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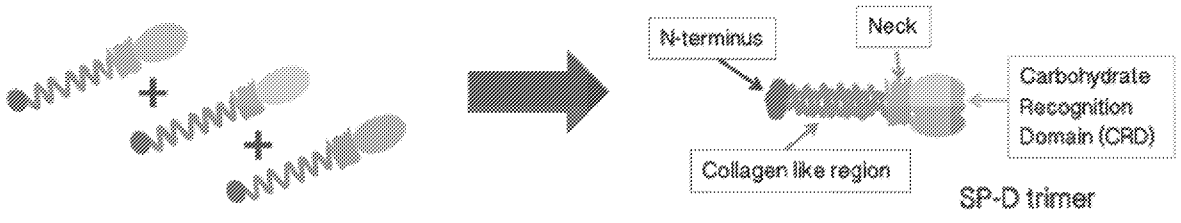


FIG. 1

(57) Abstract: Some embodiments provided herein relate to methods and pharmaceutical compositions comprising recombinant human surfactant protein D or active fragments thereof. Some such embodiments include solutions or suspensions, and lyophilized solid forms of recombinant human surfactant protein D or active fragments thereof.

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METHODS AND COMPOSITIONS COMPRISING SURFACTANT PROTEIN D (SP-D)

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Prov. App. No. 62/650142 filed March 29, 2018 entitled "METHODS AND COMPOSITIONS COMPRISING SURFACTANT PROTEIN D (SP-D)" which is incorporated by reference herein in its entirety.

REFERENCE TO SEQUENCE LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled AIRWY011WOSEQLIST, created March 20, 2019, which is approximately 7 Kb in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0003] Some embodiments provided herein relate to methods and pharmaceutical compositions comprising recombinant human surfactant protein D (SP-D) or active fragments thereof. Some such embodiments include solutions, and lyophilized solid forms of recombinant human surfactant protein D or active fragments thereof.

BACKGROUND OF THE INVENTION

[0004] Mammalian pulmonary surfactant is a mixture of proteins (10%) and lipids (90%) including the major lipid component dipalmitoylphosphatidylcholine (Zuo YY, *et al.*, Biochim Biophys Acta (2008) 1778:1947–77). The main function of the pulmonary surfactant is to ensure minimal surface tension within the lung to avoid collapse during respiration. Furthermore, by interacting with inhaled pathogens, the pulmonary surfactant also participates in host defense (Clements JA. Am Rev Respir Dis (1977) 115:67–71). Pulmonary surfactant deficiency is associated with pulmonary diseases such as asthma, bronchiolitis, respiratory distress syndrome (RDS), cystic fibrosis, and pneumonia (Griese M. Eur Respir J (1999) 13:1455–76). Surfactant formulations are indicated for the treatment of RDS, which affects

~1.5 million premature babies globally every year. Respiratory distress syndrome is a major pulmonary surfactant deficiency disease caused by the structural immaturity of the lungs in premature infants, which makes it difficult to breathe, inhibits gas exchange, and promotes alveolar collapse (Notter RH. 2000 Lung Surfactants. Basic Science and Clinical Applications. New York, NY: Marcel Dekker Inc.). Successful treatment of complex pulmonary diseases typically requires the production of surfactant formulations whose composition matches natural pulmonary surfactant as closely as possible (Robertson B, *et al.*, Biochim Biophys Acta (1998) 1408:346–61).

[0005] Pulmonary surfactant contains four different surfactant proteins. The two hydrophobic proteins, surfactant proteins B and C, are involved in the reduction of surface tension at the air-water interface, while the two hydrophilic proteins, surfactant proteins A and D, are members of the collectin family and are involved in the modulation of the host immune response and in surfactant pool recycling (Awasthi S. (2010) Recent Patents on Anti-Infective Drug Discovery, 5:115-123). Surfactant protein D (SP-D) is a C-type (Ca^{2+}-dependent) lectin that comprises four domains: a cysteine-linked N-terminal region required for the formation of intermolecular disulfide bonds, a triple-helical collagen region, an α-helical-coiled-coil trimerizing neck peptide, and a C-terminal calcium-dependent carbohydrate-recognition domain (CRD) (Crouch E. *et al.* (1994) J Biol Chem 269:17311-9). Monomers form trimers through folding of the collagenous region into triple helices and the assembly of a coiled-coil bundle of α-helices in the neck region. These trimers are stabilized by two disulfide bonds in the cysteine-rich N-terminal domain. The SP-D trimer has a total molecular weight of 129 kDa which comprises three identical 43-kDa polypeptide chains. SP-D trimers can form higher order oligomerization states which vary by size and conformation. Higher order oligomerization states may be important for SP-D function (Hakansson K, *et al.*, Protein Sci (2000) 9:1607–17; Crouch E. Respir Res (2000) 1:93–108; Crouch E. *et al.* (2006) J Biol Chem 281:18008-14).

[0006] In contrast to the other surfactant proteins, SP-D is not involved in reduction of surface tension at the air-water interface, and does not alter the biophysical properties of pulmonary surfactant (Awasthi S. (2010) Recent Patents on Anti-Infective Drug Discovery, 5:115-123). SP-D has a role in the pulmonary innate immune system by providing anti-inflammatory and antimicrobial activities that address chronic pulmonary diseases such as

asthma, cystic fibrosis, and smoking-induced emphysema (Clark H, *et al.*, Immunobiology (2002) 205:619–31). SP-D also has a critical role in regulating surfactant pool homeostasis, and absence of SP-D during development results in an emphysema phenotype (Wert, S. *et al.* (2000) Chest 117:248S; Korfhagen, T. R. *et al.* (1998) J Biol Chem 273: 28438-28443). Data based on premature newborn lambs suggest that the administration of ~2–3 mg/kg of recombinant human SP-D (rhSP-D) in combination with 100 mg/kg Survanta® (a natural surfactant available in USA) is more effective than Survanta® alone for the prevention of endotoxin shock and the reduction of lung inflammation caused by ventilation (Ikegami M, *et al.*, Am J Respir Crit Care Med (2006) 173:1342–7; Sato A, *et al.*, Am J Respir Crit Care Med (2010) 181:1098–105).

[0007] Traditionally, SP-D has been isolated from the supernatant of bronchoalveolar lavage or amniotic fluid, but most, if not all, of SP-D is lost during purification of commercial surfactant, in part due to the hydrophilic properties of SP-D (Dodagatta-Marri E, *et al.*, Methods Mol Biol (2014) 100:273–90). The use of rhSP-D to supplement pulmonary surfactant formulations can ensure therapeutic efficacy because current pulmonary surfactant formulations lack the ability to effectively modulate the host immune response in the absence of the hydrophilic surfactant proteins. An important characteristic of native SP-D that must be maintained in any pharmaceutical composition is the appropriate oligomerization state because the higher-order multimerization in the endogenous surfactant protein increases the number of SP-D-binding sites to carbohydrate ligands on the surface of pathogens, achieving potent bacterial and viral agglutination effects (White M, *et al.*, J Immunol (2008) 181:7936–43). The appropriate oligomerization state is also required for receptor recognition and receptor-mediated signal transduction for modulation of the host immune response (Yamoze M *et al.*, J Biol Chem (2008) 283:35878-35888) as well as for maintenance of surfactant homeostasis (Zhang L *et al.*, J Biol Chem (2001) 276:19214-19219).

SUMMARY OF THE INVENTION

[0008] Some embodiments include a pharmaceutical composition comprising recombinant human surfactant protein D (rhSP-D) or an active fragment thereof. Some embodiments also include a buffer, a sugar, and a calcium salt.

[0009] In some embodiments, the buffer is selected from the group consisting of acetate, citrate, glutamate, histidine, succinate, and phosphate. In some embodiments, the buffer is histidine. In some embodiments, the concentration of the histidine is from about 0.1 mM to about 100 mM. In some embodiments, the concentration of the histidine is about 5 mM.

[0010] In some embodiments, the sugar is selected from the group consisting of sucrose, maltose, lactose, glucose, fructose, galactose, mannose, arabinose, xylose, ribose, rhamnose, trehalose, sorbose, melezitose, raffinose, thioglucose, thiomannose, thiofructose, octa-O-acetyl-thiotrehalose, thiosucrose, and thiomaltose. In some embodiments, the sugar is lactose. In some embodiments, the concentration of the lactose is from 1 mM to 500 mM. In some embodiments, the concentration of the lactose is about 265 mM.

[0011] In some embodiments, the calcium salt is selected from the group consisting calcium chloride, calcium bromide, calcium acetate, calcium sulfate, and calcium citrate. In some embodiments, the calcium salt is calcium chloride. In some embodiments, the concentration of the calcium chloride is from about 0.1 mM to about 10 mM. In some embodiments, the concentration of the calcium chloride is about 5 mM.

[0012] In some embodiments, the pharmaceutical composition has a pH from about 5.0 to about 9.0. In some embodiments, the pharmaceutical composition has a pH from about 5.5 to about 6.5. In some embodiments, the pharmaceutical composition has a pH about 6.0.

[0013] In some embodiments, the concentration of the rhSP-D is from about 0.1 mg/ml to about 10 mg/ml. In some embodiments, the concentration of the rhSP-D is from about 1 mg/ml to about 4 mg/ml. In some embodiments, the concentration of the rhSP-D is about 2 mg/ml. In some embodiments, the concentration of the rhSP-D is about 4 mg/ml.

[0014] Some embodiments include a pharmaceutical composition comprising a population of rhSP-D polypeptides having oligomeric forms, wherein greater than 30% of the oligomeric forms comprise dodecamers of rhSP-D. In some embodiments, greater than 40% of the oligomeric forms comprise dodecamers of the rhSP-D. In some embodiments, less than 15% of the oligomeric forms comprise aggregates of the rhSP-D, wherein the aggregates have a radius greater than 70 nm. In some embodiments, less than 10% of the oligomeric forms comprise aggregates of the rhSP-D, wherein the aggregates have a radius greater than 70 nm. In some embodiments, less than 5% of the oligomeric forms comprise aggregates of the rhSP-D, wherein the aggregates have a radius greater than 70 nm.

[0015] Some embodiments also include a bulking agent. In some embodiments, the bulking agent is selected from the group consisting of mannitol, xylitol, sorbitol, maltitol, lactitol, glycerol, erythritol, arabitol, glycine, alanine, threonine, valine, and phenylalanine.

[0016] In some embodiments, the composition lacks a chelating agent selected from EDTA and EGTA.

[0017] In some embodiments, the rhSP-D polypeptide comprises the amino acid sequence of SEQ ID NO:02, or an active fragment thereof. In some embodiments, the rhSP-D or active fragment thereof comprises a residue at a polymorphic position corresponding to a residue selected from the group consisting of Met11, Thr160, Ser 270, and Ala 286.

[0018] Some embodiments include a pharmaceutical composition comprising 2 mg/ml rhSP-D, 5 mM histidine, 265 mM lactose, 5 mM calcium chloride, wherein the solution has a pH of 6.0. Some embodiments include a pharmaceutical composition comprising 2 mg/ml rhSP-D, 5 mM Histidine, 265 mM Lactose, 1 mM calcium chloride, wherein the solution has a pH 6.0. Some embodiments include a pharmaceutical composition comprising 1 mg/ml rhSP-D, 5 mM Histidine, 265 mM Lactose, 5 mM calcium chloride, wherein the solution has a pH 6.0. Some embodiments include a pharmaceutical composition comprising 4 mg/ml rhSP-D, 5 mM Histidine, 265 mM Lactose, 5 mM calcium chloride, wherein the solution has a pH 6.0.

[0019] Some embodiments include a lyophile of the pharmaceutical composition of any one of the foregoing pharmaceutical compositions.

[0020] Some embodiments include an article comprising a container comprising a lyophile of the pharmaceutical composition of any one of the foregoing pharmaceutical compositions.

[0021] Some embodiments include a method of preparing a lyophile comprising lyophilizing the pharmaceutical composition of any one of the foregoing pharmaceutical compositions.

[0022] Some embodiments include a method of preparing a pharmaceutical composition comprising contacting a lyophile of the pharmaceutical composition of any one of claims 1-33 with a pharmaceutically acceptable diluent to form a solution or suspension of the rhSP-D or active fragment thereof.

[0023] In some embodiments, the diluent is suitable for administration to a lung. In some embodiments, the diluent is sterile water.

[0024] In some embodiments, the solution or suspension has a concentration of the rhSP-D or active fragment thereof from about 0.1 mg/ml to about 10 mg/ml. In some embodiments, the solution or suspension has a concentration of the rhSP-D or active fragment thereof from about 1 mg/ml to about 4 mg/ml. In some embodiments, the solution or suspension has a concentration of the rhSP-D or active fragment thereof of about 2 mg/ml. In some embodiments, the solution or suspension has a concentration of the rhSP-D or active fragment thereof of about 4 mg/ml.

[0025] In some embodiments, less than about 3 % of the rhSP-D oligomeric species by mass in the solution or suspension is in a high molecular weight oligomeric form with a radius greater than 70 nm.°

[0026] In some embodiments, the solution or suspension comprises a population of rhSP-D polypeptides having oligomeric forms, wherein greater than 30% of the oligomeric forms comprise dodecamers of the rhSP-D. In some embodiments, greater than 40% of the oligomeric forms comprise dodecamers of the rhSP-D. In some embodiments, greater than 45% of the oligomeric forms comprise dodecamers of the rhSP-D.

[0027] In some embodiments, less than 10% of the oligomeric forms comprise aggregates of the rhSP-D, wherein the aggregates have a radius greater than 70 nm. In some embodiments, less than 5% of the oligomeric forms comprise aggregates of the rhSP-D, wherein the aggregates have a radius greater than 70 nm. In some embodiments, less than 3% of the oligomeric forms comprise aggregates of the rhSP-D, wherein the aggregates have a radius greater than 70 nm.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] FIG. 1 is a schematic which depicts the formation of an SP-D trimer, and structural features of the SP-D trimer.

[0029] FIG. 2 is a bar chart that depicts a 6-month lyophile stability study and shows a distribution of different SP-D oligomeric species represented by either Peak 1, Peak 2, Peak 3 or Peak 4 of an AF4-MALLS analysis for a 2 mg/ml reconstituted lyophile of rhSP-D that had been stored at either 5°C or 25°C for a period of 1, 3, or 6 months.

[0030] FIG. 3 is a set of bar chart panels that depict a 3-month lyophile stability study and shows a distribution of different SP-D oligomeric species represented by either Peak

1, Peak 2, Peak 3 or Peak 4 of an AF4-MALLS analysis for lyophiles of rhSP-D that had been reconstituted at either 2 mg/ml (upper panel) or 4 mg/ml (lower panel) and stored at either 5°C, or 25°C for a period of 1 or 3 months.

[0031] FIG. 4 is a set of bar chart panels that depict a freeze/thaw stability study and shows a distribution of different SP-D oligomeric species represented by either Peak 1, Peak 2, Peak 3 or Peak 4 of an AF4-MALLS analysis for lyophiles of rhSP-D that had been reconstituted at either 2 mg/ml (upper panel) or 4 mg/ml (lower panel), frozen by either flash liquid nitrogen freezing or in a -80°C freezer, and thawed for 1, 3, or 5 freeze/thaw cycles.

[0032] FIG. 5 is a set of bar chart panels that depict a stability study for reconstituted lyophiles and shows a distribution of different SP-D oligomeric species represented by either Peak 1, Peak 2, Peak 3 or Peak 4 of an AF4-MALLS analysis for lyophiles of rhSP-D that had been reconstituted at either 2 mg/ml (upper panel) or 4 mg/ml (lower panel), and stored at either 5°C or 25°C for a period of 1 or 3 days.

DETAILED DESCRIPTION

[0033] Surfactant protein D (SP-D) is a C-type (Ca^{2+}-dependent) lectin that comprises four domains: a cysteine-linked N-terminal region required for the formation of intermolecular disulfide bonds, a triple-helical collagen region, an α-helical-coiled-coil trimerizing neck peptide, and a C-terminal calcium-dependent carbohydrate-recognition domain (CRD) (Crouch E. *et al.* (1994) J Biol Chem 269:17311-9). Monomers form trimers through folding of the collagenous region into triple helices and the assembly of a coiled-coil bundle of α-helices in the neck region (FIG. 1). These trimers are stabilized by two disulfide bonds in the cysteine-rich N-terminal domain. The SP-D trimer has a total molecular weight of 129 kDa which comprises three identical 43-kDa polypeptide chains. SP-D trimers can form higher oligomerization states which vary by size and conformation. Higher oligomerization states may be important for SP-D function (Hakansson K, *et al.*, Protein Sci (2000) 9:1607–17; Crouch E. Respir Res (2000) 1:93–108; Crouch E. *et al.* (2006) J Biol Chem 281:18008-14). The association of SP-D trimers into higher oligomerization states is sensitive to environmental factors and conditions during purification and storage. The pathway and type of interactions involved in the formation of large oligomers of SP-D have not been previously elucidated.

[0034] Recombinant human SP-D (rhSP-D) has been produced in various mammalian systems including certain human myeloid leukemia cell lines, and Chinese hamster ovary (CHO) cells. A sample of rhSP-D can include a diverse population of different rhSP-D oligomeric forms including: trimers, hexamers, dodecamers, multimers of hexamers and dodecamers having a star-like appearance, and even larger oligomeric species identified as aggregates. The rhSP-D derived from various mammalian systems can be substantially different. For example, post-translational modifications of rhSP-D can affect the distribution of rhSP-D oligomeric forms within a given sample of the rhSP-D. In addition, rhSP-D produced in certain human myeloid leukemia cell lines can have a higher amount of rhSP-D dodecamers and a lower amount of larger oligomeric species, such as rhSP-D aggregates, compared to rhSP-D produced from CHO cells.

[0035] Applicant has discovered certain formulations of rhSP-D and active fragments thereof that provide improved shelf-life, improved stability of active rhSP-D protein, and improved stability for certain rhSP-D oligomeric forms. Some embodiments of such formulations include compositions comprising rhSP-D for administration, rhSP-D for lyophilization, lyophilized rhSP-D, reconstituted lyophilized rhSP-D, and reconstituted lyophilized rhSP-D for administration. In some embodiments, compositions are suitable for pulmonary administration, such as administration to a lung, such as a neonate lung.

Pharmaceutical compositions

[0036] Some embodiments of the compositions and methods provided herein include pharmaceutical compositions of recombinant human surfactant protein D (rhSP-D) or an active fragment thereof. In some embodiments, rhSP-D or an active fragment thereof has activity in a bacterial aggregation assay, or in a TLR4 inhibition assay. In some embodiments, the pharmaceutical composition can be an aqueous solution, a suspension, or a solid form. In some embodiments, the pharmaceutical composition of rhSP-D or an active fragment thereof is suitable for lyophilization to a solid form. In some embodiments, a solid form, such as a lyophile or powder, can be administered to a lung, and/or can be reconstituted to form a certain solution suitable for administration to a lung. In some embodiments, the pharmaceutical composition comprising the aqueous solution or suspension of rhSP-D or an active fragment thereof is suitable for administration to a lung.

[0037] Certain activities of rhSP-D, or a fragment thereof, can be readily determined using bacterial aggregation assays, Toll-like receptor 4 (TLR4) inhibition assays, and/or an asymmetric flow field-flow fractionation with multi-angle laser light scattering (AF4-MALLS) analysis. In some embodiments, the activity of rhSP-D, or an active fragment thereof, can include a biological activity, such as activity measured in a bacterial aggregation assays, or a TLR4 inhibition assay. In some embodiments, the activity of rhSP-D, or an active fragment thereof, can include the activity of a population of the rhSP-D, or active fragments thereof, to form certain oligomeric forms of the rhSP-D and/or to form a certain distribution of oligomeric forms of the rhSP-D. Example methods to identify the distribution of oligomeric forms of rhSP-D in a sample are provided herein.

[0038] SP-D comprises four domains: a cysteine-linked N-terminal region required for the formation of intermolecular disulfide bonds, a triple-helical collagen region, an α-helical-coiled-coil trimerizing neck peptide, and a C-terminal calcium-dependent carbohydrate-recognition domain (CRD). Example rhSP-D polypeptide sequences are provided in TABLE 1. Certain portions of the SP-D can have certain roles in the function of SP-D *in vivo*. For example, in SP-D knock-out mice, a recombinant fragment of human SP-D consisting of a shorter collagen region (but not the entire collagen region or the amino terminus of wild-type SP-D), the neck domain, and the CRD, inhibits certain aspects of the development of emphysema-like pathology (Knudsen L., *et al.*, (2009) The Anatomical Record 292:183-189 which is incorporated herein by reference in its entirety). The cysteine-linked N-terminal region may have activity in formation of oligomeric forms of SP-D. For example, a nucleotide change from A to C resulting in a Met to Thr substitution at position 11 in the protein (Met(11)Thr) can influence formation of oligomeric forms of SP-D. *See e.g.*, Knudsen L., *et al.*, (2013) J. Anat 223:581-592; and Winkler C., *et al.*, (2014) Exp Lung Res 40:154-163 each of which is incorporated herein by reference in its entirety. Deletion studies with a mutant rat SP-D protein lacking the rat triple-helical collagen region demonstrated that the mutant rat SP-D could still form trimers, but was unable to form higher order oligomers such as dodecamers, and lacked activity in for the regulation of pulmonary macrophage activation, airspace remodeling, and surfactant lipid homeostasis. *See* Ogasawara Y. *et al.*, (1995) J Biol Chem 270:19052-19058; and Kingma P.S., *et al.*, (2006) J Biol Chem 281:24496-24505 which are each incorporated herein by reference in its entirety. Further deletion studies with a rat SP-D

protein demonstrated that the rat cysteine-linked N-terminal region had a role in efficient viral neutralization and opsonization. *See* White M. *et al.*, (2008) J. Immunol 181:7937-7942 which is incorporated herein by reference in its entirety. Examples of human SP-D lacking certain domains are disclosed in U.S. Patent No. 8,865,643 which is incorporated by reference in its entirety.

[0039] In some embodiments, the pharmaceutical composition can include a buffer. Examples of buffers include acetate, citrate, glutamate, histidine, succinate, and phosphate. In some embodiments, the buffer is histidine. In some embodiments, the concentration of the buffer, such as histidine, is 0.1 mM, 1 mM, 2 mM, 3 mM, 4mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, or a concentration in a range between any two of the foregoing concentrations. In some embodiments, the concentration of the buffer, such as histidine, is about 0.1 mM, about 1 mM, about 2 mM, about 3 mM, 4 mM, about 5 mM, about 6 mM, about 7 mM, about 8 mM, about 9 mM, about 10 mM, about 20 mM, about 30 mM, about 40 mM, about 50 mM, about 60 mM, about 70 mM, about 80 mM, about 90 mM, about 100 mM, or a concentration in a range between any two of the foregoing concentrations.

[0040] In some embodiments, the pharmaceutical composition can include a sugar. Examples of sugars include trehalose, sucrose, maltose, lactose, glucose, fructose, galactose, mannose, arabinose, xylose, ribose, rhamnose, trehalose, sorbose, melezitose, raffinose, thioglucose, thiomannose, thiofructose, octa-O-acetyl-thiotrehalose, thiosucrose, and thiomaltose. In some embodiments, the sugar is lactose. In some embodiments, the concentration of the sugar, such as lactose, is 0.1 mM, 1 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 265 mM, 300 mM, 350 mM, 400 mM 450 mM, 500 mM, 600 mM, 700 mM, 800 mM, 900 mM, 1000 mM, or a concentration in a range between any two of the foregoing concentrations. In some embodiments, the concentration of the sugar, such as lactose, is about 0.1 mM, about 1 mM, about 10 mM, about 20 mM, about 30 mM, about 40 mM, about 50 mM, about 100 mM, about 150 mM, about 200 mM, about 250 mM, about 265 mM, about 300 mM, about 350 mM, about 400 mM about 450 mM, about 500 mM, about 600 mM, about 700 mM, about 800 mM, about 900 mM, about 1000 mM, or a concentration in a range between any two of the foregoing concentrations.

[0041] In some embodiments, the pharmaceutical composition can include a calcium salt. Examples of calcium salts include calcium chloride, calcium bromide, calcium acetate, calcium sulfate, and calcium citrate. In some embodiments, the calcium salt is calcium chloride. In some embodiments, the concentration of the calcium salt, such as calcium chloride, is 0.1 mM, 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, or a concentration in a range between any two of the foregoing concentrations. In some embodiments, the concentration of the calcium salt, such as calcium chloride, is about 0.1 mM, about 1 mM, about 2 mM, about 3 mM, 4 mM, about 5 mM, about 6 mM, about 7 mM, about 8 mM, about 9 mM, about 10 mM, about 20 mM, about 30 mM, about 40 mM, about 50 mM, about 60 mM, about 70 mM, about 80 mM, about 90 mM, about 100 mM, or a concentration in a range between any two of the foregoing concentrations.

[0042] In some embodiments, the pharmaceutical composition can include an inorganic salt or organic salt. Examples of inorganic salts include sodium chloride, potassium chloride, calcium chloride, sodium phosphate, potassium phosphate, and sodium hydrogen carbonate. Examples of organic salts include sodium citrate, potassium citrate and sodium acetate. In some embodiments, the inorganic salt is sodium chloride. In some embodiments, the concentration of the inorganic salt or organic salt, such as sodium chloride, is 0.1 mM, 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, or a concentration in a range between any two of the foregoing concentrations. In some embodiments, the concentration of the inorganic salt or organic salt, such as sodium chloride, is about 0.1 mM, about 1 mM, about 2 mM, about 3 mM, 4 mM, about 5 mM, about 6 mM, about 7 mM, about 8 mM, about 9 mM, about 10 mM, about 20 mM, about 30 mM, about 40 mM, about 50 mM, about 60 mM, about 70 mM, about 80 mM, about 90 mM, about 100 mM, or a concentration in a range between any two of the foregoing concentrations. In some embodiments, the pharmaceutical composition can lack an inorganic salt or organic salt, such as sodium chloride.

[0043] In some embodiments, the pharmaceutical composition can include a surface-active agent. Examples of surface-active agents include hexadecanol, tyloxapol, dipalmitoylphosphatidylcholine (DPPC), PG, palmitoyl-oleoyl phosphatidylglycerol, palmitic acid, tripalmitin, polysorbates such as polysorbate-20, polysorbate-80, polysorbate-21,

polysorbate-40, polysorbate-60, polysorbate-65, polysorbate-81, and polysorbate-85. More examples of surface active agents include poloxamer such as poloxamer 188, Triton such as Triton X-100, sodium dodecyl sulfate (SDS), sodium laurel sulfate, sodium octyl glycoside, lauryl-sulfobetaine, myristyl-sulfobetaine, linoleyl-sulfobetaine, stearyl-sulfobetaine, lauryl-sarcosine, myristyl-sarcosine, linoleyl-sarcosine, stearyl-sarcosine, linoleyl-betaine, myristyl-betaine,cetyl-betaine, lauroamidopropyl-betaine, cocamidopropyl-, linoleamidopropyl-betaine, myristamidopropyl-betaine, palmidopropyl-betaine, isostearamidopropyl-betaine, myristamidopropyl-dimethylamine, palmidopropyl-dimethylamine,isostearamidopropyl-dimethylamine, sodium methyl cocoyl-taurate, disodium methyl oleyl-taurate, polyethyl glycol, polypropyl glycol, and copolymers of ethylene and propylene glycol. In some embodiments, the surface-active agent is tyloxapol. In some embodiments, the concentration of the surface-active agent, such as tyloxapol, is 0.0001%, 0.0005%, 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1%, (v/v) or a concentration in a range between any two of the foregoing concentrations. In some embodiments, the concentration of the surface-active agent, such as tyloxapol, is about 0.0001%, about 0.0005%, about 0.001%, about 0.005%, about 0.01%, about 0.05%, about 0.1%, about 0.5%, about 1%, (v/v) or a concentration in a range between any two of the foregoing concentrations. In some embodiments, the pharmaceutical composition can lack a surface-active agent, such as tyloxapol.

[0044] In some embodiments, the pharmaceutical composition can have a pH of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, or a pH in a range between any two of the foregoing values. In some embodiments, the pharmaceutical composition can have a pH of about 4.0, about 4.5, about 5.0, about 5.5, about 6.0, about 6.5, about 7.0, about 7.5, about 8.0, about 8.5, about 9.0, about 9.5, about 10.0, or a pH in a range between any two of the foregoing values.

[0045] In some embodiments, the concentration of protein, such as rhSP-D or an active fragment thereof, in the pharmaceutical composition can be 0.01 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml, 5 mg/ml, 6 mg/ml, 7 mg/ml, 8 mg/ml, 9 mg/ml, 10 mg/ml, 20 mg/ml, 30 mg/ml, 40 mg/ml, 50 mg/ml, 60 mg/ml, 70 mg/ml, 80 mg/ml, 90 mg/ml, 100 mg/ml, or a concentration in a range between any two of the foregoing concentrations. In some embodiments, the concentration of protein, such as rhSP-D or an active fragment thereof, in the pharmaceutical composition can be about 0.01 mg/ml,

about 0.05 mg/ml, about 0.1 mg/ml, about 0.5 mg/ml, about 1 mg/ml, about 2 mg/ml, about 3 mg/ml, about 4 mg/ml, about 5 mg/ml, about 6 mg/ml, about 7 mg/ml, about 8 mg/ml, about 9 mg/ml, about 10 mg/ml, about 20 mg/ml, about 30 mg/ml, about 40 mg/ml, about 50 mg/ml, about 60 mg/ml, about 70 mg/ml, about 80 mg/ml, about 90 mg/ml, about 100 mg/ml, or a concentration in a range between any two of the foregoing concentrations.

[0046] In some embodiments, the pharmaceutical composition can include a bulking agent. Examples of bulking agents include a sugar disclosed herein. More examples of bulking agents include mannitol, xylitol, sorbitol, maltitol, lactitol, glycerol, erythritol, arabitol, glycerine, glycine, alanine, threonine, valine, and phenylalanine. In some embodiments, the concentration of the bulking agent, is 0.1 mM, 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, or a concentration in a range between any two of the foregoing concentrations. In some embodiments, the concentration of the bulking agent, is about 0.1 mM, about 1 mM, about 2 mM, about 3 mM, 4 mM, about 5 mM, about 6 mM, about 7 mM, about 8 mM, about 9 mM, about 10 mM, about 20 mM, about 30 mM, about 40 mM, about 50 mM, about 60 mM, about 70 mM, about 80 mM, about 90 mM, about 100 mM, or a concentration in a range between any two of the foregoing concentrations.

[0047] In some embodiments, the pharmaceutical composition can include a chelating agent. In some embodiments, the pharmaceutical composition can lack a chelating agent. Examples of chelating agents include EDTA, and EGTA.

[0048] In some embodiments, the rhSP-D comprises a wild-type human SP-D polypeptide. In some embodiments, the rhSP-D includes a polymorphism of the human SP-D polypeptide. Example SP-D polypeptide sequences are provided in TABLE 1. Polymorphisms in the human SP-D polypeptide can include: residue 11, ATG (Met) -> ACG (Thr); residue 25, AGT (Ser) -> AGC (Ser); residue 160, ACA (Thr) -> GCA (Ala); residue 270, TCT (Ser) -> ACT (Thr); and residue 286, GCT (Ala) -> GCC (Ala) in which the positions relate to a position in a mature SP-D polypeptide, such as the example polypeptide of SEQ ID NO:02. In some embodiments, the rhSP-D comprises a certain residue at a polymorphic position in which the residue selected from Met11/31, Thr160/180, Ser 270/290, and Ala 286/306 in which residue positions relate to a position in the mature SP-D polypeptide, such as example SEQ ID NO:02, and a position in the SP-D polypeptide with its leader polypeptide, such as example

SEQ ID NO:01. In some embodiments, the rhSP-D comprises Met11/31. In some embodiments, the rhSP-D comprises Met11/31, Thr160/180, Ser 270/290, and Ala 286/306. In some embodiments, the rhSP-D polypeptide has an identity with a polypeptide of SEQ ID NO:02 over the entire length of the polynucleotide of at least 80%, 90%, 95%, 99% and 100%, or any percentage in a range between any of the foregoing percentages.

TABLE 1

SEQ ID NO.	**Sequence**
SEQ ID NO:01 SP-D polypeptide including a leader sequence (underlined) and polymorphisms (underlined) at: Met 31, Thr 180, Ser 290, Ala 306.	MLLFLLSALVLLTQPLLGYLEAEMKTYSHRTMPSACTLV MCSSVESGLPGRDGRDGREGPRGEKGDPGLPGAAGQAG MPGQAGPVGPKGDNGSVGEPGPKGDTGPSGPPGPPGVPG PAGREGPLGKQGNIGPQGKPGPKGEAGPKGEVGAPGMQG SAGARGLAGPKGERGVPGERGVPGNTGAAGSAGAMGPQ GSPGARGPPGLKGDKGIPGDKGAKGESGLPDVASLRQQV EALQGQVQHLQAAFSQYKKVELFPNGQSVGEKIFKTAGF VKPFTEAQLLCTQAGGQLASPRSAAENAALQQLVVAKNE AAFLSMTDSKTEGKFTYPTGESLVYSNWAPGEPNDDGGS EDCVEIFTNGKWNDRACGEKRLVVCEF
SEQ ID NO:02 SP-D polypeptide of SEQ ID NO:01, without leader sequence, and polymorphisms (underlined) at: Met 11, Thr 160, Ser 270, Ala 286.	AEMKTYSHRTMPSACTLVMCSSVESGLPGRDGRDGREGP RGEKGDPGLPGAAGQAGMPGQAGPVGPKGDNGSVGEPG PKGDTGPSGPPGPPGVPGPAGREGPLGKQGNIGPQGKPGP KGEAGPKGEVGAPGMQGSAGARGLAGPKGERGVPGERG VPGNTGAAGSAGAMGPQGSPGARGPPGLKGDKGIPGDKG AKGESGLPDVASLRQQVEALQGQVQHLQAAFSQYKKVEL FPNGQSVGEKIFKTAGFVKPFTEAQLLCTQAGGQLASPRS AAENAALQQLVVAKNEAAFLSMTDSKTEGKFTYPTGESL VYSNWAPGEPNDDGGSEDCVEIFTNGKWNDRACGEKRL VVCEF

[0049] In some embodiments, the rhSP-D is derived from a human myeloid leukemia cell line expressing the rhSP-D from an integrated transgene. Example expression vectors, rhSP-D polypeptides, cell-lines, and methods of purifying rhSP-D from such cells, are provided in U.S 2019/0071693 and U.S. 2019/0071694 each of which is expressly incorporated by reference herein in its entirety.

[0050] In some embodiments, a pharmaceutical composition, such as a solution or suspension, comprising a population of rhSP-D polypeptides can have a certain distribution of oligomeric forms of the rhSP-D. A composition of rhSP-D can include different rhSP-D oligomeric forms including: trimers with a mass of about 130-150 kDa on SDS-PAGE which

include 3 monomers and which together can have a rod-like appearance as visualized by atomic force microscopy (AFM); hexamers with a mass of about 250 kDa on SDS-PAGE which include 6 monomers; dodecamers with a predicted mass of about 520 kDa, as measured by AF4-MALLS and which include 12 monomers and can have an X-like appearance as visualized by AFM; larger heterogeneous oligomeric species which comprise multiples of more than four trimers and can have a star-like- or star- shaped appearance with a radius of about 70 nm as visualized and identified by AFM, such oligomers are known as star-like oligomers; and even larger oligomeric species having a radius larger than 70 nm as visualized by AFM and measured by AF4-MALLS and known as aggregates.

[0051] In some embodiments, more than about 10%, 20%, 30%, 50%, 60%, 70%, 80%, 90%, or a percentage within a range between any two of the foregoing percentages, of the oligomeric forms of rhSP-D can be a dodecameric oligomeric form of rhSP-D as measured as a relative peak area (RPA) in an AF4-MALLS analysis. In some embodiments, more than about 10%, 20%, 30%, 50%, 60%, 70%, 80%, 90%, or a percentage within a range between any two of the foregoing percentages, of the mass of the oligomeric forms, such as in a solution or suspension, of rhSP-D can be a dodecameric oligomeric form of rhSP-D. In some embodiments, more than about 10%, 20%, 30%, 50%, 60%, 70%, 80%, 90%, or a percentage within a range between any two of the foregoing percentages, of the number of molecules of the oligomeric forms, such as in a solution or suspension, of rhSP-D can be a dodecameric oligomeric form of rhSP-D.

[0052] In some embodiments, less than about 0.5%, 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 50%, or a percentage within a range between any two of the foregoing percentages, of the oligomeric forms of rhSP-D can be an aggregate oligomeric form of rhSP-D as measured as an RPA or an adjusted RPA in an AF4-MALLS analysis. In some embodiments, less than about 0.5%, 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 50%, or a percentage within a range between any two of the foregoing percentages, of the mass of the oligomeric forms, such as in a solution or suspension, of rhSP-D can be an aggregate oligomeric form of rhSP-D. In some embodiments, less than about 0.5%, 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 50%, or a percentage within a range between any two of the foregoing percentages, of the number of molecules of the oligomeric forms, such as in a solution or suspension, of rhSP-D can be an aggregate oligomeric form of rhSP-D.

[0053] In some embodiments, a pharmaceutical composition consists of, consists essentially of, or comprises 1 mg/ml rhSP-D, 5 mM histidine, 265 mM lactose, 5 mM calcium chloride, having a pH of 6.0. In some embodiments, a pharmaceutical composition consists of, consists essentially of, or comprises 1 mg/ml rhSP-D, 5 mM histidine, 265 mM lactose, 1 mM calcium chloride, having a pH of 6.0. In some embodiments, a pharmaceutical composition consists of, consists essentially of, or comprises 2 mg/ml rhSP-D, 5 mM Histidine, 265 mM Lactose, 1 mM $CaCl_2$, pH 6.0. In some embodiments, a pharmaceutical composition consists of, consists essentially of, or comprises 2 mg/ml rhSP-D, 5 mM histidine, 265 mM lactose, 5 mM calcium chloride, having a pH of 6.0. In some embodiments, a pharmaceutical composition consists of, consists essentially of, or comprises 4 mg/ml rhSP-D, 5 mM histidine, 265 mM lactose, 5 mM calcium chloride, having a pH of 6.0.

[0054] In some embodiments, the pharmaceutical compositions provided herein can include an admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, or the like, and can contain auxiliary substances such as wetting or emulsifying agents, pH buffering agents, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of administration and the preparation desired. *See e.g.*, "Remington: The Science and Practice of Pharmacy", Lippincott Williams & Wilkins; 20th edition (June 1, 2003) and "Remington's Pharmaceutical Sciences," Mack Pub. Co.; 18th and 19th editions (December 1985, and June 1990, respectively). In some embodiments, such preparations can include complexing agents, metal ions, polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, dextran, and the like, liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Suitable lipids for liposomal formulation include monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. The presence of such additional components can influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance, and are thus can be chosen according to the intended application, such that the characteristics of the carrier are tailored to the selected route of administration, such as pulmonary delivery, such as delivery to a lung, such as delivery to a neonate lung.

[0055] In some embodiments, pharmaceutical compositions are suitable for intratracheal, intrabronchial or bronchoalveolar administration to a lung. In some

embodiments, intratracheal, intrabronchial or bronchoalveolar administration can include spraying, lavage, inhalation, flushing or installation, using as fluid a physiologically acceptable composition in which the pharmaceutical composition has been dissolved. Methods of administration can include the use of continuous positive airway pressure (CPAP). Methods of administration can include direct intubation. In some embodiments, pharmaceutical compositions provided herein can be delivered to the lungs while inhaling. Example forms that can be delivered include dry powders, and aerosols. A wide range of mechanical devices designed for pulmonary delivery of therapeutic products can be employed, including but not limited to nebulizers, metered dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art. These devices employ formulations suitable for the dispensing of a pharmaceutical composition. Typically, each formulation is specific to the type of device employed and can involve the use of an appropriate propellant material, in addition to diluents, adjuvants, and/or carriers useful in therapy.

Identification of oligomeric species of rhSP-D

[0056] The distribution of oligomeric forms of rhSP-D, such as the distribution of oligomeric forms of rhSP-D in a solution or suspension, can be determined by a variety of techniques. In some embodiments, a method for identifying oligomeric species of rhSP-D can include performing atomic force microscopy (AFM) on a sample of rhSP-D, identifying and quantifying oligomeric species of rhSP-D in the AFM images. *See e.g.*, Arroyo R, *et al.* (2017) Biophys J 112 (3): 503a which is incorporated by reference herein in its entirety. In an example embodiment, a sample of rhSP-D can be diluted in a buffer, such as a dilution buffer (200 mM NaCl, 20 mM Tris (pH 7.4), 1 mM EDTA) and placed on freshly cleaved mica substrate. Samples can be imaged with an atomic force microscope, such as a microscope from Nanotec (Nanotec Electrónica, Madrid, Spain) and PointProbePlus tips, type PPP-NCH (Nanosensors, Neuchâtel, Switzerland). Raw images can be subjected to general plane subtraction, flattening with background subtraction and artifact line removal. Oligomeric species that can be identified in the obtained images can include trimers, hexamers, dodecamers, and star-like oligomers. Trimers can have a rod-like appearance, and some trimers can have an average length of about 65 nm (± 8.6 nm). Dodecamers can have an X-shaped appearance, and some dodecamers can have a tip to opposite tip length of about 136 nm (± 8.1 nm). The star-like

oligomeric species can include members that have a star-like- or star- shaped appearance composed of 6-20 trimers, in which each trimer is joined to a particular member of this species through a central hub. Some star-like oligomers can have a diameter of about 140 nm, similar to the diameter of the measured dodecamers.

[0057] In some embodiments, a method for identifying oligomeric species of rhSP-D can include performing an asymmetric flow field-flow fractionation with multi-angle laser light scattering (AF4-MALLS) analysis on a sample of rhSP-D. AF4-MALLS can provide a fractogram that includes a first peak (Peak 1) which can represent rhSP-D trimers and hexamers; a second peak (Peak 2) which can represent rhSP-D dodecamers; a third peak (Peak 3) which can represent intermediate species between rhSP-D dodecamers to rhSP-D star-like oligomeric species; and a fourth peak (Peak 4) which can represent a heterogeneous mass of rhSP-D oligomers with a radius of about 70 nm. Other species of rhSP-D can be identified by AF4-MALLS having a radius larger than 70 nm which can include aggregate species.

[0058] In some embodiments, a method for identifying oligomeric species of rhSP-D can include performing a size exclusion chromatograph HPLC (SEC HPLC). In some embodiments, a method for identifying oligomeric species of rhSP-D can include performing polyacrylamide gel electrophoresis (PAGE). In some such methods, a sample of rhSP-D can be contacted with an anionic detergent, such as of SDS, and; contacted the sample with a crosslinking reagent, such as 1% glutardialdehyde; and resolving by size the species of rhSP-D, such as by PAGE. Some methods can also include identifying the species of rhSP-D, such as performing a Western blot.

Kits

[0059] Some embodiments provided herein include kits. In some embodiments, a kit can include a pharmaceutical composition provided herein. Some embodiments include a sterile container comprising a pharmaceutical composition provided herein. Some embodiments include a pharmaceutical composition provided herein in lyophilized form, and a sterile reconstituting solution. In some embodiments, a kit can include a device for administering a pharmaceutical composition provided herein, such as an inhaler, and a nebulizer.

EXAMPLES

Example 1—Lyophilization of certain formulations

[0060] Lyophilization of certain formulations without rhSP-D was investigated. Primary drying temperatures for lyophilization for various buffer formulations without rhSP-D were determined using subambient differential scanning calorimetry (DSC). The formulations are listed in TABLE 2. The initial glass transition temperature (T_g'), mid Tg′, and melting temperature for various formulations without rhSP-D were determined and are summarized in TABLE 3.

TABLE 2

Formulation	**Components**
F01-01	5 mM Histidine, 150 mM NaCl, pH 7.0
F02-01	5 mM Histidine, 150 mM NaCl, 1 mM EDTA, pH 6.0
F03-01	5mM Histidine, 50mM NaCl, 150 mM Lactose, 1 mM $CaCl_2$, pH 6.0
F04-01	5mM Histidine, 50 mM NaCl, 150 mM Lactose, 1 mM EDTA, pH 7.0
F05-01	5mM Histidine, 100mM Lactose, 1mM $CaCl_2$, pH 6.0
F06-01	5mM Histidine, 200mM NaCl, 1 mM EDTA, pH 6.0
F07-01	5mM Histidine, 200mM NaCl, 1 mM EDTA, pH 7.0

TABLE 3

Formulation	**Onset T_g'**	**Mid T_g'**	**Melting temperature (°C)**	**Melting area (J/g)**
F01-01	ND	ND	-22.5	5.5
F02-01	ND	ND	-22.7	5.9
F03-01	-36.2	-34.5	ND	ND
F04-01	-35.6	-33.9	ND	ND
F05-01	-31.8	-30.8	ND	ND
F06-01	ND	ND	-22.4	8.2
F07-01	ND	ND	-22.0	7.7
ND: not determined				

[0061] Formulations without rhSP-D and containing NaCl had a detectable initial glass transition temperature (T_g'). Melting temperature for formulations F01-01, F02-01, F06-01, and F07-01 were in the range of the eutectic temperature of NaCl. Lactose and lactose/NaCl have a $T_g' > -37$ °C. Thus, a primary drying temperature of -35 °C was feasible. Formulations without rhSP-D were lyophilized under conditions as shown in the TABLE 4.

TABLE 4

Stage	**Conditions**
Freezing:	Cool from ambient to -5°C in 60 min; Hold at -5C for 30 minutes; Cool from -5°C to -22°C at 0.5 °C/min; Hold at -22°C for 180 minutes; Cool from -22°C to -55°C at 0.5 °C/min; Hold at -55°C for 60 minutes.
Primary dry (vacuum setpoint: 65 mTorr):	Ramp from -55°C to -35°C at 0.5 °C/min; Hold at -35°C for 72 hours.
Secondary dry (vacuum setpoint: 65 mTorr):	Ramp from -35°C to 25°C at 0.05 °C/min; Hold at 25°C for 240 minutes; Ramp from 25°C to 40°C at 0.05 °C/min; Hold at 40°C for 180 minutes.
Post-run action:	Back-fill with nitrogen gas (580,000 mTorr); Stopper the vials; Lyophilization run completed.

[0062] A comparison between capacitance and Pirani pressure gauges was used to determine the completion of primary drying. The moisture content of lyophilized formulations without rhSP-D was determined by thermogravimetric analysis (TGA). The results are summarized in TABLE 5.

TABLE 5

Formulation	**Moisture (wt/vol %)**
F01-01	0.29
F02-01	0.55
F03-01	2.97
F04-01	3.17
F05-01	2.90

Formulation	Moisture (wt/vol %)
F06-01	0.42
F07-01	0.48

Example 2—Preparation and analysis of formulations containing rhSP-D

[0063] Formulations containing rhSP-D were prepared by dialysis of a solution of 1 mg/ml SP-D with a particular buffer listed in TABLE 2. rhSP-D was obtained from H9D8WT Clone P1315-2A5 (Glycotope) which is a human myeloid leukemia cell line expressing rhSP-D from an integrated transgene. Example expression vectors, rhSP-D polypeptides, cell-lines, and methods of purifying rhSP-D from such cells, are provided in U.S 2019/0071693 and U.S. 2019/0071694 each of which is expressly incorporated by reference herein in its entirety. The pH and osmolality of formulations containing rhSP-D were determined to be substantially the same as the initial buffer, confirming that dialysis was substantially complete. Formulations containing rhSP-D were lyophilized, and stored for various periods of time at various temperatures including 5°C, 25°C, and 40°C. The protein concentrations of reconstituted formulations were determined. The results are summarized in TABLE 6.

TABLE 6

Formulation	Protein concentration of formulations (mg/ml)			
	Pre-lyophilization	0 weeks post-lyophilization	2 weeks at 25°C post-lyophilization	2 weeks at 40°C post-lyophilization
F01-01	0.97	0.99	1.09	1.08
F02-01	1.00	1.01	1.09	1.10
F03-01	0.98	0.96	1.04	1.02
F04-01	0.30	0.28	0.37	0.33
F05-01	0.86	0.86	0.96	0.93
F06-01	0.94	1.00	1.06	1.07
F07-01	1.00	1.01	1.16	1.14

[0064] The turbidity of reconstituted formulations containing rhSP-D was determined by measuring absorbance at 320 nm. The results are summarized in TABLE 7.

TABLE 7

Formulation	Turbidity (A_{320} nm)			
	Pre-lyophilization	**0 weeks post-lyophilization**	**2 weeks at 25°C post-lyophilization**	**2 weeks at 40°C post-lyophilization**
F01-01	0.108	0.569	0.612	0.659
F02-01	0087	0.512	0.642	0.648
F03-01	0.088	0.114	0.110	0.104
F04-01	0.073	0.089	0.087	0.072
F05-01	0.103	0.120	0.124	0.130
F06-01	0.068	0.432	0.531	0.480
F07-01	0.062	0.507	0.530	0.569

[0065] The turbidity of the protein solutions was an indicator of protein precipitation. The low turbidity of F04-01 was likely due to the particularly low protein concentration. Formulations F03-01 and F05-01 which each contain lactose and calcium each had a relatively low turbidity. In contrast, formulations F01-01, F02-01, F06-01, and F07-01 which each contained NaCl and lacked lactose, were over five-fold more turbid after storing the lyophile for 2 weeks at 25°C than F03-01 and F05-01. Thus, the presence of lactose and calcium, and the absence of NaCl, may promote the ability of rhSP-D to form a solution after reconstitution of a lyophile.

[0066] The distribution of different oligomeric species of rhSP-D in various reconstituted formulations containing rhSP-D was determined by an asymmetric flow field-flow fractionation (AF4) with multi-angle laser light scattering (AF4-MALLS) analysis. Example methods are also provided in U.S. Prov. App. 62/650138 filed March 29, 2018 entitled "METHODS FOR CHARACTERIZING SURFACTANT PROTEIN D (SP-D) OLIGOMERS" which is incorporated by reference herein in its entirety. For the AF4-MALLS analysis, rhSP-D samples were separated by an AF4 system (Eclipse Dual Tec, Wyatt Technology Corp., Santa Barbara, CA) followed by UV (Ultimate 3000 variable wavelength detector, Dionex Corporation, Sunnyvale, CA) and MALS analysis (Dawn Heleos II detector, Wyatt Technology Corp., Santa Barbara, CA). A Dionex Ultimate 3000 HPLC system (Dionex

Corporation, Sunnyvale, CA) was used to inject the samples and deliver the mobile phase to the AF4 system. The AF4 configuration used a short channel with a 350 µm thick spacer (Wyatt Technology Corp., Santa Barbara, CA). Analysis of the data and calculations were performed using Chromeleon (Dionex Corporation, Sunnyvale, CA) and Astra (Wyatt Technology Corp., Santa Barbara, CA) software. Results from the AF4-MALLS analysis included a fractogram with several peaks. TABLE 8 lists parameters used for the AF4.

TABLE 8

Step	**Start time (min)**	**End time (min)**	**Duration (min)**	**Mode**	**X flow start (ml/min)**	**X flow end (ml/min)**
1	0	1	1	Elution		
2	1	2	1	Focus		
3	2	3	1	Focus + inject		
4	3	6	3	Focus		
5	6	6.2	0.2	Elution	0.5	3
6	6.2	9.2	3	Elution	3	3
7	9.2	19.2	10	Elution	3	0.18
8	19.2	29.2	10	Elution	0.18	0.18
9	29.2	44.2	15	Elution	0.18	0
10	44.2	54.2	10	Elution	0	0
11	54.2	59.2	5	Elution + Inject	0	0
Detector Flow: 0.5 ml/min Inject Flow: 0.2 ml/min Focus Flow: 0.5 ml/min Injection Amount: 5 µg UV detection: 214 nm Mobile phase: 20 mM Tris, 200 mM NaCl, pH 7.4 Channel: short (145 mm) Spacer: 350 µM Membrane: 10 kD PES						

[0067] Data using AF4-MALLS was analyzed to determine absolute molar mass and size of rhSP-D at a certain time during elution. The size:mass ratio was indicative of the shape of the rhSP-D. From the size to mass ratio, it was determined that in the early stages of an elution (0-34 minutes) the rhSP-D molecule had a linear or rod-shape. For rod model calculations, the software assumed that the thickness of a rod-shaped particle was insignificant (0.0 nm) compared to its length. If the thickness was significant, its thickness or approximate thickness in nm was used. Rod thickness was estimated from AFM data, and rod lengths were determined to be consistent with AFM measurements of 136 ± 8.1 nm. Arroyo R, *et al.* (2017) Biophysical Journal 112 (3): 503a which is incorporated by reference herein in its entirety. The later stages of the elution (34-45 minutes) for rhSP-D indicated that a more compact structure was being observed. A second order Debye model was employed for analysis of these stages of the elution. The second order Debye model provided better results over a wider range of molar masses, including the very large (greater than ~10^6 Daltons or ~50 nm RMS radius). For dodecamers of rhSP-D, molecular weight was determined to be 520.09 ± 4.61 kDa (N=72 determinations).

[0068] A first peak in the fractogram (Peak 1) contained rhSP-D trimers and hexamers based on mass calculations according to the rod model. A second peak in the fractogram (Peak 2) contained rhSP-D dodecamers. A third peak in the fractogram (Peak 3) contained intermediate species between rhSP-D dodecamers to rhSP-D star-like oligomers based on the intermediate molecular weight as determined by the rod model. A fourth peak in the fractogram (Peak 4) contained a heterogeneous mass of rhSP-D oligomers with constant RMS radius of about 70 nm consistent with what has been observed by AFM measurements for the star-like oligomeric species, and larger species having a radius greater than 70 nm. Beyond 36 minutes in the fractogram the RMS radius increases further, indicative of further aggregate species. The relative peak area (RPA) for each peak was determined as a percentage of the total peak area of the four peaks.

[0069] To determine the distribution of oligomeric species in solution using an AF4-MALLS analysis with a greater resolution, the species of Peak 4 were analyzed further. An adjusted RPA was determined for each of Peaks 3 and 4. Specifically, aspects of the RPA of Peak 4 that corresponded to star-like oligomeric species having a constant RMS radius of about 70 nm were determined, and these aspects were removed from the RPA of Peak 4 and

added to the RPA of Peak 3 to provide an adjusted RPA for Peak 4 and Peak 3. Thus, the RPA of Peak 1, RPA of Peak 2, adjusted RPA of Peak 3, and adjusted RPA of Peak 4 corresponded to the relative distributions of rhSP-D oligomeric species in the AF4-MALLS analysis for (1) trimers and hexamers; (2) dodecamers; (3) star-like oligomeric species having a radius of about 70 nm; and (4) aggregates having a radius greater than 70 nm, respectively.

[0070] Lyophiles of formulations containing rhSP-D were stored under various conditions, reconstituted and analyzed by AF4-MALLS. The results of an adjusted AF4-MALLS analysis are summarized in TABLE 9. Molecular weights of oligomeric species of rhSP-D were also determined for formulations stored at 5°C for 5 weeks, and are summarized in TABLE 10.

TABLE 9

Formulation	**Condition**	**AF4-MALLS Relative peak area (%)**				
		Peak 1	**Peak 2**	**Adjusted Peak 3**	**Adjusted Peak 4**	
F01-01	Pre-lyophilization	11.96	55.26	32.31	0.48	a
		2.61	2.41	3.22	0.24	sd
	0 weeks	14.92	41.21	40.30	3.57	a
		1.19	2.02	1.40	0.57	sd
	25°C, 2 weeks	20.26	44.33	32.82	2.58	a
		0.59	0.91	1.08	0.41	sd
	40°C, 2 weeks	13.48	45.15	38.28	3.09	a
		0.57	1.30	0.67	1.21	sd
	5°C, 5 weeks	11.29	52.17	32.56	3.98	a
		1.01	0.30	0.83	0.16	sd
F02-01	Pre-lyophilization	25.95	53.22	19.35	1.48	a
		1.16	1.04	1.69	0.40	sd
	0 weeks	24.87	40.84	29.15	5.15	a
		0.77	1.43	0.87	0.91	sd
	25°C, 2 weeks	38.23	38.33	20.87	2.56	a
		2.57	0.22	2.47	0.35	sd

Formulation	Condition	AF4-MALLS Relative peak area (%)				
		Peak 1	**Peak 2**	**Adjusted Peak 3**	**Adjusted Peak 4**	
	40°C, 2 weeks	34.69	33.02	29.36	2.93	a
		0.95	1.59	3.58	1.12	sd
	5°C, 5 weeks	6.39	58.40	30.36	4.84	a
		0.13	0.89	0.87	0.17	sd
F03-01	Pre-lyophilization	20.64	53.08	23.67	2.61	a
		0.52	0.21	0.77	0.27	sd
	0 weeks	4.50	38.26	53.92	3.32	a
		0.20	4.44	3.87	0.68	sd
	25°C, 2 weeks	16.09	45.33	35.89	2.70	a
		3.81	3.21	1.57	0.21	sd
	40°C, 2 weeks	8.77	46.20	43.23	1.81	a
		1.61	0.29	2.31	0.48	sd
	5°C, 5 weeks	9.00	55.83	31.89	3.28	a
		0.32	0.39	0.21	0.15	sd
F04-01	Pre-lyophilization	31.21	54.29	14.50	0.00	a
		0.33	3.78	3.82	0.00	sd
	0 weeks	17.84	68.26	13.66	0.24	a
		0.84	2.66	2.81	0.08	sd
	25°C, 2 weeks	19.54	70.76	9.48	0.23	a
		0.14	0.74	0.87	0.01	sd
	40°C, 2 weeks	19.98	57.22	21.46	1.35	a
		1.24	0.04	0.91	0.29	sd
	5°C, 5 weeks	15.95	68.45	12.84	1.16	a
		0.75	39.54	1.51	0.34	sd
F05-01	Pre-lyophilization	10.54	51.18	36.06	2.22	a
		0.45	0.75	0.82	0.19	sd
	0 weeks	6.95	43.32	46.16	3.58	a

Formulation	Condition	AF4-MALLS Relative peak area (%)				
		Peak 1	**Peak 2**	**Adjusted Peak 3**	**Adjusted Peak 4**	
		0.61	1.44	1.62	0.47	sd
	25°C, 2 weeks	6.07	48.90	42.80	2.23	a
		0.66	1.81	2.00	0.08	sd
	40°C, 2 weeks	6.03	43.08	46.28	4.61	a
		0.26	0.64	0.61	0.85	sd
	5°C, 5 weeks	8.20	57.41	29.89	4.50	a
		0.11	0.44	0.15	0.46	sd
F06-01	Pre-lyophilization	10.06	34.19	53.74	2.01	a
		0.63	2.40	2.31	0.57	sd
	0 weeks	6.04	43.52	44.48	5.96	a
		1.60	3.35	3.64	1.31	sd
	25°C, 2 weeks	8.45	45.07	42.00	4.49	a
		0.24	0.51	0.22	0.41	sd
	40°C, 2 weeks	9.63	36.51	43.63	10.23	a
		1.48	4.52	0.74	6.40	sd
	5°C, 5 weeks	7.00	49.64	37.20	6.16	a
		1.17	1.79	0.09	0.66	sd
F07-01	Pre-lyophilization	12.26	40.80	45.51	1.42	a
		0.30	0.85	0.69	0.04	sd
	0 weeks	12.17	41.12	42.25	4.46	a
		0.78	0.82	1.04	0.55	sd
	25°C, 2 weeks	12.85	34.55	48.02	4.58	a
		0.80	0.94	1.40	0.33	sd
	40°C, 2 weeks	18.49	33.36	44.02	4.13	a
		1.15	1.13	0.03	0.12	sd
	5°C, 5 weeks	12.43	46.91	36.35	4.31	a
		0.80	0.74	0.99	0.44	sd

Formulation	Condition	AF4-MALLS Relative peak area (%)				
		Peak 1	Peak 2	Adjusted Peak 3	Adjusted Peak 4	
a: average; sd: standard deviation						

TABLE 10

Formulation	Average mass (kDa)				Retention time	
	Peak 1	Peak 2	Peak 3	Peak 4		
F01-01	74.9	520.5	1894	25841		a
	25.0	2.4	99	5486		sd
F02-01	158.5	521.9	1751	30689	22.274	a
	17.6	2.5	87	16873	0.060	sd
F03-01	162.6	523.6	2445	23396	22.428	a
	62.4	2.7	76	1503	0.078	sd
F04-01	397.6	517.7	2631	88563	22.674	a
	9.1	8.3	523	34231	0.185	sd
F05-01	274.7	526.8	1971	136670	22.833	a
	124.2	5.3	447	55743	0.097	sd
F06-01	372.2	516.5	1854	39336	23.871	a
	64.1	4.9	113	2081	0.145	sd
F07-01	215.3	523.6	1889	32590	23.228	a
	19.0	4.5	59	1659	0.088	sd
a = average; sd = standard deviation						

[0071] At week 0 and after storing the lyophile for 2 weeks at 25°C, each formulation had a substantially similar distribution of RPAs indicative of a substantially similar distribution of rhSP-D oligomeric species.

[0072] With regard to adjusted Peak 4 which was indicative of aggregate oligomeric species of rhSP-D having a radius greater than 70 nm, formulation F06-01 was observed to have the largest increase with an adjusted RPA from 5.96% at 0 week to 10.23% after storing the lyophile for 2 weeks at 40°C. With regard to Peak 2 which was indicative of

rhSP-D dodecamers, formulations F01-01, F03-01, and F05-01 each showed an increase or no substantial change in the RPA for Peak 2 between 0 week and after storing the lyophile for 2 weeks at 40°C. In contrast, formulations F02-01, F04-01, F06-01, and F07-01 each showed a decrease in the RPA for Peak 2 between 0 week and after storing the lyophile for 2 weeks at 40°C. The stability of rhSP-D dodecamers in the lyophilized cake were more stable over time under all conditions examined in formulations including F03-01 and F05-01 which contained lactose and calcium. These were also the formulations that exhibited relatively low turbidity upon reconstitution as shown herein.

[0073] Formulation F04-01 had the lowest protein concentration and the lowest adjusted RPA for Peak 4 indicative of the lowest relative fraction of aggregate rhSP-D species which suggested that protein concentration could have a role in the formation of aggregates.

Example 3—Activity of formulations containing rhSP-D in a bacterial aggregation assay

[0074] The activity of reconstituted formulations containing rhSP-D was tested in a bacterial aggregation assay. In the bacterial aggregation assay, active rhSP-D aggregates bacterial cells and reduces absorbance / increases transmission through the bacterial suspension. The bacterial aggregation assay was performed by a method substantially similar to the following method. E. *coli* (ATCC: Y1088) was streaked onto a bacterial agar plate and incubated at 37°C overnight. A single colony was selected and used to inoculate an overnight culture, shaken at 37°C overnight. A 1 mL bacterial culture was pipetted into four 1.5 mL centrifuge tubes, and centrifuged at 4,000 rpm for 5 minutes. The supernatant was discarded, and the pellet re-suspended in 1 mL buffer, (150 mM HEPES, 20 mM NaCl pH 7.4). The tubes were centrifuged at 4,000 rpm for 5 minutes and the pellet was re-suspended in 7 mL buffer. Absorbance of the bacterial suspension was measured in a spectrometer at 700 nm. The bacterial suspension was adjusted to obtain an Absorbance in the range of 1.0000 to 1.1000. 1M $CaCl_2$ was added to the suspension to obtain a final concentration of 5mM $CaCl_2$. rhSP-D dilutions in placebo buffer (15 µl total volume for each dilution) were created at the following concentrations: 5, 1, 0.5, 0.25, 0.1, 0 µg/ml and added to cuvettes each containing 20 µL of the HEPES-NaCl buffer. 600 µL bacterial suspension were then added to cuvettes, and absorbance was measured every 2.5 minutes for each cuvette at 700 nm, for a total of 120 minutes.

[0075] Tested concentrations of reconstituted rhSP-D included 0 μg/ml, 0.1μg/ml, 0.25μg/ml, 0.5μg/ml, 1.0μg/ml, and 5.0 μg/ml. A percentage average aggregation at 60 minutes was calculated from the absorbance value at 60 minutes at each tested concentration, according to the following formula:

(1- abs)*100=% Aggregation

where 1 = the measured absorbance of the *E. coli* suspension without rhSP-D.
Abs = The absorbance value of the *E. coli* suspension + rhSP-D at 60 minutes.

[0076] Percentage (%) aggregation values were averaged from 3 replicates and imported, along with the standard deviation, into GraphPad Prism v7.0c, (GraphPad, La Jolla, CA 92037). The averaged values were fit using a 4-Parameter logistic curve. The resultant values for the EC_{50}, and Span were determined for each reconstituted rhSP-D sample. pEC_{50} is the $-Log_{10}$ of the EC_{50}.

[0077] Example results are presented in TABLE 11. Formulations containing rhSP-D had substantially similar potency and efficacy (curve span) before and after lyophilization in the bacterial aggregation assay, indicating that the lyophilization did not substantially affect activity of formulations tested in this assay. In addition, the formulations had substantially similar potency and efficacy under all conditions tested, indicating that although stability of the dodecamer was affected in some formulations under different conditions, this did not appear to compromise activity in the bacterial aggregation assay. *See* TABLE 11.

Example 4—Activity of formulations containing rhSP-D in TLR4 assay

[0078] The Toll-like receptors (TLRs) have a role in both the innate immune system and the adaptive immune system, and SP-D has activity to modulate signaling through TLRs, such as Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4). *See e.g.*, Haagsman HP *et al.*, (2008) Neonatology 93:288-294; Yamazoe M. *et al.*, (2008) J. Biol Chem 283:35878-35888; and Vieira F. *et al.*, (2017) Ann Anat 211:184-201 which are each incorporated by reference in its entirety. TLR4 activity may also modulate the severity of conditions, such as bronchopulmonary dysplasia (BPD) (Malash AH *et al.*, (2016) Gene 592:23-28 which is incorporated by reference in its entirety). Thus, the activity of rhSP-D to modulate TLR4 activity was measured as an indication of the effect of rhSP-D on a host

immune response. The activity of reconstituted formulations containing rhSP-D was tested in an LPS-TLR4 assay. In an LPS-TLR4 assay, reconstituted rhSP-D can inhibit lipopolysaccharide (LPS)-induced inflammatory cell responses by preventing LPS from binding/activating the Toll-like receptor 4 (TLR4). *See e.g.*, Yamazoe M. *et al.*, (2008) J. Biol Chem 283:35878-35888 which is incorporated by reference in its entirety.

[0079] The activity of reconstituted rhSP-D in a TLR4 assay was tested in a method substantially similar to the following method. HEK-Blue™ hTLR4 cells (InvivoGen, San Diego, CA, U.S.A.) were plated at a density ~20000 cells/well in 384-well plates and incubated with various concentrations of rhSP-D for 2 hours at 37°C, 5% CO_2. LPS (Escherichia coli O26:B6, L5543 Sigma Aldrich) at an EC_{80} concentration was added to each well, and the cells incubated for another 22 hours at 37°C, 5% CO_2. TLR4 activity was measured by detaching the cells from the wells, washing the suspended cells, resuspending the cells in PBS and removing any clumps by gentle pipetting. Washed cells were transferred to a 384-well plate at a density of $20e10^3$ cells/well containing HEK blue detection medium (InvivoGen, San Diego, CA, U.S.A.) that had been made up in endotoxin-free water containing 5 mM $CaCl_2$ and 1% (v/v) BSA. Cells were incubated at 37°C in 5% CO_2 for 24 hours, and activity of TLR4 was determined by measuring the activity of a secreted embryonic alkaline phosphatase (SEAP) reporter gene using a spectrophotometer at 655nm. An IC_{50} value for the rhSP-D was determined using nonlinear regression analysis by fitting the data to the four-parameter logistics equation. Because only the logarithm of the IC_{50} values are normally distributed, for the purposes of averaging numbers from a series of experiments, the pIC_{50} values were used, defined as the $-Log_{10}$ (IC_{50}). A curve span was determined from the difference between the fitted maximal response (E_{max}) and the fitted minimal response (E_{min}) and corresponds to the amplitude of the dose-response curve, or efficacy of the response.

[0080] The results of TLR4 assays with reconstituted rhSP-D, along with results of a bacterial aggregation assay described in EXAMPLE 3 are summarized in TABLE 11. The data presented in TABLE 11 for the bacterial aggregation assay includes fitted curve parameters from a single dose-response curve, composed of triplicate determinations obtained from a 60-minute time-point in three independent experiments. The data presented for the TLR4 assay includes the average ± SD of fitted curve parameters obtained from the indicated

number of independent experiments, where each dose-response curve was composed of multiple concentrations performed in triplicate.

TABLE 11

Form[n]	Condition	Bacterial aggregation assay			TLR4 receptor antagonist assay		
		pEC_{50} (mg/ml)	Curve span ± SD of the estimate	N	Avg pIC_{50} ± SD (mg/ml)	Avg curve span ± SD	N
F01-01	post lyo, 0 weeks	3.864	53.88 ±2.50	1	2.6 ± 0.2	49.1 ± 19.8	3
	25°C, 2 weeks	3.797	57.97 ±1.67	1	NR	NR	3
	40°C, 2 weeks	3.539	50.61 ±3.60	1	NR	NR	3
	5°C, 5 weeks	NT	NT		3.0 ± 0.3	43.0 ± 3.2	3
F02-01	post lyo, 0 weeks	3.823	52.93 ±3.30	1	NR	NR	1
	25°C, 2 weeks	-	-	1	NR	NR	3
	40°C, 2 weeks	3.985	58.38 ±2.142	1	NR	NR	3
	5°C, 5 weeks	NT	NT		3.3 ± 0.2	32.6 ± 21.1	3
F03-01	post lyo, 0 weeks	3.971	50.57 ±3.89	1	2.1	28.3	1
	25°C, 2 weeks	4.067	43.70 ±2.494	1	2.5 ± 0.43	48.7 ± 10.4	3
	40°C, 2 weeks	4.176	65.06 ±3.44	1	2.7 ± 0.2	49.8 ± 10.1	3
	5°C, 5 weeks	NT	NT		3.3 ± 0.04	39.3 ± 15.3	3
F04-01	post lyo, 0 weeks	3.939	56.01 ±8.25	1	NT	NT	

Form[n]	Condition	Bacterial aggregation assay			TLR4 receptor antagonist assay		
		pEC_{50} (mg/ml)	Curve span ± SD of the estimate	N	Avg pIC_{50} ± SD (mg/ml)	Avg curve span ± SD	N
	25°C, 2 weeks	3.877	55.72 ±2.85	1	NT	NT	
	40°C, 2 weeks	3.931	58.65 ±5.47	1	NT	NT	
	5°C, 5 weeks	NT	NT		NT	NT	
F05-01	post lyo, 0 weeks	3.967	54.36 ±4.37	1	2.9 ± 0.5	49.0 ± 5.8	3
	25°C, 2 weeks	4.139	61.66 ±1.43	1	2.8 ± 0.1	41.0 ± 10.0	3
	40°C, 2 weeks	3.983	50.91 ±3.97	1	2.5 ± 0.04	40.9 ± 1.6	3
	5°C, 5 weeks	NT	NT		3.4 ± 0.4	40.4 ± 3.9	3
F06-01	post lyo, 0 weeks	4.018	57.14 ±3.56	1	NR	NR	3
	25°C, 2 weeks	3.796	44.58 ±1.68	1	NR	NR	3
	40°C, 2 weeks	3.853	60.91 ±2.28	1	NR	NR	2
	5°C, 5 weeks	NT	NT		NR	NR	3
F07-01	post lyo, 0 weeks	3.622	47.05 ±4.94	1	2.4 ± 0.2	44.8 ± 8.6	4
	25°C, 2 weeks	3.889	48.11 ±2.60	1	3.0 ± 0.7	34.1 ± 16.6	3
	40°C, 2 weeks	3.73	44.30 ±2.61	1	2.9 ± 0.7	30.1 ± 12.3	3
	5°C, 5 weeks	NT	NT		NR	NR	3

Form[n]	Condition	Bacterial aggregation assay			TLR4 receptor antagonist assay		
		pEC_{50} (mg/ml)	Curve span ± SD of the estimate	N	Avg pIC_{50} ± SD (mg/ml)	Avg curve span ± SD	N
NR: no response. NT: not tested.							

[0081] All tested formulations had activity in the bacterial aggregation assay. In contrast, only a subset of formulations treated under various conditions had activity in the TLR4 assay. For F01-01, F02-01, F06-01, no result in the TLR4 assay could be determined for reconstituted lyophiles after storage for 2 weeks at 40°C. For F01-01, F02-1 no response was obtained in the TLR4 assay after two weeks in storage at either 25°C or at 40°C. For F06-01, no response was obtained in the TLR4 assay in any condition. In contrast, formulations F03-01, F05-01, and F07-01 had activity in the TLR4 assay for reconstituted lyophiles after storage for 2 weeks at 40°C. Moreover, unlike formulation F07-01, formulations F03-01, F05-01 which each contained lactose and calcium also had activity in the TLR4 assay for reconstituted lyophiles after storage for 5 weeks at 5°C. Notably, as shown in Example 2, F03-01 and F05-01 were also the only formulations that maintained stability of the dodecamer and the aggregate under all conditions tested and exhibited relatively low turbidity upon reconstitution. Taken together, the data indicated that formulations that contained lactose and $CaCl_2$ displayed better ability to stabilize the SP-D oligomeric state and maintain activity than the formulations that contained NaCl. Therefore, formulations containing lactose and $CaCl_2$ were selected for further study.

Example 5—Multivariate analysis of rhSP-D oligomeric forms

[0082] A multivariate statistical analysis was performed to determine any correlation between peaks observed in fractograms in AF4-MALLS analyses for various formulations of rhSP-D, and activity of rhSP-D samples in either the bacterial aggregation assay or the TLR4 assay. Digitized fractograms obtained from more than 40 different samples were used as a data matrix, and correlations to the measured pIC_{50} values from the TLR4 assay, or pEC_{50} results from bacterial aggregation activity assays were determined using PLS.

[0083] A full cross validation was performed on all calibration models using standard techniques. *See e.g.*, Katz, M.H. "Multivariate Analysis: A Practice Guide for Clinicians." Cambridge University Press, New York, pp. 158-162 (1999); Stahle, L. *et al.*, (1988) "Multivariate data analysis and experimental design in biomedical research. Prog. Med. Chem. 25: 291-338; Wold S. (2001) "PLS-regression: a basic tool of chemometrics." Chemom. Intell. Lab. Syst. 58: 109-130 which are each incorporated by reference in its entirety. Briefly, one sample was removed at a time, the data set was recalibrated, and a new model was constructed. This process was repeated until all the calibration samples were removed once and quantified as a validation model. Therefore, the first set, containing all samples was referred to as the calibration set and the one after cross-validation as the validation set. The jack-knife algorithm was used to determine statistical significance for any factor used in constructing partial least square (PLS) models (Martens, H. *et al.*, (2001) "Multivariate Analysis of Quality: An Introduction" Wiley and Sons, Chichester, UK).

[0084] With regard to a correlation between particular peaks observed in the AF4-MALLS analysis and activity in the bacterial aggregation assay for various formulations of rhSP-D, the majority of the activity was found to reside in Peaks 1 and 2, with some persisting throughout Peak 3. A negative correlation was found between Peak 4 and activity in the bacterial aggregation assay. With regard to a correlation between particular peaks observed in the AF4-MALLS analysis and activity in the TLR4 assay for various formulations of rhSP-D, the activity was found to localize almost exclusively in the Peak 2. Thus, activity of rhSP-D in the TLR4 assay was directly related to dodecamer species of rhSP-D. Notably, the aggregate species with radius >70 nm in adjusted Peak 4 was not associated with activity in either the bacterial aggregation assay or the TLR4 assay, confirming that this species represented inactive forms of rhSP-D.

Example 6—Formulations containing rhSP-D, lactose and $CaCl_2$

[0085] A second generation of formulations listed in TABLE 12 were prepared from a single batch of rhSP-D by dialysis of the protein solution with the formulation buffer. Formulations were lyophilized by a method substantially similar to the method described above in EXAMPLE 1.

TABLE 12

Formulation	Components	Reconstitution media after lyophilization
F01-02	1 mg/ml rhSP-D, 5 mM Histidine, 265 mM Lactose, 1 mM $CaCl_2$, pH 6.0	Water
F02-02	2 mg/ml rhSP-D, 5 mM Histidine, 265 mM Lactose, 1 mM $CaCl_2$, pH 6.0	Water
F03-02	1 mg/ml rhSP-D, 5 mM Histidine, 265 mM Lactose, 1 mM $CaCl_2$, pH 6.0, + 0.01 % Tyloxapol	Water
F04-02	1 mg/ml rhSP-D, 5 mM Histidine, 100 mM Lactose, 1 mM $CaCl_2$, pH 6.0	0.9% NaCl
F05-02	2 mg/ml rhSP-D, 5 mM Histidine, 100 mM Lactose, 1 mM $CaCl_2$, pH 6.0	0.9% NaCl
F06-02	1 mg/ml rhSP-D, 5 mM Histidine, 100 mM Lactose, 1 mM $CaCl_2$, pH 6.0, + 0.01% Tyloxapol	0.9% NaCl
F07-02	1 mg/ml rhSP-D, 5 mM Histidine, 265 mM Lactose, 5 mM $CaCl_2$, pH 6.0	Water
F08-02	1 mg/ml rhSP-D, 5 mM Histidine, 100 mM Lactose, 5 mM $CaCl_2$, pH 6.0	0.9% NaCl
F09-02	1 mg/ml rhSP-D, 5 mM Histidine, 265 mM Lactose, 1 mM $CaCl_2$, pH 7.0	Water
F10-02	1 mg/ml rhSP-D, 5 mM Histidine, 100 mM Lactose, 1 mM $CaCl_2$, pH 7.0	0.9% NaCl
F11-02	1 mg/ml rhSP-D, 5 mM Histidine, 50 mM NaCl, 170 mM Lactose, 1 mM $CaCl_2$, pH 6.0	Water
F12-02	1 mg/ml rhSP-D, 5 mM Histidine, 50 mM NaCl, 170 mM Lactose, 5 mM $CaCl_2$, pH 6.0	Water

[0086] Lyophiles were subjected to various conditions, reconstituted, and the activities of the reconstituted lyophiles were examined in the bacterial aggregation assay. The various formulations of rhSP-D were active in bacterial aggregation assay under the tested conditions for the various conditions. Example results for activities of F02-02 and F07-02 in the bacterial aggregation assay are summarized in TABLE 13.

TABLE 13

Form[n]	Condition	Bacterial aggregation assay		
		pEC_{50} (mg/ml)	**Curve span ± SD of the estimate**	**N**
F02-02	post lyo, 0 weeks	4.161	49.86±2.31	1
	40°C, 3 weeks	4.199	53.78±2.75	1
	25°C, 5 weeks	4.168	58.33±2.49	1
	5°C, 7.5 weeks	4.122	53.13±2.91	1
F07-02	post lyo, 0 weeks	3.870	58.53±2.62	1
	40°C, 3 weeks	3.900	51.3±1.66	1
	25°C, 5 weeks	4.033	54.43±4.22	1
	5°C, 7.5 weeks	4.049	50.54±3.70	1
	25°C, 10 weeks	3.809	38.36±3.91	1

[0087] The activity of formulations under various conditions in the TLR4 assay was determined by a method substantially similar as described herein. The results are summarized in TABLE 14.

[0088] The distribution of rhSP-D oligomeric species in reconstituted lyophiles was also examined by an AF4-MALLS analysis using a method substantially similar to the method described herein. As well as summarizing the results of the activity of formulations in the TLR4 assay, TABLE 14 also summarizes the relative peak area (RPA) for Peak 2 which corresponds to the relative distribution of rhSP-D dodecamers; and an adjusted RPA for Peak 4 which corresponds to the relative distribution of rhSP-D having a radius greater than 70 nm.

TABLE 14

Form n	Condition	TLR4 assay			AF4-MALLS RPA (%)	
		Avg pIC_{50} ± SD (mg/ml)	Avg curve span ± SD	N	Peak 2	Adjusted Peak 4
F01-02	0 weeks	2.4 ± 0.01	39.0 ± 22.5	2	41.79	1.26
	40°C, 3 weeks	2.6 ± 0.1	31.3 ± 12.6	4	41.93	1.79
	25°C, 5 weeks	2.6 ± 0.2	32.4 ± 15.3	4	39.83	3.51
	5°C, 7.5 weeks	2.5 ± 0.3	23.8 ± 1.7	3	45.77	2.68
	25°C, 10 weeks	2.8 ± 0.3	35.6 ± 17.0	3	38.59	3.25
F02-02	0 weeks	2.4 ± 0.2	43.0 ± 20.9	3	49.85	1.21
	40°C, 3 weeks	2.4 ± 0.1	52.7 ± 30.2	4	49.61	3.12
	25°C, 5 weeks	2.1 ± 0.2	27.7 ± 7.6	3	40.66	2.43
	5°C, 7.5 weeks	1.8 ± 0.1	29.0 ± 14.9	4	48.64	1.42
	25°C, 10 weeks	NT	NT			
F04-02	0 weeks	Activity not reproducible	Activity not reproducible	3	56.25	0.98
	40°C, 3 weeks	2.4 ± 0.8	24.3 ± 9.1	3	47.06	2.25
	25°C, 5 weeks	2.6 ± 0.2	41.0 ± 23.0	3	42.13	4.03
	5°C, 7.5 weeks	2.5 ± 0.3	36.7 ± 5.0	3	44.63	2.62
	25°C, 10 weeks	2.4 ± 0.2	33.3 ± 18.3	3	38.52	1.42

Form n	Condition	TLR4 assay			AF4-MALLS RPA (%)	
		Avg pIC_{50} ± SD (mg/ml)	Avg curve span ± SD	N	Peak 2	Adjusted Peak 4
F05-02	0 weeks	2.5 ± 0.8	68.0 ± 10.3	3	55.76	1.47
	40°C, 3 weeks	2.1 ± 0.4	28.8 ± 8.6	3	45.86	2.46
	25°C, 5 weeks	2.4 ± 0.1	34.5 ± 11.2	4	41.18	3.57
	5°C, 7.5 weeks	2.2 ± 0.4	25.3 ± 8.8	3	45.98	1.62
	25°C, 10 weeks	1.8 ± 0.4	22.7 ± 1.0	3	41.81	0.98
F07-02	0 weeks	2.7 ± 0.2	55.8 ± 10.5	3	44.10	1.21
	40°C, 3 weeks	3.0 ± 0.1	33.5 ± 8.1	4	49.30	0.73
	25°C, 5 weeks	2.4 ± 0.1	31.8 ± 15.4	3	43.42	1.61
	5°C, 7.5 weeks	2.0 ± 0.3	39.8 ± 17.3	4	49.52	1.45
	25°C, 10 weeks	2.2 ± 0.4	26.6 ± 10.4	4	45.49	2.55
F08-02	0 weeks	Activity not reproducible	Activity not reproducible	4	43.52	1.39
	40°C, 3 weeks	2.6 ± 0.5	28.6 ± 11.7	5		
	25°C, 5 weeks	2.6 ± 0.1	31.3 ± 19.4	4	40.35	2.29
	5°C, 7.5 weeks	2.6 ± 0.1	28.7 ± 3.1	3	46.53	2.16
	25°C, 10 weeks	2.0 ± 0.04	24.3 ± 9.6	3	38.82	2.19
	0 weeks	2.6 ± 0.1	65.2 ± 36.3	3	50.23	1.56

Form n	Condition	TLR4 assay			AF4-MALLS RPA (%)	
		Avg pIC_{50} ± SD (mg/ml)	Avg curve span ± SD	N	Peak 2	Adjusted Peak 4
F09-02	40°C, 3 weeks	2.7 ± 0.1	25.0 ± 9.6	4	45.52	1.70
	25°C, 5 weeks	2.5 ± 0.2	33.0± 13.0	4	42.36	2.28
	5°C, 7.5 weeks	2.7 ± 0.2	24.2 ± 10.1	3	45.48	1.34
	25°C, 10 weeks	No activity	No activity	3	39.93	2.37
F10-02	0 weeks	Activity not reproducible	Activity not reproducible	4	38.87	1.72
	40°C, 3 weeks	2.5 ± 0.2	38.0 ± 19.3	4	44.69	
	25°C, 5 weeks	2.7 ± 0.2	34.0 ± 9.9	4	41.68	1.87
	5°C, 7.5 weeks	2.4 ± 0.2	23.1 ± 3.2	3	43.14	2.06
	25°C, 10 weeks	No activity	No activity	3	44.05	2.22
F11-02	0 weeks	2.5 ± 0.60	56.3 ± 26.0	3	46.13	1.46
	40°C, 3 weeks	2.9 ± 0.3	38.4 ± 17.8	4	34.19	7.05
	25°C, 5 weeks	2.2 ± 0.1	31.3 ± 9.9	4	39.21	1.94
	5°C, 7.5 weeks	2.5 ± 0.2	25.4 ± 7.6	3	45.20	3.14
	25°C, 10 weeks	No activity	No activity	3	43.41	2.12
F12-02	0 weeks	2.8 ± 0.2	59.6 ± 11.9	2	46.74	1.58
	40°C,	NT	NT			

Form n	Condition	TLR4 assay			AF4-MALLS RPA (%)	
		Avg pIC_{50} ± SD (mg/ml)	Avg curve span ± SD	N	Peak 2	Adjusted Peak 4
	3 weeks					
	25°C, 5 weeks	2.4 ± 0.2	42.8 ± 23.6	4	37.75	1.50
	5°C, 7.5 weeks	NT	NT			
	25°C, 10 weeks	NT	NT			
NT: not tested						

[0089] Activity in the TLR4 assay could not be determined for formulations F03-02 and F06-02 which each contain tyloxapol because the formulation buffer itself exhibited antagonistic effects in the TLR4 assay which is a readout for the TLR4 assay. Formulations F09-02, F10-02, and F11-02 each lost activity in the TLR4 assay after the lyophile was stored for 10 weeks at 25°C. Formulations F10-02, F11-02 and F12-02 included the lowest observed RPAs for Peak 2. Formulation F07-02 consistently had the highest RPA for Peak 2 with the lowest RPA for the Aggregate in Peak 4 for all various conditions studied.

[0090] Lyophiles of formulations F01-02, F02-02, F07-02, that were reconstituted in water were consistently active in the TLR4 assay under various storage conditions. These formulations also maintained the stability of the dodecamer for 72 hours at 2-8°C upon reconstitution (TABLE 15). However, the levels of dodecamer (Peak 2) in the lyophile were higher over time for formulations F02-02 and F07-02 and the levels of inactive aggregate in the reconstituted solution were lower for F02-02 and F07-02 than for F01-02 after storage at 2-8°C for 72 hours (TABLE 15).

[0091] Of all of the formulations evaluated, only F02-02 and F07-02 exhibited the characteristics of consistent activity in the TLR4 assay after reconstitution, consistently higher values for a Peak 2 RPA associated with rhSP-D dodecamers, and lower values for an adjusted Peak 4 RPA associated with rhSP-D aggregates having a radius greater than 70 nm throughout

the 10 week time period, regardless of storage temperature of the lyophile. Together, the data implied that stability and activity of rhSP-D was maintained in a formulation in Histidine buffer at pH 6, with higher levels of lactose, containing at least 1 mM $CaCl_2$ in the absence of added NaCl, and with the lyophile being reconstituted in water.

[0092] A second AF4-MALLS analysis was performed to investigate the stability of reconstituted formulations. Lyophiles were subjected to various conditions, reconstituted, stored for 72 hours at 2-8 °C, and then the distribution of rhSP-D oligomeric species in reconstituted lyophiles was examined by an AF4-MALLS analysis using a method substantially similar to the method described above. TABLE 15 lists the various conditions under which the lyophiles were subjected, and lists an RPA for Peak 2 and an adjusted RPA for Peak 4 which correspond to the relative distribution of rhSP-D dodecamers and rhSP-D aggregates having a radius greater than 70 nm, respectively.

[0093] A comparison of the RPA for Peak 2 in reconstituted samples after storage for 72 hours at 2-8°C (TABLE 15) with the RPA for Peak 2 shortly after reconstitution (TABLE 14) revealed that the lyophile formulation and the reconstitution solution affected dodecamer stability. Lyophiles of formulations F04-02, F05-02, F08-02, and F10-02 that had been stored for 5 weeks at 25°C, reconstituted in saline and stored for 72 hours at 2-8 °C all exhibited a >5% increase in the RPAs for Peak 2 in comparison to the RPAs for Peak 2 that had been stored for 5 weeks at 25°C, reconstituted in saline and analyzed shortly thereafter. For the lyophiles that had been stored for 7½ weeks at 5°C, these same formulations had decreased RPAs for Peak 2 in comparison for the RPAs for Peak 2 obtained shortly after reconstitution. However, lyophiles of formulations F02-02 and F07-02 that had been stored for either 5 weeks at 25°C or 7½ weeks at 5°C, reconstituted in water and stored for 72 hours at 2-8°C, had substantially stable RPAs for Peak 2, with changes in RPA < 4%. Therefore, reconstitution in saline reduced stability of rhSP-D as demonstrated in an AF4-MALLS analysis.

TABLE 15

Formulation	Condition	AF4-MALLS RPA (%)	
		Peak 2	Adjusted Peak 4
F01-02	0 weeks	N/A	N/A
	lyophilized: 5 weeks, 25°C; then reconstituted: 72 hours, 2-8 °C	42.43	1.91
	lyophilized: 7½ weeks, 5°C; then reconstituted: 72 hours, 2-8 °C	42.75	3.08
F02-02	0 weeks	49.61	N/A
	lyophilized: 5 weeks, 25°C; then reconstituted: 72 hours, 2-8 °C	44.57	1.24
	lyophilized: 7½ weeks, 5°C; then reconstituted: 72 hours, 2-8 °C	45.92	1.93
F03-02	0 weeks	N/A	N/A
	lyophilized: 5 weeks, 25°C; then reconstituted: 72 hours, 2-8 °C	46.13	N/A
	lyophilized: 7½ weeks, 5°C; then reconstituted: 72 hours, 2-8 °C	N/A	2.06
F04-02	0 weeks	47.71	N/A
	lyophilized: 5 weeks, 25°C; then reconstituted: 72 hours, 2-8 °C	51.31	0.64
	lyophilized: 7½ weeks, 5°C; then reconstituted: 72 hours, 2-8 °C	37.44	1.97
F05-02	0 weeks	55.21	N/A
	lyophilized: 5 weeks, 25°C; then reconstituted: 72 hours, 2-8 °C	48.85	0.48
	lyophilized: 7½ weeks, 5°C; then reconstituted: 72 hours, 2-8 °C	N/A	N/A
F06-02	0 weeks	N/A	N/A
	lyophilized: 5 weeks, 25°C; then reconstituted: 72 hours, 2-8 °C	37.89	0.19

Formulation	Condition	AF4-MALLS RPA (%)	
		Peak 2	Adjusted Peak 4
	lyophilized: 7½ weeks, 5°C; then reconstituted: 72 hours, 2-8 °C	N/A	N/A
F07-02	0 weeks	35.26	N/A
	lyophilized: 5 weeks, 25°C; then reconstituted: 72 hours, 2-8 °C	41.78	3.18
	lyophilized: 7½ weeks, 5°C; then reconstituted: 72 hours, 2-8 °C	49.64	1.93
F08-02	0 weeks	36.98	N/A
	lyophilized: 5 weeks, 25°C; then reconstituted: 72 hours, 2-8 °C	53.79	0.89
	lyophilized: 7½ weeks, 5°C; then reconstituted: 72 hours, 2-8 °C	39.52	2.79
F09-02	0 weeks	49.06	N/A
	lyophilized: 5 weeks, 25°C; then reconstituted: 72 hours, 2-8 °C	49.04	0.63
	lyophilized: 7½ weeks, 5°C; then reconstituted: 72 hours, 2-8 °C	40.99	1.84
F10-02	0 weeks	30.80	N/A
	lyophilized: 5 weeks, 25°C; then reconstituted: 72 hours, 2-8 °C	46.88	1.02
	lyophilized: 7½ weeks, 5°C; then reconstituted: 72 hours, 2-8 °C	39.86	1.94
F11-02	0 weeks	49.64	N/A
	lyophilized: 5 weeks, 25°C; then reconstituted: 72 hours, 2-8 °C	40.70	N/A
	lyophilized: 7½ weeks, 5°C; then reconstituted: 72 hours, 2-8 °C	38.31	N/A
F12-02	0 weeks	42.04	N/A

Formulation	Condition	AF4-MALLS RPA (%)	
		Peak 2	Adjusted Peak 4
	lyophilized: 5 weeks, 25°C; then reconstituted: 72 hours, 2-8 °C	37.58	N/A
	lyophilized: 7½ weeks, 5°C; then reconstituted: 72 hours, 2-8 °C	N/A	N/A
N/A: not available			

Example 7—Stability of lyophilized formulations containing rhSP-D, lactose and $CaCl_2$

[0094] Formulations of rhSP-D containing either 2 mg/ml or 4 mg/ml rhSP-D, in 5 mM Histidine, 265 mM Lactose, and 5 mM $CaCl_2$, pH 6.0 were prepared from a single batch of rhSP-D by dialysis of the protein solution with buffer. Formulations were lyophilized by a method substantially similar to the method described above in EXAMPLE 1. Lyophiles were subjected to various conditions, reconstituted in water, and analyzed. The activity of reconstituted lyophiles was measured using the bacterial aggregation assay, and the TLR4 assay. The distribution of rhSP-D oligomeric species in reconstituted lyophiles was also examined by an AF4-MALLS analysis using a method substantially similar to the method described herein.

Six-month study (2 mg/ml rhSP-D)

[0095] A six-month study was performed with 2 mg/ml rhSP-D, in 5 mM Histidine, 265 mM Lactose, and 5 mM $CaCl_2$, pH 6.0. Example results for activities of reconstituted formulations in the bacterial aggregation assay are summarized in TABLE 14. Example results for activities of reconstituted formulations in the TLR4 assay are summarized in TABLE 15. Example results for the distribution of oligomeric species in reconstituted lyophiles prepared from a different batch of rhSP-D than the batch used in the bacterial aggregation assay and TLR4 assay of TABLE 14 and TABLE 15, are depicted in FIG. 2. The 3 month sample at 25°C, was held at 45°C for 1 week. In this study, oligomeric species showed a tendency to interconvert between Peaks 2 and 3, and between Peaks 1 and 2, during the first 3 months. After 3 months, the aggregation Peak 4 increased significantly. Samples held at 25°C or 5°C had substantially similar activities and distribution of oligomeric species. As shown in TABLE

15, the antagonist potency in the TLR4 assay at 3 and 6 months for this batch of rhSP-D was similar to that at t0, and within the error of the assay (data not shown). Together the data from both SP-D batches indicate that samples held for 6 months at 25°C did not perform substantially worse in the activity assays than those held for 6 months at 5°C, and that potency in the activity assays was maintained.

TABLE 14

Conditions	**Bacterial aggregation assay**			
	Avg pEC_{50} ± SD	**EC_{50} (mg/ml)**	**Avg E_{max} ± SD (Curve Span)**	**N**
2 mg/ml; pre-lyophilization	2.94	0.00115	36.98±5.142	1
2 mg/ml; 0 months post-lyophilization	2.95	0.0013	48.23±3.79	1
2 mg/ml; 3 months post-lyophilization at 5°C	3.13	0.00073	57.66±2.97	1
2 mg/ml; 3 months post-lyophilization at 25°C	3.08	0.00083	43.19±2.09	1
2 mg/ml; 6 months post-lyophilization at 5°C	2.73	0.0018	55.77±5.944	1
2 mg/ml; 6 months post-lyophilization at 25°C	2.19	0.0064	43.39±8.391	1

TABLE 15

Conditions	**TLR4 assay**			
	Avg pIC_{50} ± SD	**IC_{50} (mg/ml)**	**Avg E_{max} ± SD (Curve Span)**	**N**
2 mg/ml; pre-lyophilization	2.67 ± 0.16	0.0022	75.74 ± 50.09	3
2 mg/ml; 0 months post-lyophilization	2.57 ± 0.11	0.0028	68.65 ± 42.15	3
2 mg/ml; 3 months post-lyophilization at 5°C	2.24±0.09	0.0059	36.49±13	5
2 mg/ml; 3 months post-lyophilization at 25°C	2.14±0.11	0.0074	34.62±10.90	3

Conditions	TLR4 assay			
	Avg pIC_{50} ± SD	**IC_{50} (mg/ml)**	**Avg E_{max} ± SD (Curve Span)**	**N**
2 mg/ml; 6 months post-lyophilization at 5°C	2.71±0.26	0.0022	22.97±5.83	3
2 mg/ml; 6 months post-lyophilization at 25°C	2.76±0.09	0.0018	24.20±2.33	3

Three-month study (2 mg/ml and 4 mg/ml rhSP-D)

[0096] A three-month study was performed with 2 mg/ml rhSP-D or 4 mg/ml, in 5 mM Histidine, 265 mM Lactose, and 5 mM CaCl2, pH 6.0. Example results for activities of reconstituted formulations in the bacterial aggregation assay are summarized in TABLE 16. Example results for activities of reconstituted formulations in the TLR4 assay are summarized in TABLE 17. Example results for the distribution of oligomeric species in reconstituted lyophiles are depicted in FIG. 3 (upper panel: 2 mg/ml; lower panel: 4 mg/ml). In this study, samples held at 25°C or at 5°C had substantially similar activities and distribution of oligomeric species. Substantially no interconversion to lower order (Peak 1) species was observed. At three months, Peak 2 species tended to interconvert to Peak 3 species. Activity was maintained in both the bacterial aggregation assay and the TLR4 assay, as evidenced by similar potency values (within the error of the assays) over the entire 3 month period, regardless of storage temperature.

TABLE 16

Conditions	Bacterial aggregation assay			
	Avg pEC_{50} ± SD	**EC_{50} (mg/ml)**	**Avg E_{max} ± SD (Curve Span)**	**N**
4 mg/ml; pre-lyophilization	2.96	0.0011	39.44±5.05	1
4 mg/ml; 0 months post-lyophilization	3.05	0.0009	47.37±3.65	1
4 mg/ml; 1 month post-lyophilization at 5°C	3.08	0.00082	49.43±2.54	1
4 mg/ml; 1 month post-lyophilization at 25°C	3.23	0.00059	40.03±2.74	1

Conditions	Bacterial aggregation assay			
	Avg pEC_{50} ± SD	EC_{50} (mg/ml)	Avg E_{max} ± SD (Curve Span)	N
4 mg/ml; 3 month post-lyophilization at 5°C	2.36	0.00437	45.76±5.11	1
4 mg/ml; 3 month post-lyophilization at 25°C	2.26	0.00550	51.96±10.72	1
2 mg/ml; pre-lyophilization	2.84	0.0014	49.19±3.57	1
2 mg/ml; 0 months post-lyophilization	3.08	0.00083	50.83±3.38	1
2 mg/ml; 1 month post-lyophilization at 5°C	3.07	0.00085	50.51±2.92	1
2 mg/ml; 1 month post-lyophilization at 25°C	3.02	0.00095	46.27±3.55	1
2 mg/ml; 3 month post-lyophilization at 5°C	2.95	0.00111	50.8±3.668	1
2 mg/ml; 3 month post-lyophilization at 25°C	2.88	0.001324	45.08±4.048	1

TABLE 17

Conditions	TLR4 assay			
	Avg pIC_{50} ± SD	IC_{50} (mg/ml)	Avg E_{max} ± SD (Curve Span)	N
4 mg/ml; pre-lyophilization	1.59±0.09	0.0263	29.89±11.18	3
4 mg/ml; 0 months post-lyophilization	1.45±0.13	0.0368	26.99±10.22	3
4 mg/ml; 1 month post-lyophilization at 5°C	2.00±0.17	0.0105	45.10±24.65	4
4 mg/ml; 1 month post-lyophilization at 25°C	2.11±0.11	0.0079	45.98±16.92	4
4 mg/ml; 3 month post-lyophilization at 5°C	2.12±0.13	0.0079	19.80±0.87	3

Conditions	TLR4 assay			
	Avg pIC_{50} ± SD	**IC_{50} (mg/ml)**	**Avg E_{max} ± SD (Curve Span)**	**N**
4 mg/ml; 3 month post-lyophilization at 25°C	2.24±0.26	0.0058	19.45±2.46	3
2 mg/ml; pre-lyophilization	no response			
2 mg/ml; 0 months post-lyophilization	no response			
2 mg/ml; 1 month post-lyophilization at 5°C	2.55±0.12	0.0029	26.12±8.46	3
2 mg/ml; 1 month post-lyophilization at 25°C	2.44±0.23	0.0040	29.93±6.34	3
2 mg/ml; 3 month post-lyophilization at 5°C	2.63±0.23	0.0026	22.94±0.23	3
2 mg/ml; 3 month post-lyophilization at 25°C	2.47±0.20	0.0037	20.36±6.27	3

Example 8—Stability of formulations during freeze/thaw cycles

[0097] Formulations of rhSP-D containing either 2 mg/ml or 4 mg/ml rhSP-D, in 5 mM Histidine, 265 mM Lactose, and 5 mM $CaCl_2$, pH 6.0 were prepared. Samples were aliquoted and frozen by either flash freezing in which samples were submerged into liquid nitrogen, or by placing the sample into freezer at -80°C. A freeze/thaw cycle included freezing the aliquot and thawing at room temp. Samples were analyzed over several cycles including: t0; 1X; 3X; and 5X.

[0098] Example results for the distribution of oligomeric species in reconstituted lyophiles are depicted in FIG. 4 (upper panel: 2 mg/ml; lower panel: 4 mg/ml). Example results for activities of samples in the bacterial aggregation assay, or the TLR4 assay are summarized in TABLE 18 and TABLE 19, respectively. In this study, there were no significant differences between 2 mg/ml and 4 mg/ml samples. Freezing the samples at -80°C with five cycles, increased the Peak 4 aggregate for both 2 mg/ml and 4 mg/ml samples, compared to flash freezing with 5 cycles. Thus, the best freezing condition was flash freezing.

TABLE 18

Conditions	Bacterial aggregation assay			
	Avg pEC_{50} ± SD	EC_{50} (mg/ml)	Avg E_{max} ± SD (Curve Span)	N
2 mg/mL, FT t0	3.9	0.000125	36.95±7.411	1
4 mg/mL, FT t0	4.06	0.000087	50.48±2.752	1
2 mg/mL, 1X F/T -80˚C				
4 mg/mL, 1X F/T -80˚C	3.97	0.0001071	43.65±2.72	1
2 mg/mL, 1X F/T Flash				
4 mg/mL, 1X F/T Flash	4.0537	0.00008837	43.56±3.155	1
2 mg/mL, 3X F/T -80˚C				
4 mg/mL, 3X F/T -80˚C				
2 mg/mL, 3X F/T Flash	3.8752	0.0001333	44.08±2.165	1
4 mg/mL, 3X F/T Flash	4.1147	0.00007679	40.21±3.277	1
2 mg/mL, 5X F/T -80˚C				
4 mg/mL, 5X F/T -80˚C				
2 mg/mL, 5X F/T Flash				
4 mg/mL, 5X F/T Flash				

TABLE 19

Conditions	TLR4 assay			
	Avg pIC_{50} ± SD	IC_{50} (mg/ml)	Avg E_{max} ± SD (Curve Span)	N
2 mg/mL, FT t0	2.79±0.16	0.0017	21.71±6.90	3
4 mg/mL, FT t0	2.64±0.16	0.0024	22.26±3.46	3
2 mg/mL, 1X F/T -80˚C	2.45±0.22	0.0037	22.11±0.28	2
4 mg/mL, 1X F/T -80˚C	2.08±0.02	0.0084	16.01±2.80	2
2 mg/mL, 1X F/T Flash	2.40±0.08	0.0041	20.95±0.05	2
4 mg/mL, 1X F/T Flash	2.68±0.15	0.0022	22.41±2.13	2

Conditions	TLR4 assay			
	Avg pIC_{50} ± SD	IC_{50} (mg/ml)	Avg E_{max} ± SD (Curve Span)	N
2 mg/mL, 3X F/T -80˚C	2.42±0.09	0.0038	18.91±1.78	2
4 mg/mL, 3X F/T -80˚C	2.34±0.51	0.0064	22.11±8.36	3
2 mg/mL, 3X F/T Flash	no response	no response	no response	5
4 mg/mL, 3X F/T Flash	no response	no response	no response	5
2 mg/mL, 5X F/T -80˚C	2.23±0.06	0.006	19.23±1.79	4
4 mg/mL, 5X F/T -80˚C	2.06±0.19	0.0095	23.18±4.91	4
2 mg/mL, 5X F/T Flash	2.19±0.07	0.0065	22.66±7.46	3
4 mg/mL, 5X F/T Flash	2.12±0.14	0.0079	22.05±5.21	4

Example 9—Stability of reconstituted formulations

[0099] Formulations of rhSP-D containing rhSP-D, 5 mM Histidine, 265 mM Lactose, and 5 mM $CaCl_2$, pH 6.0 were prepared. Solutions were lyophilized and reconstituted at either 2 mg/ml or 4 mg/ml rhSP-D, then held at t0; at 2-8°C for 1 or 3 days; or at 25°C for 1 or 3 days.

[0100] Example results for the distribution of oligomeric species in reconstituted lyophiles are depicted in FIG. 5 (upper panel: 2 mg/ml; lower panel: 4 mg/ml). Example results for activities of samples in the TLR4 assay are summarized in TABLE 20. In this study, the distribution of oligomeric species was substantially stable for the various conditions for 2 mg/ml and 4 mg/ml formulations. There was no substantial difference between the distribution of oligomeric species for formulations held at either 5˚C and 25˚C for 3 days. Thus, reconstituted rhSP-D was stable at either 5˚C and 25˚C for 3 days. TABLE 20 shows that the potency of the rhSP-D in the reconstituted samples was not changed when the reconstituted sample was held for up to 3 days at either 4°C or 25°C, providing additional evidence that the physical stability of the reconstituted formulation translated to retention of biological activity.

TABLE 20

Conditions	Avg pIC_{50} ± SD	IC_{50} (mg/ml)	Avg E_{max} ± SD (Curve Span)	N
2 mg/mL, t0	2.50±0.13	0.0033	27.40±7.76	4
4 mg/mL, t0	2.47±0.03	0.0034	25.07±4.80	4
2 mg/mL, held at 4°C for 1 day	2.51±0.07	0.0031	23.00±2.51	3
2 mg/mL, held at 25°C for 1 day	2.64±0.04	0.0023	22.20±2.36	3
4 mg/mL, held at 4°C for 1 day	2.56±0.06	0.0028	22.59±3.22	4
4 mg/mL, held at 25°C for 1 day	2.46±0.18	0.0036	27.91±5.15	4
2 mg/mL, held at 4°C for 3 days	2.47±0.05	0.0034	27.02±6.99	4
2 mg/mL, held at 25°C for 3 days	2.42±0.03	0.0038	27.55±8.74	3
4 mg/mL, held at 4°C for 3 days	2.57±0.07	0.0027	35.29±1.35	4
4 mg/mL, held at 25°C for 3 days	2.39±0.05	0.0041	22.74±2.88	4

Example 10—Chemical stability of lyophilized formulations

[0101] An RP HPLC assay was used to evaluate the stability of lyophilized SP-D samples. Twelve different formulations were prepared. The RP HPLC assay included the use of an Agilent PLRP column, 300 Å, 3 µm, 2.1 x 150 mm (PL1912-3301). The first gradient (Gradient 1) went from a fairly shallow gradient (1.7% B per min to 6% B per minute after six minutes.The result was detection of a main peak, as well minor peaks eluting both before and after the main peak for a reduced sample.

[0102] All of the samples displayed purities near 95% at t0 when analyzing reduced samples (TABLE 21). After five weeks of storage 25° C, there was no substantial change in the RP HPLC profiles in most of the sample; F11-02 and F12-02 which both contained NaCl showed the largest changes (TABLE 22). TABLE 21 lists relative areas of the peaks in the RP HPLC chormatograms of reduced SP-D samples for formulations at t0. TABLE 22 lists relative areas of peaks in RP HPLC chormatograms of reduced SP-D samples for formulations at t5 (five weeks at 25°C).

TABLE 21

Formulation	Main Peak	Pre-Peak	Post-Peaks
F01-02	95.4 %	1.2 %	3.4 %
F02-02	95.8 %	0.3 %	3.9 %
F03-02	95.0 %	1.0 %	3.9 %
F04-02	95.7 %	0.7 %	3.6 %
F05-02	96.2 %	0.3 %	3.5 %
F06-02	94.7 %	0.4 %	4.9 %
F07-02	95.9 %	1.0 %	3.2 %
F08-02	95.5 %	0.8 %	3.7 %
F09-02	95.3 %	1.1 %	3.6 %
F10-02	95.7 %	1.3 %	3.0 %
F11-02	96.5 %	0.4 %	3.1 %
F12-02	96.5 %	0.7 %	2.9 %

TABLE 22

Formulation	Main Peak	Pre-Peak	Post-Peaks
F01-02	95.3 %	1.3 %	3.5 %
F02-02	95.6 %	0.4 %	4.1 %
F03-02	95.4 %	0.7 %	3.9 %
F04-02	95.6 %	0.9 %	3.6 %
F05-02	95.8 %	0.5 %	3.8 %
F06-02	95.9 %	0.4 %	3.6 %
F07-02	95.9 %	0.9 %	3.2 %
F08-02	96.0 %	0.9 %	3.2 %
F09-02	95.3 %	1.1 %	3.7 %
F10-02	95.3 %	1.0 %	3.7 %
F11-02	96.0 %	0.8 %	3.2 %

Formulation	Main Peak	Pre-Peak	Post-Peaks
F12-02	95.2 %	1.0 %	3.8 %

[0103] The term "comprising" as used herein is synonymous with "including," "containing," or "characterized by," and is inclusive or open-ended and does not exclude additional, unrecited elements or method steps.

[0104] The above description discloses several methods and materials of the present invention. This invention is susceptible to modifications in the methods and materials, as well as alterations in the fabrication methods and equipment. Such modifications will become apparent to those skilled in the art from a consideration of this disclosure or practice of the invention disclosed herein. Consequently, it is not intended that this invention be limited to the specific embodiments disclosed herein, but that it cover all modifications and alternatives coming within the true scope and spirit of the invention.

[0105] All references cited herein, including but not limited to published and unpublished applications, patents, and literature references, are incorporated herein by reference in their entirety and are hereby made a part of this specification. To the extent publications and patents or patent applications incorporated by reference contradict the disclosure contained in the specification, the specification is intended to supersede and/or take precedence over any such contradictory material.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising recombinant human surfactant protein D (rhSP-D) or an active fragment thereof, a buffer, a sugar, and a calcium salt.

2. The pharmaceutical composition of claim 1, wherein the buffer is selected from the group consisting of acetate, citrate, glutamate, histidine, succinate, and phosphate.

3. The pharmaceutical composition of claim 1 or 2, wherein the buffer is histidine.

4. The pharmaceutical composition of claim 3, wherein the concentration of the histidine is from about 0.1 mM to about 100 mM.

5. The pharmaceutical composition of claim 4, wherein the concentration of the histidine is from about 1 mM to about 10 mM.

6. The pharmaceutical composition of claim 5, wherein the concentration of the histidine is from about 3 mM to about 7 mM.

7. The pharmaceutical composition of claim 6, wherein the concentration of the histidine is about 5 mM.

8. The pharmaceutical composition of any one of claims 1-7, wherein the sugar is selected from the group consisting of sucrose, maltose, lactose, glucose, fructose, galactose, mannose, arabinose, xylose, ribose, rhamnose, trehalose, sorbose, melezitose, raffinose, thioglucose, thiomannose, thiofructose, octa-O-acetyl-thiotrehalose, thiosucrose, and thiomaltose.

9. The pharmaceutical composition of claim 8, wherein the sugar is lactose.

10. The pharmaceutical composition of claim 9, wherein the concentration of the lactose is from 1 mM to 500 mM.

11. The pharmaceutical composition of claim 10, wherein the concentration of the lactose is from 200 mM to 300 mM.

12. The pharmaceutical composition of claim 11, wherein the concentration of the lactose is about 265 mM.

13. The pharmaceutical composition of any one of claims 1-12, wherein the calcium salt is selected from the group consisting calcium chloride, calcium bromide, calcium acetate, calcium sulfate, and calcium citrate.

14. The pharmaceutical composition of claim 13, wherein the calcium salt is calcium chloride.

15. The pharmaceutical composition of claim 14, wherein the concentration of the calcium chloride is from about 0.1 mM to about 10 mM.

16. The pharmaceutical composition of claim 15, wherein the concentration of the calcium chloride is from about 1 mM to about 10 mM.

17. The pharmaceutical composition of claim 16, wherein the concentration of the calcium chloride is from about 3 mM to about 7 mM.

18. The pharmaceutical composition of claim 17, wherein the concentration of the calcium chloride is about 5 mM.

19. The pharmaceutical composition of any one of claims 1-18, having a pH from about 4.0 to about 9.0.

20. The pharmaceutical composition of claim 19, having a pH from about 5.0 to about 7.0.

21. The pharmaceutical composition of claim 20, having a pH from about 5.5 to about 6.5.

22. The pharmaceutical composition of claim 21, having a pH about 6.0.

23. The pharmaceutical composition of any one of claims 1-22, wherein the concentration of the rhSP-D is from about 0.1 mg/ml to about 10 mg/ml.

24. The pharmaceutical composition of claim 23, wherein the concentration of the rhSP-D is from about 1 mg/ml to about 6 mg/ml.

25. The pharmaceutical composition of claim 24, wherein the concentration of the rhSP-D is from about 3 mg/ml to about 5 mg/ml.

26. The pharmaceutical composition of claim 25, wherein the concentration of the rhSP-D is about 4 mg/ml.

27. The pharmaceutical composition of claim 24, wherein the concentration of the rhSP-D is from about 1 mg/ml to about 3 mg/ml.

28. The pharmaceutical composition of claim 27, wherein the concentration of the rhSP-D is about 2 mg/ml.

29. The pharmaceutical composition of any one of claims 1-28 comprising a population of rhSP-D polypeptides having oligomeric forms, wherein greater than 30% of the oligomeric forms comprise dodecamers of rhSP-D.

30. The pharmaceutical composition of claim 29, wherein greater than 35% of the oligomeric forms comprise dodecamers of rhSP-D.

31. The pharmaceutical composition of claim 30, wherein greater than 40% of the oligomeric forms comprise dodecamers of the rhSP-D.

32. The pharmaceutical composition of any one of claims 1-31 comprising a population of rhSP-D polypeptides having oligomeric forms,, wherein less than 15% of the oligomeric forms comprise aggregates of the rhSP-D, wherein the aggregates have a radius greater than 70 nm.

33. The pharmaceutical composition of claim 32, wherein less than 10% of the oligomeric forms comprise aggregates of the rhSP-D, wherein the aggregates have a radius greater than 70 nm.

34. The pharmaceutical composition of claim 333, wherein less than 5% of the oligomeric forms comprise aggregates of the rhSP-D, wherein the aggregates have a radius greater than 70 nm.

35. The pharmaceutical composition of any one of claims 1-34, further comprising a bulking agent.

36. The pharmaceutical composition of claim 35, wherein the bulking agent is selected from the group consisting of mannitol, xylitol, sorbitol, maltitol, lactitol, glycerol, erythritol, arabitol, glycine, alanine, threonine, valine, and phenylalanine.

37. The pharmaceutical composition of any one of claims 1-36, wherein the composition lacks a chelating agent.

38. The pharmaceutical composition of claim 37, wherein the chelating agent is selected from EDTA and EGTA.

39. The pharmaceutical composition of claim 1 comprising 1 mg/ml rhSP-D, 5 mM Histidine, 265 mM Lactose, 5 mM calcium chloride, wherein the solution has a pH 6.0.

40. The pharmaceutical composition of claim 1 comprising: 2 mg/ml rhSP-D, 5 mM histidine, 265 mM lactose, 5 mM calcium chloride, wherein the solution has a pH of 6.0.

41. The pharmaceutical composition of claim 1 comprising 2 mg/ml rhSP-D, 5 mM Histidine, 265 mM Lactose, 1 mM calcium chloride, wherein the solution has a pH 6.0.

42. The pharmaceutical composition of claim 1 comprising 4 mg/ml rhSP-D, 5 mM Histidine, 265 mM Lactose, 5 mM calcium chloride, wherein the solution has a pH 6.0

43. The pharmaceutical composition of any one of claims 1-42, wherein the rhSP-D polypeptide comprises the amino acid sequence of SEQ ID NO:02, or an active fragment thereof.

44. The pharmaceutical composition of claim 43, wherein the rhSP-D or active fragment thereof comprises a residue at a polymorphic position corresponding to a residue selected from the group consisting of Met11, Thr160, Ser 270, and Ala 286.

45. The pharmaceutical composition of any one of claims 1-44, wherein the rhSP-D polypeptide or an active fragment thereof has activity in a bacterial aggregation assay, or in a TLR4 inhibition assay.

46. A lyophile of the pharmaceutical composition of any one of claims 1-45.

47. An article comprising a container comprising a lyophile of the pharmaceutical composition of any one of claims 1-45.

48. A method of preparing a lyophile comprising lyophilizing the pharmaceutical composition of any one of claims 1-45.

49. A method of preparing a pharmaceutical composition comprising contacting a lyophile of the pharmaceutical composition of any one of claims 1-45 with a pharmaceutically acceptable diluent to form a solution or suspension of the rhSP-D or active fragment thereof.

50. The method of claim 49, wherein the diluent is suitable for administration to a lung.

51. The method of claim 49 or 50, wherein the diluent is sterile water.

52. The method of any one of claims 49-51, wherein the solution or suspension has a concentration of the rhSP-D or active fragment thereof from about 0.1 mg/ml to about 10 mg/ml.

53. The method of claim 52, wherein the solution or suspension has a concentration of the rhSP-D or active fragment thereof from about 1 mg/ml to about 6 mg/ml.

54. The method of claim 53, wherein the solution or suspension has a concentration of the rhSP-D or active fragment thereof from about 3 mg/ml to about 5 mg/ml.

55. The method of claim 54, wherein the solution or suspension has a concentration of the rhSP-D or active fragment thereof of about 4 mg/ml.

56. The method of claim 53, wherein the solution or suspension has a concentration of the rhSP-D or active fragment thereof from about 1 mg/ml to about 3 mg/ml.

57. The method of claim 56, wherein the solution or suspension has a concentration of the rhSP-D or active fragment thereof of about 2 mg/ml.

58. The method of any one of claims 49-57, wherein the solution or suspension comprises a population of rhSP-D polypeptides having oligomeric forms,

59. The method of claim 58, wherein greater than 30% of the oligomeric forms comprise dodecamers of the rhSP-D.

60. The method of claim 59, wherein greater than 35% of the oligomeric forms comprise dodecamers of the rhSP-D.

61. The method of claim 60, wherein greater than 40% of the oligomeric forms comprise dodecamers of the rhSP-D.

62. The method of claim 61, wherein greater than 45% of the oligomeric forms comprise dodecamers of the rhSP-D.

63. The method of any one of claims 58-62, wherein less than 10% of the oligomeric forms comprise aggregates of the rhSP-D, wherein the aggregates have a radius greater than 70 nm.

64. The method of claim 63, wherein less than 5% of the oligomeric forms comprise aggregates of the rhSP-D, wherein the aggregates have a radius greater than 70 nm.

65. The method of claim 64, wherein less than 3% of the oligomeric forms comprise aggregates of the rhSP-D, wherein the aggregates have a radius greater than 70 nm.

66. The method of any one of claims 58-65, wherein less than about 3% of the rhSP-D oligomeric species by mass in the solution or suspension is in a high molecular weight oligomeric form with a radius greater than 70 nm.

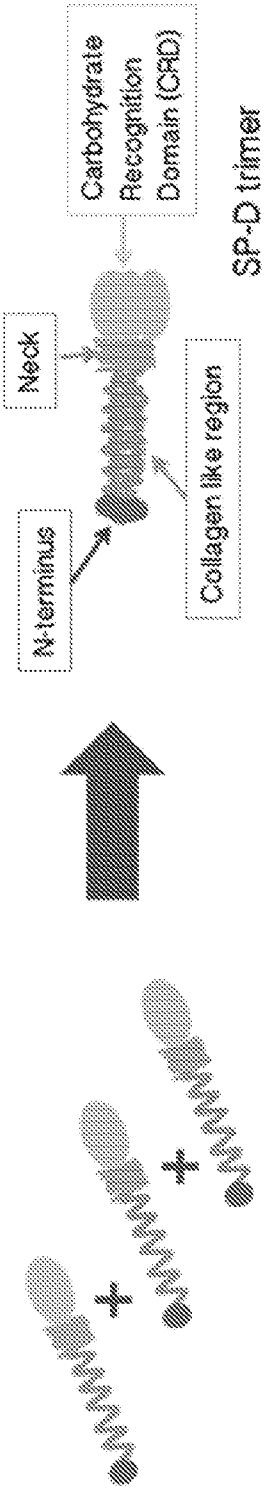

Carbohydrate Recognition Domain (CRD)
SP-D trimer
Neck
Collagen like region
N-terminus

FIG. 1

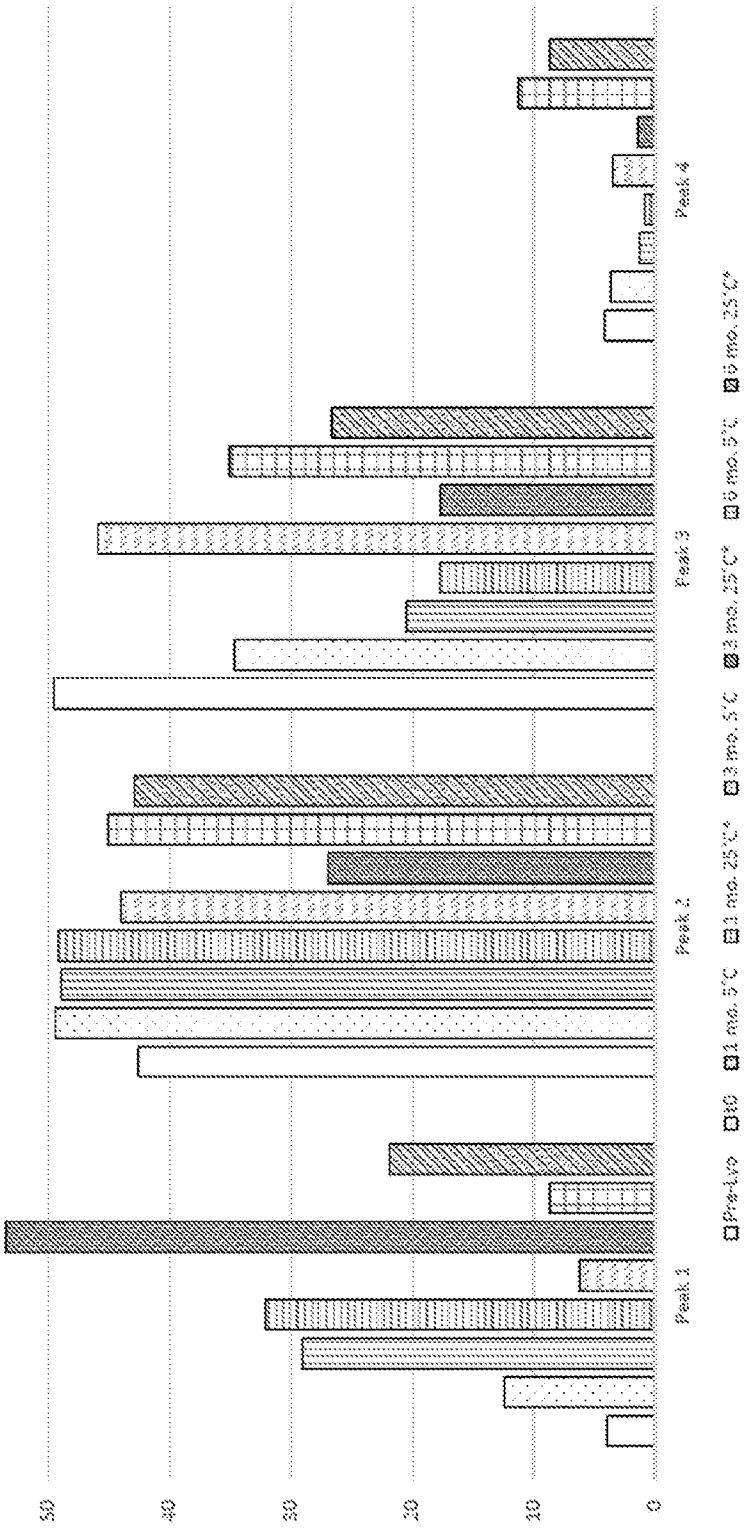

50
40
30
20
10
0
Peak 1
Peak 2
Peak 3
Peak 4
Pre-Lyo
t0
1 mo. 5°C
1 mo. 25°C
3 mo. 5°C
3 mo. 25°C
6 mo. 5°C
6 mo. 25°C

FIG. 2

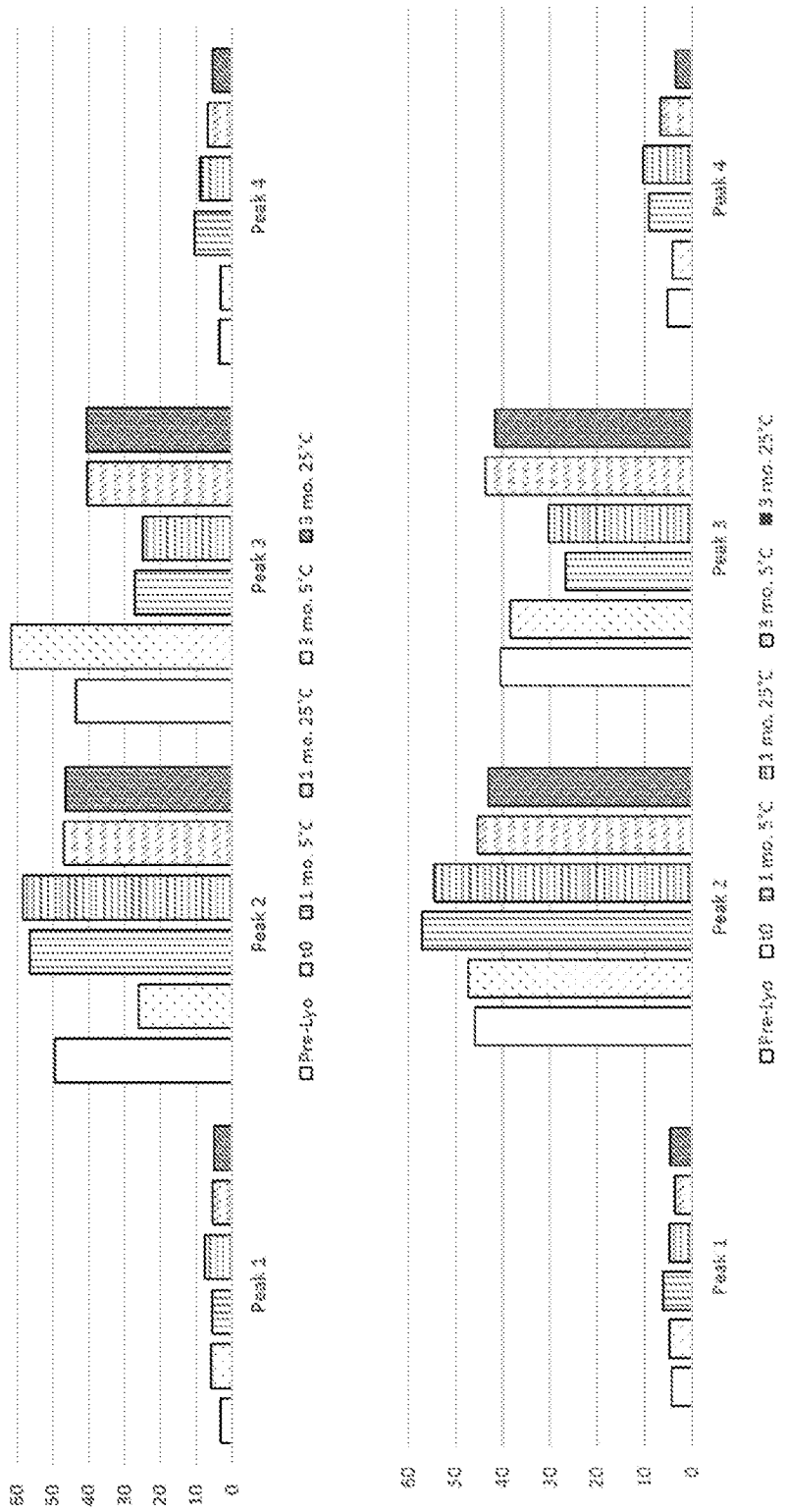

60
50
40
30
20
10
0
Peak 1
Peak 2
Peak 3
Peak 4
Pre-Lyo
t0
1 mo. 5°C
1 mo. 25°C
3 mo. 5°C
3 mo. 25°C

FIG. 3

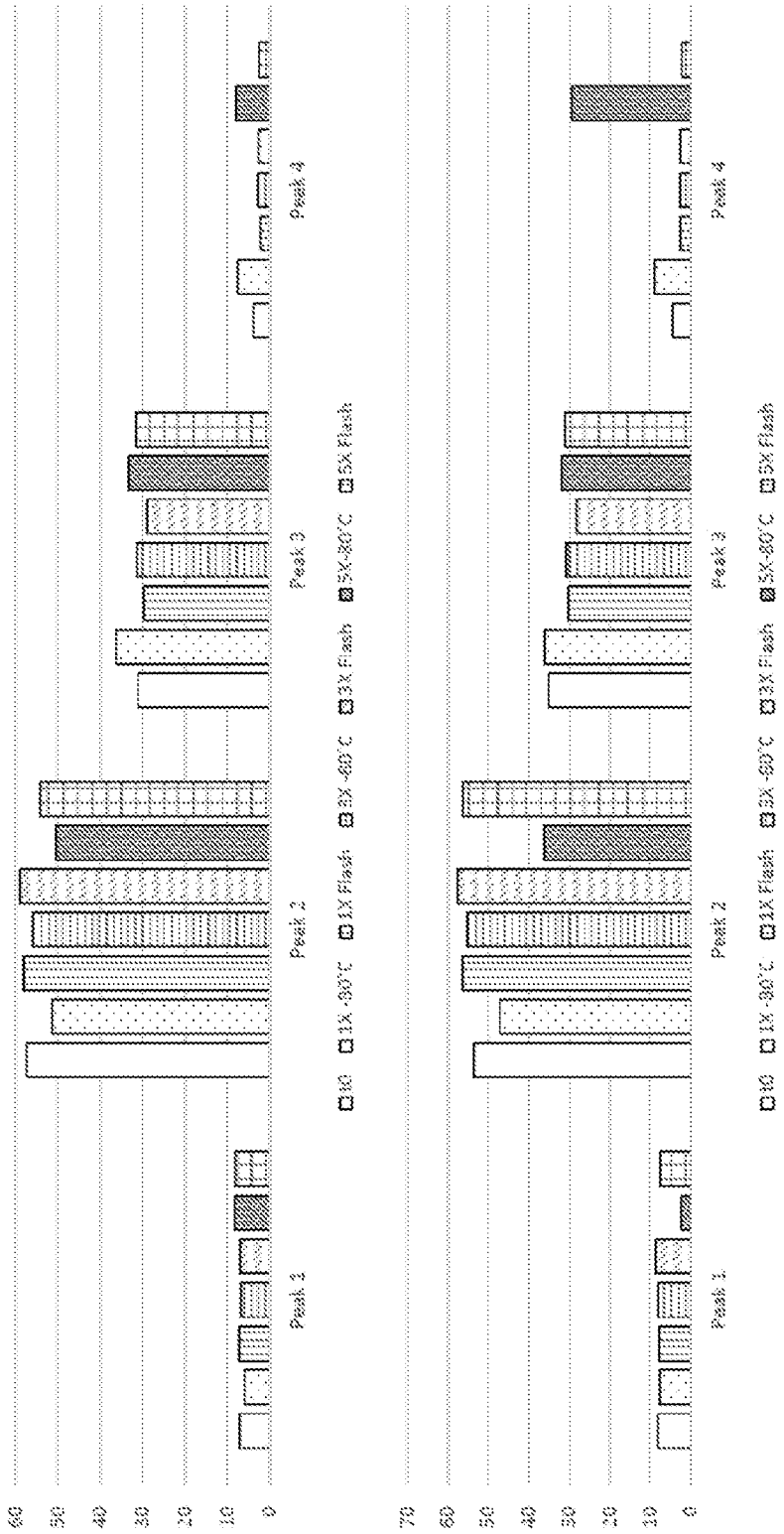

Peak 1
Peak 2
Peak 3
Peak 4
0
10
20
30
40
50
60
70
1X -80°C
1X Flash
3X -80°C
3X Flash
5X -80°C
5X Flash

FIG. 4

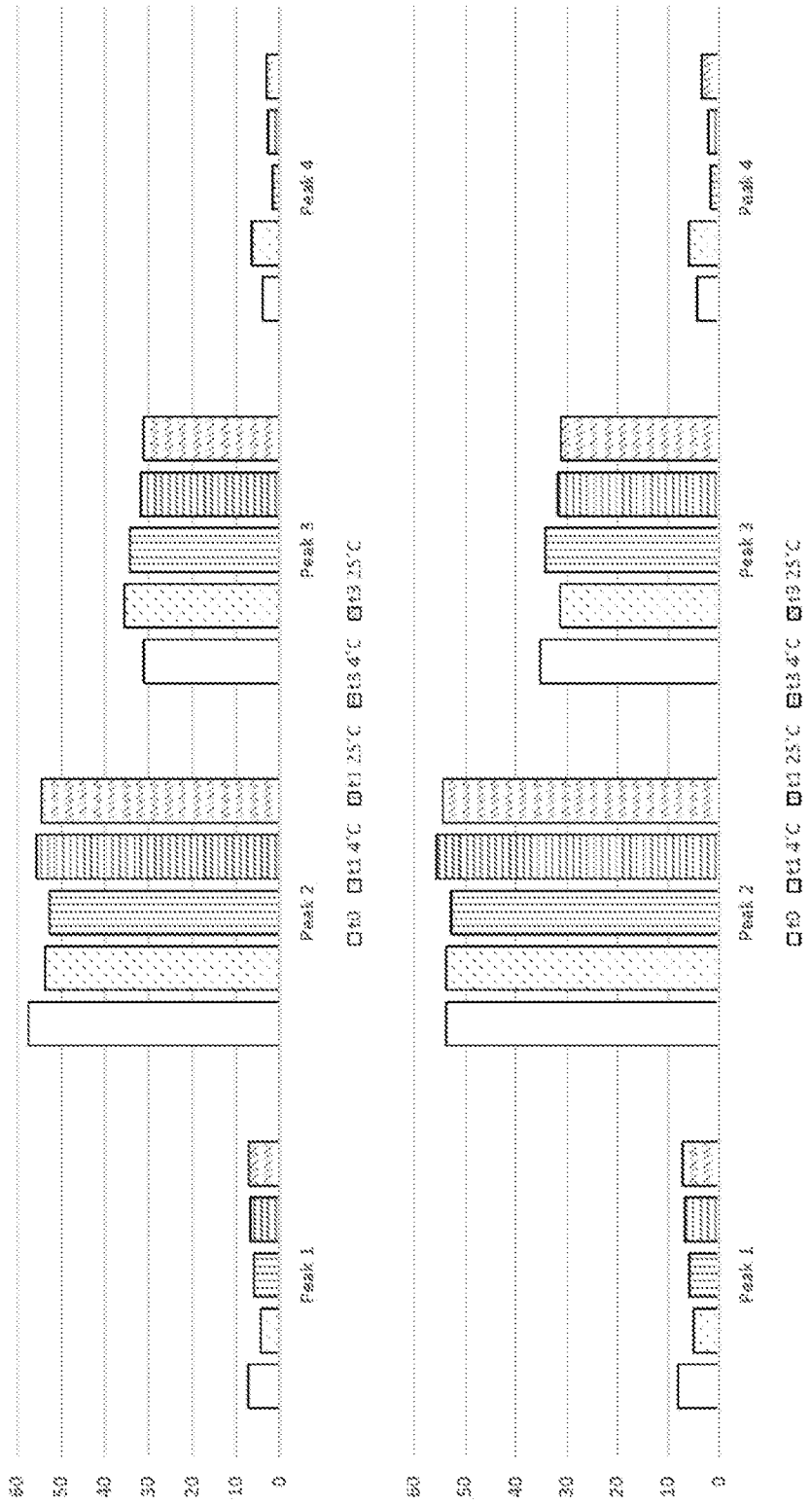

60
50
40
30
20
10
0
Peak 1
Peak 2
Peak 3
Peak 4
40
41.4°C
41.25°C
43.4°C
43.25°C

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/24305

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 14/785, C12N 5/09, C12N 15/85 (2019.01)
CPC - C07K 14/785, C12N 5/0694, C12N 15/85, C12N 2015/8518

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 9,370,555 B2 (CHILDREN'S HOSPITAL MEDICAL CENTER) 21 June 2016 (21.06.2016); col 3, ln 50-57; col 10, ln 56-60; col 12, ln 5-9; col 14, ln 16-35	1-7, 39-42
Y	US 2017/0189527 A1 (ARES TRADING SA) 6 July 2017 (06.07.2017); abstract; para [0086], [0124], [0168]	1-7, 39-42

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
20 May 2019	25 JUN 2019

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/24305

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

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

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/24305

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

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

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

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

3. ☒ Claims Nos.: 8-38, 43-66
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.