyophilization process time of the pre-lyophilization formulation is reduced with respect to the lyophilization process time of producing a lyophilate powder using a lyophilization method comprising more than one drying step.