

LYOPHILIZED AND SPRAYABLE SILK FIBROIN AND MODIFIED SILK FIBROIN FORMS

FIELD

5 The disclosure relates to peptide compositions, e.g., silk fibroin derived peptide composition.

BACKGROUND

10 Silk is a natural polymer produced by a variety of insects and spiders, and comprises a filament core protein, silk fibroin, and a glue-like coating consisting of a non-filamentous protein, sericin.

SUMMARY

15 Embodiments of the present disclosure provide a plurality of substantially solid silk fibroin particles comprising silk fibroin fragments, the particles being characterized by at least one of: bulk density, surface area, pore size, pore volume, aspect ratio, and/or Hausner ratio. In some embodiments, the substantially solid silk fibroin particles are annealed. In some embodiments, the substantially solid silk fibroin particles are grounded. In some embodiments, the substantially solid silk
20 fibroin particles have a bulk density of less than 0.03 g/ml, less than 0.04 g/ml, less than 0.05 g/ml, less than 0.06 g/ml, less than 0.07 g/ml, less than 0.08 g/ml, less than 0.09 g/ml, less than 0.10 g/ml, less than 0.11 g/ml, less than 0.12 g/ml, less than 0.13 g/ml, less than 0.14 g/ml, less than 0.15 g/ml, less than 0.16 g/ml, less than 0.17 g/ml, less than 0.18 g/ml, less than 0.19 g/ml, less than 0.20 g/ml, less than 0.21 g/ml, less
25 than 0.22 g/ml, less than 0.23 g/ml, less than 0.24 g/ml, or less than 0.25 g/ml, less than 0.26 g/ml, less than 0.27 g/ml, less than 0.28 g/ml, less than 0.29 g/ml, less than 0.30 g/ml, less than 0.31 g/ml, less than 0.32 g/ml, less than 0.33 g/ml, less than 0.34 g/ml, or less than 0.35 g/ml. In some embodiments, the substantially solid silk fibroin particles have an average bulk density of about 0.03 g/ml, about 0.04 g/ml, or about
30 0.05 g/ml, about 0.03 g/ml, about 0.04 g/ml, about 0.05 g/ml, about 0.06 g/ml, about 0.07 g/ml, about 0.08 g/ml, about 0.09 g/ml, about 0.10 g/ml, about 0.11 g/ml, about 0.12 g/ml, about 0.13 g/ml, about 0.14 g/ml, about 0.15 g/ml, about 0.16 g/ml, about 0.17 g/ml, about 0.18 g/ml, about 0.19 g/ml, about 0.20 g/ml, about 0.21 g/ml, about 0.22 g/ml, about 0.23 g/ml, about 0.24 g/ml, about 0.25 g/ml, about 0.26 g/ml, about
35 0.27 g/ml, about 0.28 g/ml, about 0.29 g/ml, about 0.30 g/ml, about 0.31 g/ml, about

0.32 g/ml, about 0.33 g/ml, about 0.34 g/ml, or about 0.35 g/ml. In some embodiments, the substantially solid silk fibroin particles have a Hausner ratio of between 1.00 and 1.11, between 1.12 and 1.18, between 1.19 and 1.25, between 1.26 and 1.34, or between 1.35 and 1.45. In some embodiments, the substantially solid silk fibroin particles have an average diameter of between about 3 mm and about 10 mm. In some embodiments, the substantially solid silk fibroin particles have an average diameter of about 1 mm, about 2 mm, about 3 mm, about 4 mm, about 5 mm, about 6 mm, about 7 mm, about 8 mm, about 9 mm, about 10 mm, about 11 mm, about 12 mm, about 13 mm, about 14 mm, about 15 mm, about 16 mm, about 17 mm, about 18 mm, about 19 mm, or about 20 mm. In some embodiments, the substantially solid silk fibroin particles have an aspect ratio between 1 and about 1.45. In some embodiments, the substantially solid silk fibroin particles have an aspect ratio of 1, about 1.10, about 1.15, about 1.20, about 1.25, about 1.30, about 1.35, about 1.40, or about 1.45. In some embodiments, the substantially solid silk fibroin particles have an aspect ratio of 1, about 1.10, about 1.11, about 1.12, about 1.13, about 1.14, about 1.15, about 1.16, about 1.17, about 1.18, about 1.19, about 1.20, about 1.21, about 1.22, about 1.23, about 1.24, about 1.25, about 1.26, about 1.27, about 1.28, about 1.29, about 1.30, about 1.31, about 1.32, about 1.33, about 1.34, about 1.35, about 1.36, about 1.37, about 1.38, about 1.39, about 1.40, about 1.41, about 1.42, about 1.43, about 1.44, about 1.45, about 1.46, about 1.47, about 1.48, about 1.49, or about 1.50. In some embodiments, the substantially solid silk fibroin particles are substantially spherical. In some embodiments, the substantially solid silk fibroin particles are mesoporous. In some embodiments, the substantially solid silk fibroin particles have a BET (Brunauer–Emmett–Teller) surface area between about 2.50 m²/g and about 6.50 m²/g. In some embodiments, the substantially solid silk fibroin particles have a BET (Brunauer–Emmett–Teller) surface area of between about 2.50 m²/g and about 3.00 m²/g, about 3.00 m²/g and about 3.50 m²/g, about 3.50 m²/g and about 4.00 m²/g, about 4.00 m²/g and about 4.50 m²/g, about 4.50 m²/g and about 5.00 m²/g, about 5.00 m²/g and about 5.50 m²/g, about 5.50 m²/g and about 6.00 m²/g, or about 6.00 m²/g and about 6.50 m²/g. In some embodiments, the substantially solid silk fibroin particles have an average pore size between about 25 Å and about 500 Å. In some embodiments, the substantially solid silk fibroin particles have an average pore size of about 25 Å to about 30 Å, about 30 Å to about 35 Å, about 35 Å to about 40 Å, about 40 Å to about 45 Å, about 45 Å to about 50 Å, about 50 Å to about 55 Å,

about 55 Å to about 60 Å, about 60 Å to about 65 Å, about 65 Å to about 70 Å, about 70 Å to about 75 Å, about 75 Å to about 80 Å, about 80 Å to about 85 Å, about 85 Å to about 90 Å, about 90 Å to about 95 Å, about 95 Å to about 100 Å, about 100 Å to about 105 Å, about 105 Å to about 110 Å, about 110 Å to about 115 Å, about 115 Å to about 120 Å, about 120 Å to about 125 Å, about 125 Å to about 130 Å, about 130 Å to about 135 Å, about 135 Å to about 140 Å, about 140 Å to about 145 Å, or about 145 Å to about 150 Å. In some embodiments, a substantially solid silk fibroin particle comprises a plurality of radially orientated microchannels. In some embodiments, the substantially solid silk fibroin particle comprises an emulsifier, a surfactant, a buffering agent, an amino acid, or a sugar. In some embodiments, the substantially solid silk fibroin particle comprises a polysaccharide, a polysorbate, a glycoside, PBS, arginine, trehalose, glucose, or sucrose. In some embodiments, the substantially solid silk fibroin particle comprises a surfactant selected from sucrose ester, cetearyl glucoside, caprylyl/capryl glucoside, sucrose laurate, sucrose palmitate, sucrose stearate, sucrose cocoate, sorbitan monostearate, and combinations thereof. In some embodiments, the substantially solid silk fibroin particle comprises an additional protein or peptide, a C12-C24 fatty alcohol, a glycolipid, or a lipid. In some embodiments, the substantially solid silk fibroin particle comprises a protein, a peptide, a sugar surfactant, a biosurfactant, a lipid, or a combination. In some embodiments, the substantially solid silk fibroin particle comprises a sugar fatty acid ester, a sugar fatty acid monoester, a sugar fatty diester, a sugar fatty triester, or a sugar fatty and polyester. In some embodiments, the substantially solid silk fibroin particle comprises a sucrose fatty acid ester, a sorbitan or sorbitol fatty acid ester, an alkyl glucoside, an alkyl polyglucoside, or a combination thereof. In some embodiments, the substantially solid silk fibroin particle comprises KCl, NaCl, MgCl₂, CaCl₂, PBS, Tris, Polysorbate 20, Polysorbate 80, Capryl Glucoside, Sucrose, Histidine, Glycine, or Arginine. In some embodiments, a stabilizer is selected from a polysaccharide, e.g., and without limitation, maltodextrins, dextran, a surfactant, e.g., and without limitation, polysorbate (20, 60, 80), capryl glucoside, a buffering agent, e.g., and without limitation, PBS, tris acetate, sodium phosphate, an amino acid, e.g., and without limitation, arginine, glycine, cysteine, histidine, lysine, serine, and/or a sugar, e.g., and without limitation, trehalose, glucose, sucrose, maltose, fucose.

Embodiments of the present disclosure provide a silk fibroin nanoclay composite or film, comprising silk fibroin fragments and a clay, wherein the % (w/w) of clay in the composite is from about 1% to about 99%. In some embodiments, the clay is a bentonite clay. In some embodiments, the concentration of clay in the composite or film is from about 20% (w/w) to about 33% (w/w), from about 33% (w/w) to about 50% (w/w), or from about 50% (w/w) to about 67% (w/w). In some embodiments, the water vapor permeance (WVP) of the composite is inversely proportional to the concentration of clay in the composite. In some embodiments, water vapor permeance (WVP, $\text{g/m}^2 \cdot \text{Pa} \cdot 24\text{h}$) of the composite is from about 0.20 to about 0.30, from about 0.30 to about 0.35, from about 0.35 to about 0.40, from about 0.40 to about 0.45, from about 0.45 to about 0.50, from about 0.50 to about 0.55, from about 0.55 to about 0.60, from about 0.60 to about 0.65, from about 0.65 to about 0.70, from about 0.70 to about 0.75, from about 0.75 to about 0.80, or from about 0.80 to about 0.85.

Embodiments of the present disclosure provide a stabilized silk fibroin solution comprising silk fibroin fragments and a stabilizer, wherein the solution has a z-average value lower than a substantially similar silk fibroin solution comprising silk fibroin fragments but excluding the stabilizer, and/or the solution has a z-average plateau value lower than a substantially similar silk fibroin solution comprising silk fibroin fragments but excluding the stabilizer. In some embodiments, the z-average is measured after a period of time after the silk fibroin fragments and the stabilizer are co-formulated, wherein the period time ranges from 1 hour to 250 hours, from 1 hour to 350 hours, from 1 hour to 450 hours, from 1 hour to 550 hours, from 1 hour to 650 hours, or from 1 hour to 1000 hours. In some embodiments, the z-average is measured after a period of time after the silk fibroin fragments and the stabilizer are co-formulated, wherein the period time ranges from 1 minute to 10 minutes, from 1 minute to 20 minutes, from 1 minute to 30 minutes, from 1 minute to 40 minutes, from 1 minute to 50 minutes, from 1 minute to 60 minutes, from 1 minute to 70 minutes, or from 1 minute to 80 minutes. In some embodiments, the stabilizer is an emulsifier, a surfactant, a buffering agent, an amino acid, or a sugar. In some embodiments, the stabilizer is a polysaccharide, a polysorbate, a glycoside, PBS, arginine, trehalose, glucose, or sucrose. In some embodiments, the stabilizer is a surfactant selected from sucrose ester, cetearyl glucoside, caprylyl/capryl glucoside, sucrose laurate, sucrose palmitate, sucrose stearate, sucrose cocoate, sorbitan

monostearate, and combinations thereof. In some embodiments, the stabilizer is an additional protein or peptide, a C12-C24 fatty alcohol, a glycolipid, or a lipid. In some embodiments, the stabilizer is a protein, a peptide, a sugar surfactant, a biosurfactant, a lipid, or a combination. In some embodiments, the stabilizer is a sugar fatty acid ester, a sugar fatty acid monoester, a sugar fatty diester, a sugar fatty triester, or a sugar fatty and polyester. In some embodiments, the stabilizer is a sucrose fatty acid ester, a sorbitan or sorbitol fatty acid ester, an alkyl glucoside, an alkyl polyglucoside, or a combination thereof. In some embodiments, the stabilizer is KCl, NaCl, MgCl₂, CaCl₂, PBS, Tris, Polysorbate 20, Polysorbate 80, Capryl Glucoside, Sucrose, Histidine, Glycine, or Arginine. In some embodiments, a stabilizer is selected from a polysaccharide, e.g., and without limitation, maltodextrins, dextran, a surfactant, e.g., and without limitation, polysorbate (20, 60, 80), capryl glucoside, a buffering agent, e.g., and without limitation, PBS, tris acetate, sodium phosphate, an amino acid, e.g., and without limitation, arginine, glycine, cysteine, histidine, lysine, serine, and/or a sugar, e.g., and without limitation, trehalose, glucose, sucrose, maltose, fucose.

In some embodiments, the solution is sprayable. Embodiments of the present disclosure provide a liquid in air suspension comprising a plurality of droplets comprising the stabilized silk fibroin solution described above, wherein the droplets are sufficiently stable after being sprayed, for a period of time necessary to reach a surface. In some embodiments, the droplets or drops are sufficiently stable after being formed, for a period of time necessary to reach a surface.

In some embodiments, the silk fibroin fragments in any of the embodiments of the plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets described herein, have a weight average molecular weight selected from between about 1 kDa and about 5 kDa, from between about 5 kDa and about 10 kDa, from between about 6 kDa and about 17 kDa, from between about 10 kDa and about 15 kDa, from between about 14 kDa and about 30 kDa, from between about 15 kDa and about 20 kDa, from between about 17 kDa and about 39 kDa, from between about 20 kDa and about 25 kDa, from between about 25 kDa and about 30 kDa, from between about 30 kDa and about 35 kDa, from between about 35 kDa and about 40 kDa, from between about 39 kDa and about 54 kDa, from between about 39 kDa and about 80 kDa, from between about 40 kDa and about 45 kDa, from between about 45 kDa and about 50 kDa, from between about 50 kDa and about 55

kDa, from between about 55 kDa and about 60 kDa, from between about 60 kDa and about 100 kDa, from between about 80 kDa and about 144 kDa, from between about 144 kDa and about 250 kDa, or from between about 250 kDa and about 350 kDa, and a polydispersity from 1 to about 5. In some embodiments, the polydispersity of any of the above mentioned embodiments is from 1 to about 1.5, from about 1.5 to about 2.0, from about 2.0 to about 2.5, from about 2.5 to about 3.0, from about 3.0 to about 3.5, from about 3.5 to about 4.0, from about 4.0 to about 4.5, or from about 4.5 to about 5.0. Any of the above mentioned embodiments may further comprise about 0.001% (w/w) to about 10% (w/w) sericin relative to the silk fibroin fragments. In any of the above mentioned embodiments, the silk fibroin fragments do not spontaneously or gradually gelate and do not visibly change in color or turbidity when in an aqueous solution for at least 10 days prior to being formulated into the substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, or the stabilized silk fibroin solution.

In any of the embodiments of the plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets described herein, the silk fibroin fragments may comprise a plurality of amino acids selected from M, R, V, K, T, F, I, L, C, A, Q, Y, N, D, E, G, S, H, P, and W, wherein at least one of the amino acids is modified, substituted, or replaced. In any of the above mentioned embodiments, the fibroin is a fibroin heavy chain, a fibroin light chain, or a fibrohexamerin. In any of the above mentioned embodiments, the silk fibroin fragment comprises between about 2 and about 100 amino acids. In any of the above mentioned embodiments, silk fibroin fragment comprises between one and five modifications, substitutions, and/or replacements. In any of the above mentioned embodiments, a modification, substitution, and/or replacement is selected from an asparagine to aspartic acid modification, substitution, and/or replacement, a glutamine to glutamic acid modification, substitution, and/or replacement, and a methionine to methionine oxide modification, substitution, and/or replacement. In any of the above mentioned embodiments, the fibroin is a fibroin heavy chain, and wherein a modification, substitution, and/or replacement is at a position corresponding to any one position from 1 to 5263 of the fibroin heavy chain. In any of the above mentioned embodiments, a modification, substitution, and/or replacement is at Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, and/or

N5262. In any of the above mentioned embodiments, the fibroin is a fibroin light chain, and wherein a modification, substitution, and/or replacement is at a position corresponding to any one position from 1 to 262 of the fibroin light chain. In any of the above mentioned embodiments, a modification, substitution, and/or replacement is at N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, and/or Q255. In any of the above mentioned embodiments, the fibroin is a fibrohexamerin (p25), and wherein a modification, substitution, and/or replacement is at a position corresponding to any one position from 1 to 220 of the fibrohexamerin (p25). In any of the above mentioned embodiments, a modification, substitution, and/or replacement is at Q62, N93, M120, N149, N172, N174, and/or N202. In any of the above mentioned embodiments, each modification, substitution, and/or replacement is independently ranging between about 1% to about 99% in the silk fibroin fragments portion of the substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, or the stabilized silk fibroin solution composition. In any of the above mentioned embodiments, a % modification, substitution, and/or replacement is defined as (number of peptide or protein fragments comprising a modification, substitution, and/or replacement at a specific position, divided by the total number of peptide or protein fragments which include the specific position, whether comprising a modification, substitution, and/or replacement, or not) x 100.

In any of the plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets described herein, the silk fibroin fragments are included in one or more fractions, each fraction independently comprising a plurality of fibroin heavy chain fragments, a plurality of fibroin light chain fragments, and/or a plurality of fibrohexamerin (p25) fragments. In any of the embodiments, the silk fibroin fragments have a weight average molecular weight (M_w) selected from between about 1 kDa and about 20 kDa, from between about 20 kDa and about 40 kDa, from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, from between about 120 kDa and about 140 kDa, or from between about 140 kDa and about 160 kDa, from between about 160 kDa and about 180 kDa, from between about 180 kDa and about 200 kDa, or from between about 200 kDa and about 250 kDa, and a polydispersity between 1 and about 1.7. In any of the above mentioned embodiments, the silk fibroin fragments

have a weight average molecular weight (M_w) selected from between about 10 kDa and about 20 kDa, from between about 20 kDa and about 40 kDa, from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, from between about 120 kDa and about 140 kDa, from between about 140 kDa and about 160 kDa, or from between about 160 kDa and about 180 kDa, and a polydispersity between 1 and about 1.1, or 1 and about 1.2. In any of the above mentioned embodiments, the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from between about 10 kDa and about 20 kDa, from between about 20 kDa and about 40 kDa, from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, or from between about 120 kDa and about 140 kDa, and a polydispersity between 1 and about 1.1, or 1 and about 1.2. In any of the above mentioned embodiments, the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, or from between about 100 kDa and about 120 kDa, and a polydispersity between 1 and about 1.1. In any of the above mentioned embodiments, the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from between about 10 kDa and about 20 kDa, from between about 20 kDa and about 40 kDa, from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, or from between about 100 kDa and about 110 kDa, and a polydispersity between 1 and about 1.1, or 1 and about 1.2. In any of the above mentioned embodiments, the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, or from between about 120 kDa and about 140 kDa, and a polydispersity between 1 and about 1.1. In any of the above mentioned embodiments, the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from between about 20 kDa and about 40 kDa, or from between about 40 kDa and about 60 kDa, and a polydispersity between 1 and about 1.1, or 1 and about 1.2. In any of the above mentioned embodiments, the one or more fractions are selected from AS77, AS78, AS79, AS80, and AS81. In any of the above mentioned embodiments, the one or more fractions are selected from

AS82, AS83, AS84, AS85, AS86, AS87, AS88, and AS89. In any of the above mentioned embodiments, the one or more fractions are selected from AS90, AS91, AS92, AS93, and AS94. In any of the above mentioned embodiments, the one or more fractions are selected from AS95, AS96, AS97, AS98, AS99, and AS100. In any of the above mentioned embodiments, the silk fibroin fragments have a weight average molecular weight (M_w) selected from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, from between about 120 kDa and about 140 kDa, from between about 140 kDa and about 160 kDa, from between about 160 kDa and about 180 kDa, from between about 180 kDa and about 200 kDa, or from between about 200 kDa and about 220 kDa, and a polydispersity between 1 and about 1.7. In any of the above mentioned embodiments, the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, from between about 120 kDa and about 140 kDa, from between about 140 kDa and about 160 kDa, from between about 160 kDa and about 180 kDa, from between about 180 kDa and about 200 kDa, or from between about 200 kDa and about 210 kDa, and a polydispersity between 1 and about 1.2, or 1 and about 1.3. In any of the above mentioned embodiments, the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, or from between about 100 kDa and about 110 kDa, and a polydispersity between 1 and about 1.1, or 1 and about 1.2. In any of the above mentioned embodiments, the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, from between about 120 kDa and about 140 kDa, from between about 140 kDa and about 160 kDa, from between about 160 kDa and about 180 kDa, from between about 180 kDa and about 200 kDa, or from between about 200 kDa and about 210 kDa, and a polydispersity between 1 and about 1.2, or 1 and about 1.3. In any of the above mentioned embodiments, the one or more fractions are selected from AS101, AS102, AS103, AS104, and AS105. In any of the above mentioned embodiments, the one or more fractions are selected from AS106, AS107, AS108,

AS109, AS110, and AS111. In any of the above mentioned embodiments, the silk fibroin fragments comprise one or more amino acid modifications, substitutions, or replacements of an amino acid is selected from M, R, V, K, T, F, I, L, C, A, Q, Y, N, D, E, G, S, H, P, and W. In any of the above mentioned embodiments, wherein the silk fibroin fragments comprise between about 2 and about 100 amino acids. In any of the above mentioned embodiments, a silk fibroin fragment comprise between one and five modifications, substitutions, and/or replacements. In any of the above mentioned embodiments, the fibroin is a fibroin heavy chain, and wherein a modification, substitution, and/or replacement is at a position corresponding to any one position from 1 to 5263 of the fibroin heavy chain. In any of the above mentioned embodiments, the fibroin is a fibroin light chain, and wherein a modification, substitution, and/or replacement is at a position corresponding to any one position from 1 to 262 of the fibroin light chain. In any of the above mentioned embodiments, the fibroin is a fibrohexamerin (p25) chain, and wherein a modification, substitution, and/or replacement is at a position corresponding to any one position from 1 to 220 of the fibrohexamerin (p25) chain. In any of the above mentioned embodiments, a modification, substitution, and/or replacement is selected from an asparagine to aspartic acid modification, substitution, and/or replacement, a glutamine to glutamic acid modification, substitution, and/or replacement, and a methionine to methionine oxide modification, substitution, and/or replacement. In any of the above mentioned embodiments, a modification, substitution, and/or replacement is at fibroin heavy chain position selected from Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, and/or N5262. In any of the above mentioned embodiments, a modification, substitution, and/or replacement is at fibroin light chain position selected from N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, and/or Q255. In any of the above mentioned embodiments, a modification, substitution, and/or replacement is at fibrohexamerin (p25) position selected from Q62, N93, M120, N149, N172, N174, and/or N202. In any of the above mentioned embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 1% to about 99%. In any of the above mentioned embodiments, the % modification, substitution, and/or replacement is defined as (number of peptide or protein fragments comprising a modification, substitution, and/or replacement at a specific position, divided by the

total number of peptide or protein fragments which include the specific position, whether comprising a modification, substitution, and/or replacement, or not) x 100.

In any of the above mentioned embodiments, molecular weight is determined by MALS.

5 Embodiments of the present disclosure provide a pouch or a laundry pod comprising the plurality of substantially solid silk fibroin particles of any of the above mentioned embodiments. In some embodiments, the plurality of substantially solid silk fibroin particles are compressed in a multi-particulate puck. In some
10 embodiments, the laundry pod further comprises a dissolvable enclosure comprising polyvinylalcohol (PVA) or a derivative of PVA. In some embodiments, the laundry pod further comprises an enclosure comprising one or more of nylon, polyglycolide (PGA), polylactic acid (PLA), poly(lactide-co-glycolide) (PLGA), polycaprolactone (PCL), poly(butylene succinate) (PBS), polybutylene succinate adipate, poly(p-dioxanone) (PPDO), poly(butylene adipate-co-terephthalate) (PBAT), a copolyester
15 of terephthalic acid and lactic acid, a copolyester of terephthalic acid and glycolic acid, a copolyester of terephthalic acid and succinic acid, poly(hydroxybutyrate), poly(hydroxyvalerate), polyhydroxyhexanoate, a poly(hydroxyalkanoate) (PHA), polymethylene adipate/terephthalate.

 Embodiments of the present disclosure provide a method of making the
20 plurality of substantially solid silk fibroin particles of any of the above mentioned embodiments, the method comprising dripping a solution comprising a plurality of the silk fibroin fragments into liquid nitrogen. In some embodiments, the method further comprises a lyophilization step. In some embodiments, the concentration of silk fibroin fragments in the solution is from about 3% (w/w) to about 50% (w/w). In
25 some embodiments, the concentration of silk fibroin fragments in the solution is from about 6% (w/w) to about 25% (w/w). In some embodiments, the concentration of silk fibroin fragments in the solution is from about 6% (w/w) to about 20% (w/w). In some embodiments, the concentration of silk fibroin fragments in the solution is about
30 3% (w/w), about 4% (w/w), about 5% (w/w), about 6% (w/w), about 7% (w/w), about 8% (w/w), about 9% (w/w), about 10% (w/w), about 11% (w/w), about 12% (w/w), about 13% (w/w), about 14% (w/w), about 15% (w/w), about 16% (w/w), about 17% (w/w), about 18% (w/w), about 19% (w/w), about 20% (w/w), about 21% (w/w), about 22% (w/w), about 23% (w/w), about 24% (w/w), or about 25% (w/w). In some embodiments, the silk fibroin fragments comprise one or more of a molecular weight,

polydispersity, and/or a modification, substitution, and/or replacement at a specific amino acid position, as defined in any one of the above mentioned embodiments. In some embodiments, the solution is stabilized as defined in any one the above mentioned embodiments.

5 In any of the above mentioned embodiments, the particles have a reconstitution yield of more than 90%. In any of the above mentioned embodiments, the particles have a reconstitution yield in DI water of more than 90%.

 In some embodiments, a reconstitution rate of at least 90% is preserved after a stability testing comprising simulated ageing of a plurality of substantially solid silk
10 fibroin particles of any one of claims 1 to 92, the ageing comprising storage a temperature between about 40 °C and about 60 °C, for a period of time ranging from about 400 days to about 650 days. In some embodiments, the simulated age ranges from about 4 years to about 15 years.

15 BRIEF DESCRIPTION OF THE DRAWINGS

 The presently disclosed embodiments will be further explained with reference to the attached drawings. The drawings shown are not necessarily to scale, with emphasis instead generally being placed upon illustrating the principles of the presently disclosed embodiments.

20 **Figure 1.** Ion Exchange Fractionation Schemes for the isolation of the populations that constitute Low and Mid Skid silk/modified polypeptide compositions. Low and Mid Skid silk/modified polypeptide compositions contains silk/modified polypeptides that are negatively, positively charged, or neutral. Using Q anion exchange chromatography (A) these populations were isolated.

25 **Figure 2.** Chromatogram of Low Skid silk/modified polypeptide composition loaded in a Q-Sepharose HP column (Cytiva). The flow through contains the silk/modified polypeptides that do not get captured in the column and are the depleted in negatively charged amino acids. After the column is loaded with Low or Mid Skid silk/modified polypeptide compositions and the flow through is collected, the column
30 is washed until the UV-280 absorbance becomes less than 200 AU. The captured negatively charged silk/modified polypeptides are eluted with high salt concentration (1M NaCl) and constitute AS11 and AS22. The chromatography is performed in Tris-containing buffers but the flow through and the Q-elution were finally dialyzed in water.

Figure 3. Analytical Size Exclusion Chromatography of Low, Mid Skid silk/modified silk compositions and their constituent AS compositions. Average molecular weight in kDa and polydispersity measurements are shown.

Figures 4A- 4B. Analytical Size Exclusion Chromatography of the Low and Mid skid silk/modified peptide compositions and their components (see table 1 for more details). **Fig. 4A**, Molecular weight of the various Activated Silk new compositions described in this study. **Fig. 4B**, Polydispersity (PDI) of the various Activated Silk new compositions described in this study. AS24 reconstitutes the average molecular weight and polydispersity of the Low skid silk/modified peptide composition and it consists of 50% AS12 and 50% AS22 (see table 1 for details). AS6 reconstitutes the average molecular weight and polydispersity of the Mid skid silk/modified peptide composition and it consists of 50% AS1 and 50% AS11 (see table 1 for details).

Figure 5. Isoelectric Focusing Electrophoresis of Low Skid silk/modified polypeptide compositions. Lanes 2, 7; Low Skid silk different amounts loaded. Lanes 3, 5, 8, 10; AS12 silk, different preparations different amounts loaded. Lanes 4, 6, 9, 11; AS22 silk, different preparations different amounts loaded.

Figures 6A- 6B. Self-assembly reactions of the of the Low and Mid skid silk/modified peptide compositions and their components (see table 1 for more details). Both graphs depict the kinetic parameters of gel formation during self-assembly of silk. On graph A calculation of the three self-assembly kinetic parameters is shown, $t_{0.5}$, A_{max} and SARF. For more details look at the text.

Figures 7A- 7C. Self-assembly kinetics of the Low and Mid skid silk/modified peptide compositions and their components (see table 1 for more details). **Fig. 7A**, the Self-assembly Rate Factor shows how fast the self-assembly reaction proceeds once it is initiated and the self-assembly nuclei are organized. **Fig. 7B**, Maximum Gel Yield shows how dense the silk gel is after self-assembly is complete. **Fig. 7C**, Time required for the self-assembly reaction to produce half of the maximum gel amount.

Figure 8. the Low and Mid skid silk/modified peptide compositions and their components (see table 1 for more details). The Self Assembly Factor reflects the average propensity of silk to self-assemble and form gels.

While the above-identified drawings set forth presently disclosed embodiments, other embodiments are also contemplated, as noted in the discussion.

This disclosure presents illustrative embodiments by way of representation and not limitation. Numerous other modifications and embodiments can be devised by those skilled in the art which fall within the scope and spirit of the principles of the presently disclosed embodiments.

5 **Figure 9** is a graph of Weight average molecular weight (i.e., average molecular weight average or average MW) using Size exclusion chromatography with a refractive index detector (SEC-RI) plotted as a function of time for solubilized fibroin in 9.3 M LiBr at 100 °C - 103 °C (i.e., 3 degree Celsius temperature gradient between 100 °C and 103 °C).

10 **Figure 10** is a graph of weight average molecular weight (i.e., average molecular weight average or average MW) using Size exclusion chromatography with a refractive index detector (SEC-RI) plotted as a function of time for solubilized fibroin in 9.3 M LiBr at 122 °C - 125 °C (i.e., 3 degree Celsius temperature gradient between 122 °C and 125 °C).

15 **Figure 11** is a graph illustrating percentage of amino acid modification in silk.

Figures 12A-12C are graphs illustrating percentage of amino acid modifications in Low Skid Silk and Mid Skid silk. **Fig. 12A** illustrates heavy chain modifications, **Fig. 12B** illustrates light chain modifications, and **Fig. 12C** illustrates fibrohexamerin modifications. N are Asparagines that become aspartic acid and Q are
20 Glutamines that become deamidated. M corresponds to Methionies that become oxidized. The numbers after each amino acid show its position along the amino acid chain from the corresponding protein.

Figures 13A- 13B are graphs illustrating percentage of amino acid modifications in Low Skid Silk and Mid Skid silk produced and lyophilized. **Fig. 13A**
25 illustrates heavy chain modifications and **Fig. 13B** illustrates light chain modifications. N are Asparagines that become aspartic acid and Q are Glutamines that become deamidated. M corresponds to Methionies that become oxidized. The numbers after each amino acid show its position along the amino acid chain from the corresponding protein.

30 **Figures 14A- 14B** are graphs illustrating percentage of amino acid modifications in Low Skid silk produced in Walpole and Medford using the Skid process with differing process parameters and variable levels. **Fig. 14A** illustrates

heavy chain modifications and **Fig. 14B** illustrates light chain modifications. N are Asparagines that become aspartic acid and Q are Glutamines that become deamidated. M corresponds to Methionies that become oxidized. The numbers after each amino acid show its position along the amino acid chain from the corresponding protein.

5 **Figures 15A- 15D** are graphs illustrating percentage of amino acid modifications in Low and Mid silk produced in Skid and Benchtop processes. N are Asparagines that become aspartic acid and Q are Glutamines that become deamidated. M corresponds to Methionies that become oxidized. The numbers after each amino acid show its position along the amino acid chain from the corresponding protein.

10 **Figure 16** is an explanation of the method used to calculate percentage ratios of modified amino acids at specific locations along the sequence of each peptide.

Figure 17 illustrates an Anion exchange chromatography and size exclusion chromatography scheme of the isolation of Low Skid silk/modified peptide compositions. Low Skid silk/modified polypeptide compositions is composed of a variety of peptide populations, in a wide range of sizes and charge. Using Q-Sephadex anion exchange chromatography as a first step, and HiLoad Superdex 200 size exclusion chromatography as a second purification step, distinct populations of Low Skid silk/modified polypeptide compositions were separated. The Q-Sephadex eluate was loaded onto HiLoad Superdex 200 size exclusion chromatography, which resulted in negatively charged silk compositions/modified peptides fractionated by size.

Figures 18A and 18B are chromatograms of the anion exchange chromatography and the following size exclusion chromatography of the eluate (Q-eluate) of Low Skid silk/modified polypeptide compositions. **Fig. 18A:** Anion exchange chromatography was performed with a Q-Sephadex column (Cytiva). Low Skid silk/modified peptide compositions were separated to uncharged peptide population (flowthrough – light blue background) and eluted negatively charged silk compositions (eluate – light pink background) by anion exchange chromatography. Light yellow background indicates column wash with 50 mM Tris pH=8.0 before eluting the charged peptide population. **Fig. 18B:** The negatively charged eluate was loaded onto the Superdex 200 column and was flowed through the column with 50 mM Tris, 200 mM CaCl₂, pH=8.0. When the UV-280 absorbance started to increase fractions were collected to separate the Low Skid silk/modified peptide compositions

by size. The relative elution volume of silk compositions AS77 and AS81 are indicated on the chromatogram.

Figures 19A and 19B illustrates the Analytical Size Exclusion Chromatography of Low Skid silk/modified silk compositions and their constituent AS compositions. **Fig. 19A.** Average molecular weight in kDa of Low Skid silk (LS) and AS77-AS81 are shown. **Fig. 19B.** Polydispersity (PDI) measurements are shown. The numerical data is presented in Table 7.

Figure 20 is a SDS polyacrylamide gel electrophoresis of Low Skid silk/modified polypeptide compositions. Lanes are indicated by fraction number, at the order of elution from the Superdex 200 column, and their respective silk composition: fraction 6 is AS77, fraction 7 is AS78, fraction 8 is AS79, fraction 9 is AS80, and fraction 10 is AS81.

Figures 21A and 21B are graphs illustrating self-assembly reactions of the of the Low Skid silk/modified peptide compositions. Mid Skid Silk reaction was used as a positive control. **Fig. 21A.** Illustrate kinetic parameters of gel formation during self-assembly of silk. Self-Assembly parameters of Mid Skid silk: Amax is 0.6780 (Abs), SARF is 8.676, T0.5 is 3.668 h, and the FSAF is 3.08 (Abs/min). **Fig. 21B.** Is a snapshot of a later time point of the same self-assembly assay, 12 days after setting the assay. None of the tested fraction has self-assembled over time.

Figures 22A and 22B illustrate the characterization of Low Skid silk compositions by Dynamic Light Scattering. Low skid silk/modified peptide compositions were diluted to a concentration of 1 mg/mL, filtered, and analyzed by the Zetasizer Pro to estimate the diameter particle size of each silk composition. **Fig. 22A.** Illustrates intensity diameter particle size distribution measured for silk compositions AS77, AS78, AS79, AS80, and AS81. **Fig. 22B.** Illustrate correlogram functions of silk compositions AS77, AS78, AS79, AS80, AS81.

Figure 23 illustrates size exclusion chromatography scheme of the isolation of Low Skid silk/modified peptide compositions. Low Skid silk/modified polypeptide compositions is composed of a variety of peptide populations, in a wide range of sizes, using HiLoad Superdex 200 size exclusion chromatography, distinct populations of Low Skid silk/modified polypeptide compositions were separated.

Figure 24 is a chromatogram of Low Skid silk/modified polypeptide compositions loaded onto a Superdex 200 gel filtration column. Low Skid silk/modified peptide compositions were loaded onto the Superdex 200 column and were flowed through the column with 50 mM Tris, 200 mM CaCl₂, pH=8.0. When the UV-280 absorbance started to increase fractions were collected to separate the Low Skid silk/modified peptide compositions by size. The relative elution volume of silk compositions AS82, AS86, and AS87 are indicated on the chromatogram.

Figures 25A and 25B illustrate Analytical Size Exclusion Chromatography of Low Skid silk/modified silk compositions and their constituent AS compositions. **Fig. 25A.** Illustrates average molecular weight in kDa of Low Skid silk (LS) and AS82-AS89 are shown. **Fig. 25B.** Polydispersity (PDI) measurements are shown. The numerical data is presented in Table 9.

Figure 26 is an SDS polyacrylamide gel electrophoresis of Low Skid silk/modified polypeptide compositions. Lanes are indicated by fraction number, at the order of elution from the Superdex 200 column, and their respective silk composition: fraction 6 is AS82, fraction 7 is AS83, fraction 8 is AS84, fraction 9 is AS85, and fraction 10 is AS86.

Figures 27A and 27B are graphs illustrating self-assembly reactions of the of the Low Skid silk/modified peptide compositions. Mid Skid Silk reaction was used as a positive control. **Fig. 27A.** Illustrates kinetic parameters of gel formation during self-assembly of silk. Self-Assembly parameters of Mid Skid silk: Amax is 0.6978 (Abs), SARF is 8.591, T_{0.5} is 3.361 h, and the FSAF is 3.46 (Abs/min). **Fig. 27B.** Is a snapshot of a later time point of the same self-assembly assay, 18 days after setting the assay. AS87, AS88, and AS89 demonstrate gel formation at this time point, that was already observed five days post assay (LS, Low Skid silk; MS, Mid Skid silk).

Figures 28A- 28C are graphs showing characterization of Low Skid silk compositions by Dynamic Light Scattering. Low skid silk/modified peptide compositions were diluted to a concentration of 1 mg/mL, filtered, and analyzed by the Zetasizer Pro to estimate particle size of each silk composition. **Fig. 28A.** Shows intensity particle size distribution measured for silk compositions AS82, AS83, AS84, AS85, AS86, AS87, AS88, and AS89. **Fig. 28B.** Shows intensity particle size distribution measured for silk compositions AS82, Low Skid silk/modified peptide

compositions (LS), and Mid Skid silk/modified peptide compositions (MS). **Fig. 28C.** Shows correlogram functions of silk compositions AS82, AS83, AS84, AS85, AS86, AS87, AS88, AS89, Low Skid silk/modified peptide compositions (LS), Mid Skid silk/modified peptide compositions (MS).

5 **Figure 29** illustrates anion exchange chromatography (Q), hydrophobic interaction chromatography (HIC), and size exclusion chromatography (SEC) scheme of the isolation of Low Skid silk/modified peptide compositions. Low Skid silk/modified polypeptide compositions is composed of a variety of peptide populations, in a wide range of sizes and charge. Using Q-Sepharose anion exchange
10 chromatography as a first step, Butyl ImpRes Hydrophobic interactions resin as a second step, and HiLoad Superdex 200 size exclusion chromatography as a third purification step, distinct populations of Low Skid silk/modified polypeptide compositions were isolated. The Q-Sepharose eluate was loaded onto a Butyl ImpRes (HIC) column, and the HIC-eluate was loaded onto a HiLoad Superdex 200 size
15 exclusion chromatography, which resulted in fractionation of negatively charged silk compositions/modified peptides with hydrophobicity characteristics fractionated by size. The Q-Sepharose eluate contained negatively charged peptides in all sizes. Resolving these peptides by Butyl ImpRes column resulted in elution of high-molecular-weight, negatively charged, somewhat hydrophobic silk
20 compositions/modified peptides. The smaller negatively charged peptides were washed as flowthrough and did not bind the Butyl ImpRes column. The Q-HIC(elution) was loaded into Superdex 200 and was separated by size.

Figures 30A- 30E are chromatograms of anion exchange chromatography, hydrophobic interactions chromatography, and the following size exclusion
25 chromatography of Low Skid silk/modified polypeptide compositions. **Fig.30A.** illustrates anion exchange chromatography was performed with a Q-Sepharose column. Low Skid silk/modified peptide compositions were separated to uncharged peptide population (flowthrough – light blue background) and eluted negatively charged silk compositions (eluate – light pink background) by anion exchange
30 chromatography. Light yellow background indicates column wash with 50 mM Tris pH=8.0 before eluting the charged peptide population. **Fig.30B.** illustrates the negatively charged eluate (Q-elution) was loaded onto a Butyl ImpRes column, in the presence of 300 mM ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$, to expose hydrophobic

domains of the silk peptides, which allows binding to the column. The highly charged peptide population did not bind the column (flowthrough), highlighted in light blue. The column was washed until OD280 was reduced to ~100 units (light yellow). Then, the bound silk peptides (Q-HIC(elution)) were eluted by using 50 mM Tris, pH=8.0 without ammonium sulfate (light pink). **Fig.30C.** illustrates the Q-HIC(elution) was further fractionated by size exclusion chromatography (SEC), using the gel filtration column Superdex 200. The Q-HIC(elution) fraction was flowed through the column with 50 mM Tris, 200 mM CaCl₂, pH=8.0. When the UV-280 absorbance started to increase fractions were collected to separate the Low Skid silk/modified peptide compositions by size. The relative elution volume of silk compositions AS90 and AS94 are indicated on the chromatogram. **Fig.30D.** the Q-HIC(flowthrough) fraction was further fractionated by SEC, using the Superdex 200 column, at the same procedure as in (VC). The relative elution volume of silk compositions AS95 and AS100 are indicated on the chromatogram. **Fig.30E.** illustrates the superimposition of chromatograms (VC) and (VD). the Q-HIC(elution) fraction has a higher-molecular-weight range compared to the Q-HIC(flowthrough) fractions, which elutes later in SEC, and has lower-molecular-weight range.

Figures 31A- 31B are graphs showing analytical Size Exclusion Chromatography of Low Skid silk/modified silk compositions and their constituent AS compositions. **Fig.31A.** Average molecular weight in kDa of Low Skid silk (LS) and AS90-AS100 are shown. **Fig.31B.** Polydispersity (PDI) measurements are shown. The numerical data is presented in Table 11.

Figures 32A- 32B are SDS polyacrylamide gel electrophoresis of Low Skid silk/modified polypeptide compositions. **Fig. 32A.** Q-HIC(elution) SEC fractions. **Fig. 32B.** Q-HIC(flowthrough) SEC fractions. Lanes are indicated by fraction number, at the order of elution from the Superdex 200 column, and their respective silk composition: in **Fig. 32A**, fraction 6 is AS90, fraction 7 is AS91, fraction 8 is AS92, fraction 9 is AS93, and fraction 10 is AS94. In **Fig. 32B**, fraction 8 is AS95, fraction 9 is AS96, fraction 10 is AS97, fraction 11 is AS98, fraction 12 is AS99, and fraction 13 is AS100.

Figure 33 illustrates self-assembly reactions of the of the Low Skid silk/modified peptide compositions. Mid Skid Silk reaction was used as a positive

control. kinetic parameters of gel formation during self-assembly of silk. Q-HIC(elution) is the elution fraction that was eluted from the Butyl ImpRes column, prior to SEC purification; LS, Low Skid silk; MS, Mid Skid silk. Self-Assembly parameters of Mid Skid silk: Amax is 0.6974 (Abs), SARF is 8.661, T0.5 is 3.834 h, and the FSAF is 3.03 (Abs/min).

Figures 34A- 34F illustrate the characterization of Low Skid silk compositions by Dynamic Light Scattering. Low and Mid skid silk/modified peptide compositions were diluted to a concentration of 1 mg/mL, filtered, and analyzed by the Zetasizer Pro to estimate the diameter particle size of each silk composition. **Fig. 34A.** Intensity diameter particle size distribution measured for silk compositions AS90, Q-HIC(elution) fraction (prior to fractionation by SEC), Low Skid silk (LS), and Mid Skid silk (MS). **Fig. 34B.** Correlogram functions of silk compositions presented in (34A). **Fig. 34C.** Intensity diameter particle size distribution measured for silk compositions AS90-AS94, derived from Q-HIC(elution)-SEC fractionation process. **Fig. 34D.** Correlogram functions of silk compositions presented in (34C). **Fig. 34E.** Intensity diameter particle size distribution measured for silk compositions AS95-AS100, derived from Q-HIC(flowthrough)-SEC fractionation process. **Fig. 34F.** Correlogram functions of silk compositions presented in (34E).

Figure 35. illustrates size exclusion chromatography scheme of the isolation of Mid Skid silk/modified peptide compositions. Mid Skid silk/modified polypeptide compositions is composed of a variety of peptide populations, in a wide range of sizes, using HiLoad Superdex 200 size exclusion chromatography, distinct populations of Mid Skid silk/modified polypeptide compositions were able to be separated.

Figure 36. Is a chromatogram of Mid Skid silk/modified polypeptide compositions loaded onto a Superdex 200 gel filtration column. Mid Skid silk/modified peptide compositions were loaded onto the Superdex 200 column and were flowed through the column with 50 mM Tris, 200 mM CaCl₂, pH=8.0. When the UV-280 absorbance started to increase fractions were collected to separate the Mid Skid silk/modified peptide compositions by size. The relative elution volume of silk compositions AS107 and AS111 are indicated on the chromatogram.

Figures 37A- 37B. Illustrates analytical Size Exclusion Chromatography of Mid Skid silk/modified silk compositions and their constituent AS compositions. **Fig. 37A.** Average molecular weight in kDa of Mid Skid silk (MS) and AS106-AS111 are shown. **Fig. 37B.** Polydispersity (PDI) measurements are shown. The numerical data is presented in Table 14.

Figure 38. Is a SDS polyacrylamide gel electrophoresis of Mid Skid silk/modified polypeptide compositions. Lanes are indicated by fraction number, at the order of elution from the Superdex 200 column, and their respective silk composition: fraction 6 is AS107, fraction 7 is AS108, fraction 8 is AS109, fraction 9 is AS110, and fraction 10 is AS111.

Figure 39. Illustrates self-assembly reactions of the of the Mid Skid silk/modified peptide compositions. Kinetic parameters of gel formation during self-assembly of silk. Dashed red lines show how the self-assembly parameters Amax, SARF, and T0.5 were calculated for unfractionated Mid Skid silk (MS). These numerical calculated parameters of silk compositions AS106-AS111 can be found in Table 16. Low Skid silk (LS) was used as a negative control. LS, Low Skid silk; MS, Mid Skid silk.

Figures 40A-40B. Illustrates characterization of Mid Skid silk compositions by Dynamic Light Scattering. Mid skid silk/modified peptide compositions were diluted to a concentration of 1 mg/mL, filtered, and analyzed by the Zetasizer Pro to estimate particle size of each silk composition. **Fig. 40A.** Intensity particle size distribution measured for silk compositions AS106, AS107, AS108, AS109, AS110, AS111, and Mid Skid (MS). **Fig. 50B.** Correlation functions of silk compositions presented in (40A).

Figure 41. Illustrates anion exchange chromatography and size exclusion chromatography scheme of the isolation of Mid Skid silk/modified peptide compositions. Mid Skid silk/modified polypeptide compositions is composed of a variety of peptide populations, in a wide range of sizes and charge. Using Q-Sepharose anion exchange chromatography as a first step, and HiLoad Superdex 200 size exclusion chromatography as a second purification step, distinct populations of Mid Skid silk/modified polypeptide compositions were separated. The Q-Sepharose eluate was loaded onto HiLoad Superdex 200 size exclusion chromatography, which

resulted in negatively charged silk compositions/modified peptides fractionated by size.

Figures 42A-42B. Are chromatograms of the anion exchange chromatography and the following size exclusion chromatography of the eluate (Q-eluate) of Mid Skid silk/modified polypeptide compositions. **Fig. 42A.** Anion exchange chromatography was performed with a Q-Sepharose column (Cytiva). Mid Skid silk/modified peptide compositions were separated to uncharged peptide population (flowthrough – light blue background) and eluted negatively charged silk compositions (eluate – light pink background) by anion exchange chromatography. Light yellow background indicates column wash with 50 mM Tris pH=8.0 before eluting the charged peptide population. **Fig. 42B.** The negatively charged eluate (Q-elution) was loaded onto the Superdex 200 column and was flowed through the column with 50 mM Tris, 200 mM CaCl₂, pH=8.0. When the UV-280 absorbance started to increase fractions were collected to separate the Mid Skid silk/modified peptide compositions by size. The relative elution volume of silk compositions AS101 and AS105 are indicated on the chromatogram.

Figures 43A-43B. Illustrate analytical Size Exclusion Chromatography of Mid Skid silk/modified silk compositions and their constituent AS compositions. **Fig. 43A.** Average molecular weight in kDa of Mid Skid silk (MS) and AS101-AS105 are shown. **Fig. 43B.** Polydispersity (PDI) measurements are shown. The numerical data is presented in Table 16.

Figure 44A. Is a SDS polyacrylamide gel electrophoresis of Mid Skid silk/modified polypeptide compositions. Lanes are indicated by fraction number, at the order of elution from the Superdex 200 column, and their respective silk composition: fraction 6 is AS101, fraction 7 is AS102, fraction 8 is AS103, fraction 9 is AS104, and fraction 10 is AS105.

Figure 44B illustrates self-assembly reactions of the of the Mid Skid silk/modified peptide compositions. Low Skid Silk reaction was used as a negative control. Kinetic parameters of gel formation during self-assembly of silk are shown. Red dotted lines are shown to clarify the calculations of A_{max}, SARF (Self-Assembly Rate Factor), and T_{0.5} parameters in Table 17.

Figures 45A, 45B, and 45C. Are graphs illustrating characterization of Mid Skid silk compositions by Dynamic Light Scattering. Mid skid silk/modified peptide compositions were diluted to a concentration of 1 mg/mL, filtered, and analyzed by

the Zetasizer Pro (Malvern) to estimate the diameter particle size of each silk composition. **Fig. 45A.** Intensity diameter particle size distribution by intensity measured for silk compositions AS101, AS102, AS103, AS104, and AS105. **Fig. 45B.** Intensity diameter particle size distribution by intensity measured for silk compositions AS101, AS105, and Mid Skid silk (MS), to emphasize the size difference between AS101 and AS105. **Fig. 45C.** Correlogram functions of silk compositions AS101, AS102, AS103, AS104, AS105, and Mid Skid silk (MS).

Figure 46. is an illustration of the values for three molar mass moments (M_n , M_w , and M_z) as it relates to molar mass and the number of molecules at each molar mass. This example is applicable to a polydisperse sample; for a monodisperse sample, $M_n = M_w = M_z$.

Figures 47A- 47B are analytical SEC-MALS of Low, Mid and High Molecular Weight Silk. **Fig. 47A.** Weight Average Molecular Weight in kDa of Low, Mid, and High Molecular Weight Silk. **Fig. 47B.** Polydispersity Index (PDI) measurements of Low, Mid, and High Molecular Weight Silk are shown.

Figures 48A- 48B. Are analytical SEC-MALS of Low, Mid and High Molecular Weight Silk organized by Silk Type produced by different process parameters and variable levels. Individual data points are shown and the mean is represented by the height of the box. The bars encompass one standard deviation. **Fig. 48A.** Weight-Average Molecular Weight Ranges for Low, Mid, and High Molecular Weight Silk. **Fig. 48B.** PDI Ranges for Low, Mid, and High Molecular Weight Silk.

Figures 49A- 49B are analytical SEC-MALS of Low Skid silk/modified silk compositions and the constituent AS compositions as separated by Q-SEC (Q-eluent). **Fig. 49A.** Average molecular weight in kDa of Low Skid silk (LS) and AS77-AS81 are shown. **Fig. 49B.** Polydispersity (PDI) measurements are shown. The numerical data is presented in Table 24.

Figures 50A- 50B are analytical SEC-MALS of Low Skid silk/modified silk compositions and the constituent AS compositions as separated by SEC. **Fig. 50A.** Average molecular weight in kDa of Low Skid silk (LS) and AS82-AS89 are shown. **Fig. 50B.** Polydispersity (PDI) measurements are shown. The numerical data is presented in Table 25.

Figures 51A- 51B are analytical SEC-MALS of Low Skid silk/modified silk compositions and the constituent AS compositions as separated by Q-HIC-SEC (Q-HIC-Eluent). **Fig. 51A.** Average molecular weight in kDa of Low Skid silk (LS) and

AS90-AS94 are shown. **Fig. 51B.** Polydispersity (PDI) measurements are shown. The numerical data is presented in Table 26.

Figures 52A- 52B are analytical SEC-MALS of Low Skid silk/modified silk compositions and the constituent AS compositions as separated by Q-HIC-SEC (Q-HIC-Flowthrough). **Fig. 52A.** Average molecular weight in kDa of Low Skid silk (LS) and AS95-AS100 are shown. **Fig. 52B.** Polydispersity (PDI) measurements are shown. The numerical data is presented in Table 26.

Figures 53A- 53B are analytical SEC-MALS of Mid Skid silk/modified silk compositions and the constituent AS compositions as separated by Q--SEC (Q-flow through). **Fig. 53A.** Average molecular weight in kDa of Mid Skid silk (MS) and AS101-AS105 are shown. **Fig. 53B.** Polydispersity (PDI) measurements are shown. The numerical data is presented in Table 27.

Figures 54A- 54B are analytical SEC-MALS of Mid Skid silk/modified silk compositions and the constituent AS compositions as separated by SEC. **Fig. 54A.** Average molecular weight in kDa of Mid Skid silk (MS) and AS106-AS111 are shown. **Fig. 54B.** Polydispersity (PDI) measurements are shown. The numerical data is presented in Table 28.

Figures 55A, 55B, and 55C show the sequence listing for fibroin heavy chain.

Figure 56 shows the sequence listing for fibroin light chain.

Figure 57 shows the sequence listing for fibrohexamerin.

Figure 58 illustrates three chromatography principles of silk fractionalization.

Figure 59 illustrates the anion exchange chromatography followed by size exclusion chromatography of silk fractionalization.

Figure 60 illustrates the anion exchange chromatography followed by hydrophobic interactions chromatography and size exclusion chromatography.

Figure 61 is a chart including assays for characterizing silk fractions.

Figure 62 shows graphs presenting the data of Tables 44 and 45. Data shown with standard deviation. "Nanoclay" refers to Elementis Bentone Hydroclay.

Figure 63 is as diagram of the typical bentonite clay structure.

Figure 64 shows SEM images of cross-sectional area of film cast with Elementis Bentone Hydroclay 2001. Highly-ordered stacking of clay layers is visible.

Figure 65 is an SEM image of cross-sectional area of film cast with pure RSF.

Figure 66 is an SEM image of cross-sectional area of 1:1 RSF/2001 film cast under neutral (pH ~7.0) conditions. The layered structure of the clay is retained.

Figure 67 is an SEM image of cross-sectional area of 1:1 RSF/2001 film cast under acidic (pH ~3.5) conditions. Note the ribbon-like structure.

5 **Figure 68** illustrates the increased diffusive pathway created by the RSF/nanoclay composite.

10 **Figure 69** is an FTIR scan of the amide I region of RSF/2001 films cast under neutral conditions. The concentration of nanoclay is varied from 0% (red) to 70% (yellow). As nanoclay content increases, the amide I peak shifts left, away from the beta sheet region.

Figure 70 is a flow chart showing various embodiments for producing pure silk fibroin-based protein fragments (SPFs) of the present disclosure.

15 **Figure 71** is a flow chart showing various parameters that can be modified during the process of producing SPFs of the present disclosure during the extraction and the dissolution steps.

Figures 72 and 73 are graphs representing the effect of extraction volume on % mass loss.

20 **Figure 74** is a graph summarizing the effect of Extraction Time on Molecular Weight of silk processed under the conditions of 100 °C Extraction Temperature, 100 °C LiBr and 100 °C Oven Dissolution (Oven/Dissolution Time was varied).

Figure 75 is a graph summarizing the effect of Extraction Time on Molecular Weight of silk processed under the conditions of 100 °C Extraction Temperature, boiling LiBr and 60 °C Oven Dissolution (Oven/Dissolution Time was varied).

25 **Figure 76** is a graph summarizing the effect of Extraction Time on Molecular Weight of silk processed under the conditions of 100 °C Extraction Temperature, 60 °C LiBr and 60 °C Oven Dissolution (Oven/Dissolution Time was varied).

Figure 77 is a graph summarizing the effect of Extraction Time on Molecular Weight of silk processed under the conditions of 100 °C Extraction Temperature, 80 °C LiBr and 80 °C Oven Dissolution (Oven/Dissolution Time was varied).

30 **Figure 78** is a graph summarizing the effect of Extraction Time on Molecular Weight of silk processed under the conditions of 100 °C Extraction Temperature, 80 °C LiBr and 60 °C Oven Dissolution (Oven/Dissolution Time was varied).

Figure 79 is a graph summarizing the effect of Extraction Time on Molecular Weight of silk processed under the conditions of 100 °C Extraction Temperature, 100 °C LiBr and 60 °C Oven Dissolution (Oven/Dissolution Time was varied).

Figure 80 is a graph summarizing the effect of Extraction Time on Molecular Weight of silk processed under the conditions of 100 °C Extraction Temperature, 140 °C LiBr and 140 °C Oven Dissolution (Oven/Dissolution Time was varied).

Figure 81 is a graph summarizing the effect of Extraction Temperature on Molecular Weight of silk processed under the conditions of 60 minute Extraction Time, 100 °C LiBr and 100 °C Oven Dissolution (Oven/Dissolution Time was varied).

Figure 82 is a graph summarizing the effect of LiBr Temperature on Molecular Weight of silk processed under the conditions of 60 minute Extraction Time, 100 °C Extraction Temperature and 60 °C Oven Dissolution (Oven/Dissolution Time was varied).

Figure 83 is a graph summarizing the effect of LiBr Temperature on Molecular Weight of silk processed under the conditions of 30 minute Extraction Time, 100 °C Extraction Temperature and 60 °C Oven Dissolution (Oven/Dissolution Time was varied).

Figure 84 is a graph summarizing the effect of Oven/Dissolution Temperature on Molecular Weight of silk processed under the conditions of 100 °C Extraction Temperature, 30 minute Extraction Time, and 100 °C Lithium Bromide (Oven/Dissolution Time was varied).

Figure 85 is a graph summarizing the effect of Oven/Dissolution Temperature on Molecular Weight of silk processed under the conditions of 100 °C Extraction Temperature, 60 minute Extraction Time, and 100 °C Lithium Bromide. (Oven/Dissolution Time was varied).

Figure 86 is a graph summarizing the effect of Oven/Dissolution Temperature on Molecular Weight of silk processed under the conditions of 100 °C Extraction Temperature, 60 minute Extraction Time, and 140 °C Lithium Bromide (Oven/Dissolution Time was varied).

Figure 87 is a graph summarizing the effect of Oven/Dissolution Temperature on Molecular Weight of silk processed under the conditions of 100 °C Extraction Temperature, 30 minute Extraction Time, and 140 °C Lithium Bromide (Oven/Dissolution Time was varied).

Figure 88 is a graph summarizing the effect of Oven/Dissolution Temperature on Molecular Weight of silk processed under the conditions of 100 °C Extraction Temperature, 60 minute Extraction Time, and 80 °C Lithium Bromide (Oven/Dissolution Time was varied).

5 **Figure 89** is a graph summarizing the Molecular Weights of silk processed under varying conditions including Extraction Time, Extraction Temperature, Lithium Bromide (LiBr) Temperature, Oven Temperature for Dissolution, Oven Time for Dissolution.

10 **Figure 90** is a graph summarizing the Molecular Weights of silk processed under conditions in which Oven/Dissolution Temperature is equal to LiBr Temperature.

15 **Figures 91A-91C** illustrate Low-MW silk solid resulted from lyophilization described herein at different stages of grinding. **Fig. 91A** illustrates the coarse particles of the Low-MW silk solid immediate after removal from the lyophilization bottle. **Fig. 91B** illustrates the reduced size particle midway through grinding. **Fig. 91C** illustrates the fine particles with even size distribution at the completion grinding.

Figure 92 illustrates solid particles of Mid-MW silk solid.

20 **Figure 93** illustrates example of two different particle size solid silk particles formed during thin film evaporation described herein.

Figures 94A and 94B illustrate examples of microparticles prepared by a solution precipitation process described herein.

Figure 95 illustrates milled silk powder for uses described herein.

25 **Figures 96A-96B** illustrate a pouch containing the solid formulation comprising silk fibroin fragments described herein. **Fig. 96A** illustrates a pouch described herein containing loose silk fibroin fragments. **Fig. 96B** illustrates a pouch described herein containing a disc of cryo-pelletized silk fibroin fragments.

Figures 97A illustrates lyophilized silk pellets. **Fig. 97B** is a graph illustrating activated silk reconstitution yield.

30 **Figure 98A** shows lyophilized silk. **Fig. 98B** is a graph showing lyophilization temperatures.

Figure 99A shows cryo-pelletized silk. **Fig. 99B** is a graph showing lyophilization temperatures.

Figure 100 illustrates lyophilized beads at various concentrations.

Figures 101A- 101G are SEM images of Lyophilized Silk with Different Processing Conditions. **Fig. 101A** shows silk lyophilized at 6% with no annealing. **Fig. 101B** shows silk lyophilized at 6% with annealing. **Fig. 101C** shows silk lyophilized at 6% with no annealing. **Fig. 101D** shows silk lyophilized at 6% with annealing. **Fig. 101E** shows 16% silk with no annealing. **Fig. 101F** shows 16% silk with annealing at 4 hours. **Fig. 101G** shows 16% silk with annealing at 20 hours.

Figures 102A- 102B are images of powder lyophilized silk from densified lyophilized pellets. **Fig. 102A** shows 10% Mid Mw Activated Silk™ grounded from densified pellets. **Fig. 102B** shows 16% Low Mw Activated Silk™ grounded from densified pellets.

Figure 103 is an images showing how particle size and reconstitution method impacts reconstitution yield. The finer grind silk powder stayed above liquid level and did not wet out for reconstitution using static method vs. the rougher grind.

Figures 104A- 104C are images demonstrating the ability to spray Activated Silk™. **Fig. 104A** is a close up of aerosol nozzle during spraying. **Fig. 104B** illustrates a spray mist stream. **Fig. 104C** illustrates the spray pattern using aerosol can 6" above surface.

Figure 105A is an image illustrating bulk "heaving" away from tray during lyophilization.

Figure 105B is an image illustrating meltback.

Figure 106 is a graph illustrating a bulk lyophilization example profile.

Figure 107 is an image of product from Bulk freeze lyophilization.

Figure 108 is an image of product meltback.

Figure 109 is an image showing the discoloration of the final product.

Figure 110 is a graph illustrating Profile 5 results.

Figures 111A- 111C are images of Cryo Lyophilized Pellets. **Fig. 111A** is 6% Cryo Lyophilized Pellets. **Fig. 111B** is 10% Cryo Lyophilized Pellets. **Fig. 111C** is 17% Cryo Lyophilized Pellets.

Figure 112 is a graph illustrating a BET Isotherm linear plot.

Figure 113 are SEM image of Lyophilized Activated Silk™ via cryopelletizing method.

Figure 114 is a graph illustrating "z-average" particle size.

Figure 115 is an image showing samples of the silk solution.

Figure 116 are graphs showing Sample output for both Intensity and Z-Average Particle Size. Over time, particle size distribution shifts towards higher size regimes, increasing intensity peaks in that region (Left). This is summarized by an increase in the Z-Average (Right). The Python program allows for quick comparison of data across timepoints and samples.

Figure 117 is an image showing the workflow for Dynamic Light Scattering, Data Processing in Python and JMP.

Figure 118 is a graph illustrating in silk-only controls, 27P is unsurprisingly orders of magnitude more stable than 33B over a range of temperatures and concentrations (Below Left, Top Right). However, 33B's faster aggregation makes it a useful model system in which to study excipient impact on aggregation rate (Below Right). (n = 3 replicates unless otherwise noted).

Figure 118 are graphs illustrating baseline system aggregation for both 27P and 33B. The graphs show that 27P is orders of magnitude more stable than 33B over a range of temperatures and concentrations.

Figure 119A is a graph illustrating baseline aggregation of 27P.

Figure 119B is a graph illustrating the main effects on aggregation rate of the baseline system.

Figure 120 is a graph illustrating z- average for various silk solutions.

Figure 121 is a graph illustrating z- average for various silk solutions.

Figure 122 are graphs illustrating z- average for various silk solutions.

Figure 123 is an image showing soluble silk, gel silk, and particulates in solution.

Figure 124 is a graph illustrating z- average for various silk solutions.

Figure 125 is a graph illustrating z- average for various silk solutions.

Figure 126 is a graph illustrating z- average for various silk solutions.

Figures 127A-127C are graphs illustrating testing stability of concentrated 33B solutions. **Fig. 127A** shows results from DLS testing of 33B samples. **Fig. 127B** shows the z-average for various silk solutions at 4 °C. **Fig. 127C** shows incubation curves at 70 °C. These graphs highlight the vast differences in aggregation profile with temperature, and further shows the impressive performance of PBS buffer in slowing aggregation.

Figures 128A- 128B shows results of excipients from 40C Testing. **Fig. 128A** Arginine HCl, MgCl₂ > NaCl, KCl > PBS > CaCl₂. **Fig. 128B** The two best

performers (ArgHCl, MgCl₂ have not changed pH nor aggregated after 1085hr (45 days). Equivalent 33B/DI water fully gelled after 10 days at this temperature.

Figures 129A- 129B are scans taken of solutions stored at 40° C over the course of about 45 days (**Fig. 129A**) show a similar aggregation profile as solutions continuously scanned at 70C over the course of 1hr (**Fig. 129B**).

Figures 130A- 130C are graphs illustrating how the normalized relative aggregation rates seem to correlate between temperatures.

Figures 131A- 131B are graphs showing best-performing excipients at 40C also show marked improvements at 70C compared to DI water.

Figure 132 shows the equation for modeling 70C aggregation in prism.

Figure 133A- 133C show 70C curves that were analyzed via the One-Phase Association model in Prism software to extract better quantitative data from samples.

Figure 134 is a graph showing the results of preservative screening, specifically the continuous z-average measurements at 70C.

Figure 135 is a bar graph showing the effect of various preservatives on aggravation.

Figure 136 is a graph showing that salts have potential to stabilize otherwise aggregation-prone solutions.

Figure 137 is a bar graph showing that salts have potential to stabilize otherwise aggregation-prone solutions.

DETAILED DESCRIPTION

The disclosure provides a lyophilized or sprayable peptide or protein fragment comprising a plurality of amino acids selected from M, R, V, K, T, F, I, L, C, A, Q, Y, N, D, E, G, S, H, P, and W, wherein at least one of the amino acids is modified, substituted, or replaced as disclosed herein.

The disclosure provides a pouch comprising a plurality of holes, wherein the pouch encloses a substantially solid formulation comprising silk fibroin fragments. The disclosure further provides a method of reconstituting a substantially solid formulation comprising silk fibroin fragments in a solvent. The disclosure also provides a method of making a pouch enclosing a substantially solid formulation of silk fibroin fragments.

Silk is a natural polymer produced by a variety of insects and spiders. Silk produced by *Bombyx mori* (silkworm) comprises a filament core protein, silk fibroin,

and a glue-like coating consisting of a nonfilamentous protein, sericin. Silk fibroin is a FDA approved, edible, non-toxic, and relative inexpensive silkworm cocoon derived proteins. The structure and content of amino acids in the silk fibroin protein are very similar to the tissue of the human body.

5 Methods of making silk fibroin or silk fibroin-based protein fragments are known and are described for example in U.S. Patents Nos. 9,187,538, 9,511,012, 9,517,191, 9,522,107, 9,522,108, 9,545,369, and 10,166,177, all of which are incorporated herein in their entireties.

Definitions

10 As used in the preceding sections and throughout the rest of this specification, unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one skilled in the art to which this disclosure belongs. All patents and publications referred to herein are incorporated by reference in their entireties.

15 All percentages, parts and ratios are based upon the total weight of the eye care compositions of the present disclosure, unless otherwise specified. All such weights as they pertain to listed ingredients are based on the active level and, therefore, do not include solvents or by-products that may be included in commercially available materials, unless otherwise specified. The term “weight
20 percent” may be denoted as “wt. %” or % w/w herein.

 As used herein, the term “a”, “an”, or “the” generally is construed to cover both the singular and the plural forms.

 The term “about” as used herein, generally refers to a particular numeric value that include variation and an acceptable error range as determined by one of ordinary
25 skill in the art, which will depend in part on how the numeric value is measured or determined, i.e., the limitations of the measurement system. For example, “about” can mean zero variation, and a range of $\pm 20\%$, $\pm 10\%$, or $\pm 5\%$ of a given numeric value.

 As used herein, the term “dermatologically acceptable carrier” means a carrier suitable for use in contact with mammalian keratinous tissue without causing any
30 adverse effects such as undue toxicity, incompatibility, instability, allergic response, for example. A dermatologically acceptable carrier may include, without limitations, water, liquid or solid emollients, humectants, solvents, and the like.

As used herein, the term “hydrophilic-lipophilic balance” (HLB) of a surfactant is a measure of the degree to which it is hydrophilic or hydrophobic, as determined by calculating values for the different regions of the molecule, as described by Griffin’s method $HLB = 20 * M_h/M$, where M_h is the molecular mass of the hydrophilic portion of the surfactant, and M is the molecular mass of the entire surfactant molecule, giving a result on a scale of 0 to 20. A HLB value of 0 corresponds to a completely lipophilic molecule, and a value of 20 corresponds to a completely hydrophilic molecule. The HLB value can be used to predict the surfactant properties of a molecule: $HLB < 10$: Lipid-soluble (water-insoluble), $HLB > 10$: Water-soluble (lipid-insoluble), $HLB = 1-3$: anti-foaming agent, 3-6: W/O (water-in-oil) emulsifier, 7-9: wetting and spreading agent, 8-16: O/W (oil-in-water) emulsifier, 13-16: detergent, 16-18: solubilizer or hydrotrope.

As used herein, “average weight average molecular weight” refers to an average of two or more values of weight average molecular weight of silk fibroin or fragments thereof of the same compositions, the two or more values determined by two or more separate experimental readings.

As used herein, the term polymer “polydispersity (PD)” is generally used as a measure of the broadness of a molecular weight distribution of a polymer, and is defined by the formula $polydispersity\ PD = \frac{Mw}{Mn}$.

As used herein, the term “substantially homogeneous” may refer to silk fibroin- based protein fragments that are distributed in a normal distribution about an identified molecular weight. As used herein, the term “substantially homogeneous” may refer to an even distribution of a component or an additive, for example, silk fibroin fragments, dermatologically acceptable carrier, etc., throughout a composition of the present disclosure.

As used herein, the terms “silk fibroin peptide,” “silk fibroin protein fragment,” and “silk fibroin fragment” are used interchangeably. Molecular weight or number of amino acids units are defined when molecular size becomes an important parameter.

As used herein, the term “fast-dissolving solid forms” refers to fast-dissolving solid forms including freeze dried forms (cakes, wafers, thin films), and compressed tablets.

As used herein, the terms “peptide” or “protein” refers to a chain of amino acids that are held together by peptide bonds (also called amide bonds). The basic distinguishing factors for proteins and peptides are size and structure. Peptides are smaller than proteins. Traditionally, peptides are defined as molecules that consist of
5 between 2 and 50 amino acids, whereas proteins are made up of 50 or more amino acids. In addition, peptides tend to be less well defined in structure than proteins, which can adopt complex conformations known as secondary, tertiary, and quaternary structures.

As used herein, the term “fibroin” or “silk protein” is a type of structural
10 protein produced by certain spider and insect species that produce silk (See definition provided in WIPO Pearl-WIPO’s Multilingual Terminology Portal database, <https://wipopearl.wipo.int/en/linguistic>). Fibroin may include silkworm fibroin, insect or spider silk protein (e.g., spidroin), recombinant spider protein, silk proteins present in other spider silk types, e.g., tubuliform silk protein (TuSP), flagelliform silk
15 protein, minor ampullate silk proteins, aciniform silk protein, pyriform silk protein, aggregate silk glue), silkworm fibroin produced by genetically modified silkworm, or recombinant silkworm fibroin.

As used herein, the term “silk fibroin” refers to silkworm fibroin, silk fibroin produced by genetically modified silkworm, or recombinant silkworm fibroin (See (1)
20 Narayan Ed., Encyclopedia of Biomedical Engineering, Vol. 2, Elsevier, 2019; (2) Kobayashi et al. Eds, Encyclopedia of Polymeric Nanomaterials, Springer, 2014, https://link.springer.com/referenceworkentry/10.1007%2F978-3-642-36199-9_323-1). In an embodiment, silk fibroin is obtained from *Bombyx mori*.

The term “solid solution” as used herein, refers to the active agent molecularly
25 dissolved in the solid excipient matrix such as hydrophobic polymers, wherein the active agent is miscible with the polymer matrix excipient.

The term “solid dispersion” as used herein, refers to the active agent dispersed as crystalline or amorphous particles, wherein the active agent is dispersed in an amorphous polymer and is distributed at random between the polymer matrix
30 excipient.

As used herein, the term “substantially homogeneous” may refer to silk fibroin-based protein fragments that are distributed in a normal distribution about an identified molecular weight. As used herein, the term “substantially homogeneous” may also refer to an even distribution of a component or an additive, for example, silk

fibroin-based protein fragments, dermatologically acceptable carrier, etc., throughout a silk composition or formulation.

As used herein, the term “surface tension” refers to the tendency of fluid surfaces to shrink into the minimum surface area possible. At liquid–air interfaces, surface tension results from the greater attraction of liquid molecules to each other (due to cohesion) than to the molecules in the air (due to adhesion). The net effect is an inward force at its surface that causes the liquid to behave as if its surface were covered with a stretched elastic membrane. Because of the relatively high attraction of water molecules to each other through a web of hydrogen bonds, water has a higher surface tension (72.8 mN/m at 20 °C) than most other liquids.

SPF Definitions and Properties

As used herein, “silk protein fragments” (SPF) include, without limitation, one or more of: “silk fibroin fragments” as defined herein; “recombinant silk fragments” as defined herein; “spider silk fragments” as defined herein; “silk fibroin-like protein fragments” as defined herein; “chemically modified silk fragments” as defined herein; and/or “sericin or sericin fragments” as defined herein. SPF may have any molecular weight values or ranges described herein, and any polydispersity values or ranges described herein. As used herein, in some embodiments the term “silk protein fragment” also refers to a silk protein that comprises or consists of at least two identical repetitive units which each independently selected from naturally-occurring silk polypeptides or of variations thereof, amino acid sequences of naturally-occurring silk polypeptides, or of combinations of both.

SPF Molecular Weight and Polydispersity

In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 1 to about 5 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 5 to about 10 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 10 to about 15 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 15 to about 20 kDa. In an embodiment, a composition of the present

disclosure includes SPF having an average weight average molecular weight selected from between about 14 to about 30 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 20 to about 25 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 25 to about 30 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 30 to about 35 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 35 to about 40 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 39 to about 54 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 40 to about 45 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 45 to about 50 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 50 to about 55 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 55 to about 60 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 60 to about 65 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 65 to about 70 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 70 to about 75 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 75 to about 80 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 80 to about 85 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 85 to about 90 kDa. In an embodiment, a composition of the present disclosure includes SPF having

an average weight average molecular weight selected from between about 90 to about 95 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 95 to about 100 kDa. In an embodiment, a composition of the present disclosure includes

5 SPF having an average weight average molecular weight selected from between about 100 to about 105 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 105 to about 110 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected

10 from between about 110 to about 115 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 115 to about 120 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 120 to about 125 kDa. In an embodiment, a

15 composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 125 to about 130 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 130 to about 135 kDa. In an embodiment, a composition of the present disclosure includes SPF having an

20 average weight average molecular weight selected from between about 135 to about 140 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 140 to about 145 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about

25 145 to about 150 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 150 to about 155 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 155 to about 160 kDa. In an embodiment, a composition of the

30 present disclosure includes SPF having an average weight average molecular weight selected from between about 160 to about 165 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 165 to about 170 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average

molecular weight selected from between about 170 to about 175 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 175 to about 180 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 180 to about 185 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 185 to about 190 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 190 to about 195 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 195 to about 200 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 200 to about 205 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 205 to about 210 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 210 to about 215 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 215 to about 220 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 220 to about 225 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 225 to about 230 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 230 to about 235 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 235 to about 240 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 240 to about 245 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 245 to about 250 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight

selected from between about 250 to about 255 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 255 to about 260 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 260 to about 265 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 265 to about 270 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 270 to about 275 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 275 to about 280 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 280 to about 285 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 285 to about 290 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 290 to about 295 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 295 to about 300 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 300 to about 305 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 305 to about 310 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 310 to about 315 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 315 to about 320 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 320 to about 325 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 325 to about 330 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from

between about 330 to about 335 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 335 to about 340 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 340 to about 345 kDa. In an embodiment, a composition of the present disclosure includes SPF having an average weight average molecular weight selected from between about 345 to about 350 kDa.

In some embodiments, compositions of the present disclosure include SPF compositions selected from compositions #1001 to #3500, having weight average molecular weights selected from about 1 kDa to about 250 kDa, and a polydispersity selected from between 1 and about 5 (including, without limitation, a polydispersity of 1), between 1 and about 1.5 (including, without limitation, a polydispersity of 1), between about 1.5 and about 2, between about 1.5 and about 3, between about 2 and about 2.5, between about 2.5 and about 3, between about 3 and about 3.5, between about 3.5 and about 4, between about 4 and about 4.5, and between about 4.5 and about 5:

PDI (about) MW (about)	1-5	1-1.5	1.5-2	1.5-3	2-2.5	2.5-3	3-3.5	3.5-4	4-4.5	4.5-5
1 kDa	1001	1002	1003	1004	1005	1006	1007	1008	1009	1010
2 kDa	1011	1012	1013	1014	1015	1016	1017	1018	1019	1020
3 kDa	1021	1022	1023	1024	1025	1026	1027	1028	1029	1030
4 kDa	1031	1032	1033	1034	1035	1036	1037	1038	1039	1040
5 kDa	1041	1042	1043	1044	1045	1046	1047	1048	1049	1050
6 kDa	1051	1052	1053	1054	1055	1056	1057	1058	1059	1060
7 kDa	1061	1062	1063	1064	1065	1066	1067	1068	1069	1070
8 kDa	1071	1072	1073	1074	1075	1076	1077	1078	1079	1080
9 kDa	1081	1082	1083	1084	1085	1086	1087	1088	1089	1090
10 kDa	1091	1092	1093	1094	1095	1096	1097	1098	1099	1100
11 kDa	1101	1102	1103	1104	1105	1106	1107	1108	1109	1110
12 kDa	1111	1112	1113	1114	1115	1116	1117	1118	1119	1120
13 kDa	1121	1122	1123	1124	1125	1126	1127	1128	1129	1130
14 kDa	1131	1132	1133	1134	1135	1136	1137	1138	1139	1140
15 kDa	1141	1142	1143	1144	1145	1146	1147	1148	1149	1150
16 kDa	1151	1152	1153	1154	1155	1156	1157	1158	1159	1160
17 kDa	1161	1162	1163	1164	1165	1166	1167	1168	1169	1170
18 kDa	1171	1172	1173	1174	1175	1176	1177	1178	1179	1180

19 kDa	1181	1182	1183	1184	1185	1186	1187	1188	1189	1190
20 kDa	1191	1192	1193	1194	1195	1196	1197	1198	1199	1200
21 kDa	1201	1202	1203	1204	1205	1206	1207	1208	1209	1210
22 kDa	1211	1212	1213	1214	1215	1216	1217	1218	1219	1220
23 kDa	1221	1222	1223	1224	1225	1226	1227	1228	1229	1230
24 kDa	1231	1232	1233	1234	1235	1236	1237	1238	1239	1240
25 kDa	1241	1242	1243	1244	1245	1246	1247	1248	1249	1250
26 kDa	1251	1252	1253	1254	1255	1256	1257	1258	1259	1260
27 kDa	1261	1262	1263	1264	1265	1266	1267	1268	1269	1270
28 kDa	1271	1272	1273	1274	1275	1276	1277	1278	1279	1280
29 kDa	1281	1282	1283	1284	1285	1286	1287	1288	1289	1290
30 kDa	1291	1292	1293	1294	1295	1296	1297	1298	1299	1300
31 kDa	1301	1302	1303	1304	1305	1306	1307	1308	1309	1310
32 kDa	1311	1312	1313	1314	1315	1316	1317	1318	1319	1320
33 kDa	1321	1322	1323	1324	1325	1326	1327	1328	1329	1330
34 kDa	1331	1332	1333	1334	1335	1336	1337	1338	1339	1340
35 kDa	1341	1342	1343	1344	1345	1346	1347	1348	1349	1350
36 kDa	1351	1352	1353	1354	1355	1356	1357	1358	1359	1360
37 kDa	1361	1362	1363	1364	1365	1366	1367	1368	1369	1370
38 kDa	1371	1372	1373	1374	1375	1376	1377	1378	1379	1380
39 kDa	1381	1382	1383	1384	1385	1386	1387	1388	1389	1390
40 kDa	1391	1392	1393	1394	1395	1396	1397	1398	1399	1400
41 kDa	1401	1402	1403	1404	1405	1406	1407	1408	1409	1410
42 kDa	1411	1412	1413	1414	1415	1416	1417	1418	1419	1420
43 kDa	1421	1422	1423	1424	1425	1426	1427	1428	1429	1430
44 kDa	1431	1432	1433	1434	1435	1436	1437	1438	1439	1440
45 kDa	1441	1442	1443	1444	1445	1446	1447	1448	1449	1450
46 kDa	1451	1452	1453	1454	1455	1456	1457	1458	1459	1460
47 kDa	1461	1462	1463	1464	1465	1466	1467	1468	1469	1470
48 kDa	1471	1472	1473	1474	1475	1476	1477	1478	1479	1480
49 kDa	1481	1482	1483	1484	1485	1486	1487	1488	1489	1490
50 kDa	1491	1492	1493	1494	1495	1496	1497	1498	1499	1500
51 kDa	1501	1502	1503	1504	1505	1506	1507	1508	1509	1510
52 kDa	1511	1512	1513	1514	1515	1516	1517	1518	1519	1520
53 kDa	1521	1522	1523	1524	1525	1526	1527	1528	1529	1530
54 kDa	1531	1532	1533	1534	1535	1536	1537	1538	1539	1540
55 kDa	1541	1542	1543	1544	1545	1546	1547	1548	1549	1550
56 kDa	1551	1552	1553	1554	1555	1556	1557	1558	1559	1560
57 kDa	1561	1562	1563	1564	1565	1566	1567	1568	1569	1570
58 kDa	1571	1572	1573	1574	1575	1576	1577	1578	1579	1580
59 kDa	1581	1582	1583	1584	1585	1586	1587	1588	1589	1590
60 kDa	1591	1592	1593	1594	1595	1596	1597	1598	1599	1600
61 kDa	1601	1602	1603	1604	1605	1606	1607	1608	1609	1610
62 kDa	1611	1612	1613	1614	1615	1616	1617	1618	1619	1620
63 kDa	1621	1622	1623	1624	1625	1626	1627	1628	1629	1630
64 kDa	1631	1632	1633	1634	1635	1636	1637	1638	1639	1640

65 kDa	1641	1642	1643	1644	1645	1646	1647	1648	1649	1650
66 kDa	1651	1652	1653	1654	1655	1656	1657	1658	1659	1660
67 kDa	1661	1662	1663	1664	1665	1666	1667	1668	1669	1670
68 kDa	1671	1672	1673	1674	1675	1676	1677	1678	1679	1680
69 kDa	1681	1682	1683	1684	1685	1686	1687	1688	1689	1690
70 kDa	1691	1692	1693	1694	1695	1696	1697	1698	1699	1700
71 kDa	1701	1702	1703	1704	1705	1706	1707	1708	1709	1710
72 kDa	1711	1712	1713	1714	1715	1716	1717	1718	1719	1720
73 kDa	1721	1722	1723	1724	1725	1726	1727	1728	1729	1730
74 kDa	1731	1732	1733	1734	1735	1736	1737	1738	1739	1740
75 kDa	1741	1742	1743	1744	1745	1746	1747	1748	1749	1750
76 kDa	1751	1752	1753	1754	1755	1756	1757	1758	1759	1760
77 kDa	1761	1762	1763	1764	1765	1766	1767	1768	1769	1770
78 kDa	1771	1772	1773	1774	1775	1776	1777	1778	1779	1780
79 kDa	1781	1782	1783	1784	1785	1786	1787	1788	1789	1790
80 kDa	1791	1792	1793	1794	1795	1796	1797	1798	1799	1800
81 kDa	1801	1802	1803	1804	1805	1806	1807	1808	1809	1810
82 kDa	1811	1812	1813	1814	1815	1816	1817	1818	1819	1820
83 kDa	1821	1822	1823	1824	1825	1826	1827	1828	1829	1830
84 kDa	1831	1832	1833	1834	1835	1836	1837	1838	1839	1840
85 kDa	1841	1842	1843	1844	1845	1846	1847	1848	1849	1850
86 kDa	1851	1852	1853	1854	1855	1856	1857	1858	1859	1860
87 kDa	1861	1862	1863	1864	1865	1866	1867	1868	1869	1870
88 kDa	1871	1872	1873	1874	1875	1876	1877	1878	1879	1880
89 kDa	1881	1882	1883	1884	1885	1886	1887	1888	1889	1890
90 kDa	1891	1892	1893	1894	1895	1896	1897	1898	1899	1900
91 kDa	1901	1902	1903	1904	1905	1906	1907	1908	1909	1910
92 kDa	1911	1912	1913	1914	1915	1916	1917	1918	1919	1920
93 kDa	1921	1922	1923	1924	1925	1926	1927	1928	1929	1930
94 kDa	1931	1932	1933	1934	1935	1936	1937	1938	1939	1940
95 kDa	1941	1942	1943	1944	1945	1946	1947	1948	1949	1950
96 kDa	1951	1952	1953	1954	1955	1956	1957	1958	1959	1960
97 kDa	1961	1962	1963	1964	1965	1966	1967	1968	1969	1970
98 kDa	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980
99 kDa	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990
100 kDa	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000
101 kDa	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
102 kDa	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020
103 kDa	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030
104 kDa	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040
105 kDa	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050
106 kDa	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060
107 kDa	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070
108 kDa	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080
109 kDa	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090
110 kDa	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100

111 kDa	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110
112 kDa	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120
113 kDa	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130
114 kDa	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140
115 kDa	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150
116 kDa	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160
117 kDa	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170
118 kDa	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180
119 kDa	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190
120 kDa	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200
121 kDa	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210
122 kDa	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220
123 kDa	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230
124 kDa	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240
125 kDa	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250
126 kDa	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260
127 kDa	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270
128 kDa	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280
129 kDa	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290
130 kDa	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300
131 kDa	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310
132 kDa	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320
133 kDa	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330
134 kDa	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340
135 kDa	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350
136 kDa	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360
137 kDa	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370
138 kDa	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380
139 kDa	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390
140 kDa	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400
141 kDa	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410
142 kDa	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420
143 kDa	2421	2422	2423	2424	2425	2426	2427	2428	2429	2430
144 kDa	2431	2432	2433	2434	2435	2436	2437	2438	2439	2440
145 kDa	2441	2442	2443	2444	2445	2446	2447	2448	2449	2450
146 kDa	2451	2452	2453	2454	2455	2456	2457	2458	2459	2460
147 kDa	2461	2462	2463	2464	2465	2466	2467	2468	2469	2470
148 kDa	2471	2472	2473	2474	2475	2476	2477	2478	2479	2480
149 kDa	2481	2482	2483	2484	2485	2486	2487	2488	2489	2490
150 kDa	2491	2492	2493	2494	2495	2496	2497	2498	2499	2500
151 kDa	2501	2502	2503	2504	2505	2506	2507	2508	2509	2510
152 kDa	2511	2512	2513	2514	2515	2516	2517	2518	2519	2520
153 kDa	2521	2522	2523	2524	2525	2526	2527	2528	2529	2530
154 kDa	2531	2532	2533	2534	2535	2536	2537	2538	2539	2540
155 kDa	2541	2542	2543	2544	2545	2546	2547	2548	2549	2550
156 kDa	2551	2552	2553	2554	2555	2556	2557	2558	2559	2560

157 kDa	2561	2562	2563	2564	2565	2566	2567	2568	2569	2570
158 kDa	2571	2572	2573	2574	2575	2576	2577	2578	2579	2580
159 kDa	2581	2582	2583	2584	2585	2586	2587	2588	2589	2590
160 kDa	2591	2592	2593	2594	2595	2596	2597	2598	2599	2600
161 kDa	2601	2602	2603	2604	2605	2606	2607	2608	2609	2610
162 kDa	2611	2612	2613	2614	2615	2616	2617	2618	2619	2620
163 kDa	2621	2622	2623	2624	2625	2626	2627	2628	2629	2630
164 kDa	2631	2632	2633	2634	2635	2636	2637	2638	2639	2640
165 kDa	2641	2642	2643	2644	2645	2646	2647	2648	2649	2650
166 kDa	2651	2652	2653	2654	2655	2656	2657	2658	2659	2660
167 kDa	2661	2662	2663	2664	2665	2666	2667	2668	2669	2670
168 kDa	2671	2672	2673	2674	2675	2676	2677	2678	2679	2680
169 kDa	2681	2682	2683	2684	2685	2686	2687	2688	2689	2690
170 kDa	2691	2692	2693	2694	2695	2696	2697	2698	2699	2700
171 kDa	2701	2702	2703	2704	2705	2706	2707	2708	2709	2710
172 kDa	2711	2712	2713	2714	2715	2716	2717	2718	2719	2720
173 kDa	2721	2722	2723	2724	2725	2726	2727	2728	2729	2730
174 kDa	2731	2732	2733	2734	2735	2736	2737	2738	2739	2740
175 kDa	2741	2742	2743	2744	2745	2746	2747	2748	2749	2750
176 kDa	2751	2752	2753	2754	2755	2756	2757	2758	2759	2760
177 kDa	2761	2762	2763	2764	2765	2766	2767	2768	2769	2770
178 kDa	2771	2772	2773	2774	2775	2776	2777	2778	2779	2780
179 kDa	2781	2782	2783	2784	2785	2786	2787	2788	2789	2790
180 kDa	2791	2792	2793	2794	2795	2796	2797	2798	2799	2800
181 kDa	2801	2802	2803	2804	2805	2806	2807	2808	2809	2810
182 kDa	2811	2812	2813	2814	2815	2816	2817	2818	2819	2820
183 kDa	2821	2822	2823	2824	2825	2826	2827	2828	2829	2830
184 kDa	2831	2832	2833	2834	2835	2836	2837	2838	2839	2840
185 kDa	2841	2842	2843	2844	2845	2846	2847	2848	2849	2850
186 kDa	2851	2852	2853	2854	2855	2856	2857	2858	2859	2860
187 kDa	2861	2862	2863	2864	2865	2866	2867	2868	2869	2870
188 kDa	2871	2872	2873	2874	2875	2876	2877	2878	2879	2880
189 kDa	2881	2882	2883	2884	2885	2886	2887	2888	2889	2890
190 kDa	2891	2892	2893	2894	2895	2896	2897	2898	2899	2900
191 kDa	2901	2902	2903	2904	2905	2906	2907	2908	2909	2910
192 kDa	2911	2912	2913	2914	2915	2916	2917	2918	2919	2920
193 kDa	2921	2922	2923	2924	2925	2926	2927	2928	2929	2930
194 kDa	2931	2932	2933	2934	2935	2936	2937	2938	2939	2940
195 kDa	2941	2942	2943	2944	2945	2946	2947	2948	2949	2950
196 kDa	2951	2952	2953	2954	2955	2956	2957	2958	2959	2960
197 kDa	2961	2962	2963	2964	2965	2966	2967	2968	2969	2970
198 kDa	2971	2972	2973	2974	2975	2976	2977	2978	2979	2980
199 kDa	2981	2982	2983	2984	2985	2986	2987	2988	2989	2990
200 kDa	2991	2992	2993	2994	2995	2996	2997	2998	2999	3000
201 kDa	3001	3002	3003	3004	3005	3006	3007	3008	3009	3010
202 kDa	3011	3012	3013	3014	3015	3016	3017	3018	3019	3020

203 kDa	3021	3022	3023	3024	3025	3026	3027	3028	3029	3030
204 kDa	3031	3032	3033	3034	3035	3036	3037	3038	3039	3040
205 kDa	3041	3042	3043	3044	3045	3046	3047	3048	3049	3050
206 kDa	3051	3052	3053	3054	3055	3056	3057	3058	3059	3060
207 kDa	3061	3062	3063	3064	3065	3066	3067	3068	3069	3070
208 kDa	3071	3072	3073	3074	3075	3076	3077	3078	3079	3080
209 kDa	3081	3082	3083	3084	3085	3086	3087	3088	3089	3090
210 kDa	3091	3092	3093	3094	3095	3096	3097	3098	3099	3100
211 kDa	3101	3102	3103	3104	3105	3106	3107	3108	3109	3110
212 kDa	3111	3112	3113	3114	3115	3116	3117	3118	3119	3120
213 kDa	3121	3122	3123	3124	3125	3126	3127	3128	3129	3130
214 kDa	3131	3132	3133	3134	3135	3136	3137	3138	3139	3140
215 kDa	3141	3142	3143	3144	3145	3146	3147	3148	3149	3150
216 kDa	3151	3152	3153	3154	3155	3156	3157	3158	3159	3160
217 kDa	3161	3162	3163	3164	3165	3166	3167	3168	3169	3170
218 kDa	3171	3172	3173	3174	3175	3176	3177	3178	3179	3180
219 kDa	3181	3182	3183	3184	3185	3186	3187	3188	3189	3190
220 kDa	3191	3192	3193	3194	3195	3196	3197	3198	3199	3200
221 kDa	3201	3202	3203	3204	3205	3206	3207	3208	3209	3210
222 kDa	3211	3212	3213	3214	3215	3216	3217	3218	3219	3220
223 kDa	3221	3222	3223	3224	3225	3226	3227	3228	3229	3230
224 kDa	3231	3232	3233	3234	3235	3236	3237	3238	3239	3240
225 kDa	3241	3242	3243	3244	3245	3246	3247	3248	3249	3250
226 kDa	3251	3252	3253	3254	3255	3256	3257	3258	3259	3260
227 kDa	3261	3262	3263	3264	3265	3266	3267	3268	3269	3270
228 kDa	3271	3272	3273	3274	3275	3276	3277	3278	3279	3280
229 kDa	3281	3282	3283	3284	3285	3286	3287	3288	3289	3290
230 kDa	3291	3292	3293	3294	3295	3296	3297	3298	3299	3300
231 kDa	3301	3302	3303	3304	3305	3306	3307	3308	3309	3310
232 kDa	3311	3312	3313	3314	3315	3316	3317	3318	3319	3320
233 kDa	3321	3322	3323	3324	3325	3326	3327	3328	3329	3330
234 kDa	3331	3332	3333	3334	3335	3336	3337	3338	3339	3340
235 kDa	3341	3342	3343	3344	3345	3346	3347	3348	3349	3350
236 kDa	3351	3352	3353	3354	3355	3356	3357	3358	3359	3360
237 kDa	3361	3362	3363	3364	3365	3366	3367	3368	3369	3370
238 kDa	3371	3372	3373	3374	3375	3376	3377	3378	3379	3380
239 kDa	3381	3382	3383	3384	3385	3386	3387	3388	3389	3390
240 kDa	3391	3392	3393	3394	3395	3396	3397	3398	3399	3400
241 kDa	3401	3402	3403	3404	3405	3406	3407	3408	3409	3410
242 kDa	3411	3412	3413	3414	3415	3416	3417	3418	3419	3420
243 kDa	3421	3422	3423	3424	3425	3426	3427	3428	3429	3430
244 kDa	3431	3432	3433	3434	3435	3436	3437	3438	3439	3440
245 kDa	3441	3442	3443	3444	3445	3446	3447	3448	3449	3450
246 kDa	3451	3452	3453	3454	3455	3456	3457	3458	3459	3460
247 kDa	3461	3462	3463	3464	3465	3466	3467	3468	3469	3470
248 kDa	3471	3472	3473	3474	3475	3476	3477	3478	3479	3480

249 kDa	3481	3482	3483	3484	3485	3486	3487	3488	3489	3490
250 kDa	3491	3492	3493	3494	3495	3496	3497	3498	3499	3500

As used herein, “low molecular weight,” “low MW,” or “low-MW” SPF may include SPF having a weight average molecular weight, or average weight average molecular weight selected from between about 5 kDa to about 38 kDa, about 14 kDa to about 30 kDa, or about 6 kDa to about 17 kDa. In some embodiments, a target low molecular weight for certain SPF may be weight average molecular weight of about 5 kDa, about 6 kDa, about 7 kDa, about 8 kDa, about 9 kDa, about 10 kDa, about 11 kDa, about 12 kDa, about 13 kDa, about 14 kDa, about 15 kDa, about 16 kDa, about 17 kDa, about 18 kDa, about 19 kDa, about 20 kDa, about 21 kDa, about 22 kDa, about 23 kDa, about 24 kDa, about 25 kDa, about 26 kDa, about 27 kDa, about 28 kDa, about 29 kDa, about 30 kDa, about 31 kDa, about 32 kDa, about 33 kDa, about 34 kDa, about 35 kDa, about 36 kDa, about 37 kDa, or about 38 kDa.

As used herein, “medium molecular weight,” “medium MW,” or “mid-MW” SPF may include SPF having a weight average molecular weight, or average weight average molecular weight selected from between about 31 kDa to about 55 kDa, or about 39 kDa to about 54 kDa. In some embodiments, a target medium molecular weight for certain SPF may be weight average molecular weight of about 31 kDa, about 32 kDa, about 33 kDa, about 34 kDa, about 35 kDa, about 36 kDa, about 37 kDa, about 38 kDa, about 39 kDa, about 40 kDa, about 41 kDa, about 42 kDa, about 43 kDa, about 44 kDa, about 45 kDa, about 46 kDa, about 47 kDa, about 48 kDa, about 49 kDa, about 50 kDa, about 51 kDa, about 52 kDa, about 53 kDa, about 54 kDa, or about 55 kDa.

As used herein, “high molecular weight,” “high MW,” or “high-MW” SPF may include SPF having a weight average molecular weight, or average weight average molecular weight selected from between about 55 kDa to about 150 kDa. In some embodiments, a target high molecular weight for certain SPF may be about 55 kDa, about 56 kDa, about 57 kDa, about 58 kDa, about 59 kDa, about 60 kDa, about 61 kDa, about 62 kDa, about 63 kDa, about 64 kDa, about 65 kDa, about 66 kDa, about 67 kDa, about 68 kDa, about 69 kDa, about 70 kDa, about 71 kDa, about 72 kDa, about 73 kDa, about 74 kDa, about 75 kDa, about 76 kDa, about 77 kDa, about 78 kDa, about 79 kDa, or about 80 kDa.

In some embodiments, the molecular weights described herein (e.g., low molecular weight silk, medium molecular weight silk, high molecular weight silk) may be converted to the approximate number of amino acids contained within the respective SPF, as would be understood by a person having ordinary skill in the art.

- 5 For example, the average weight of an amino acid may be about 110 Daltons (i.e., 110 g/mol). Therefore, in some embodiments, dividing the molecular weight of a linear protein by 110 Daltons may be used to approximate the number of amino acid residues contained therein.

- In an embodiment, SPF in a composition of the present disclosure have a
10 polydispersity selected from between 1 to about 5.0, including, without limitation, a polydispersity of 1. In an embodiment, SPF in a composition of the present disclosure have a polydispersity selected from between about 1.5 to about 3.0. In an embodiment, SPF in a composition of the present disclosure have a polydispersity selected from between 1 to about 1.5, including, without limitation, a polydispersity
15 of 1. In an embodiment, SPF in a composition of the present disclosure have a polydispersity selected from between about 1.5 to about 2.0. In an embodiment, SPF in a composition of the present disclosure have a polydispersity selected from between about 2.0 to about 2.5. In an embodiment, SPF in a composition of the present disclosure have a polydispersity selected from between about 2.5 to about 3.0.
20 In an embodiment, SPF in a composition of the present disclosure have a polydispersity selected from between about 3.0 to about 3.5. In an embodiment, SPF in a composition of the present disclosure have a polydispersity selected from between about 3.5 to about 4.0. In an embodiment, SPF in a composition of the present disclosure have a polydispersity selected from between about 4.0 to about 4.5.
25 In an embodiment, SPF in a composition of the present disclosure have a polydispersity selected from between about 4.5 to about 5.0.

- In an embodiment, SPF in a composition of the present disclosure have a polydispersity of 1. In an embodiment, SPF in a composition of the present disclosure have a polydispersity of about 1.1. In an embodiment, SPF in a composition of the
30 present disclosure have a polydispersity of about 1.2. In an embodiment, SPF in a composition of the present disclosure have a polydispersity of about 1.3. In an embodiment, SPF in a composition of the present disclosure have a polydispersity of about 1.4. In an embodiment, SPF in a composition of the present disclosure have a polydispersity of about 1.5. In an embodiment, SPF in a composition of the present

[illegible]

polydispersity of about 4.3. In an embodiment, SPF in a composition of the present disclosure have a polydispersity of about 4.4. In an embodiment, SPF in a composition of the present disclosure have a polydispersity of about 4.5. In an embodiment, SPF in a composition of the present disclosure have a polydispersity of about 4.6. In an embodiment, SPF in a composition of the present disclosure have a polydispersity of about 4.7. In an embodiment, SPF in a composition of the present disclosure have a polydispersity of about 4.8. In an embodiment, SPF in a composition of the present disclosure have a polydispersity of about 4.9. In an embodiment, SPF in a composition of the present disclosure have a polydispersity of about 5.0.

In some embodiments, in compositions described herein having combinations of low, medium, and/or high molecular weight SPF, such low, medium, and/or high molecular weight SPF may have the same or different polydispersities.

Silk Fibroin Fragments

Methods of making silk fibroin or silk fibroin protein fragments and their applications in various fields are known and are described for example in U.S. Patents Nos. 9,187,538, 9,511,012, 9,517,191, 9,522,107, 9,522,108, 9,545,369, and 10,166,177, 10,287,728 and 10,301,768, all of which are incorporated herein in their entireties. Raw silk from silkworm *Bombyx mori* is composed of two primary proteins: silk fibroin (approximately 75%) and sericin (approximately 25%). Silk fibroin is a fibrous protein with a semi-crystalline structure that provides stiffness and strength. As used herein, the term “silk fibroin” means the fibers of the cocoon of *Bombyx mori* having a weight average molecular weight of about 370,000 Da. The crude silkworm fiber consists of a double thread of fibroin. The adhesive substance holding these double fibers together is sericin. The silk fibroin is composed of a heavy chain having a weight average molecular weight of about 350,000 Da (H chain), and a light chain having a weight average molecular weight about 25,000 Da (L chain). Silk fibroin is an amphiphilic polymer with large hydrophobic domains occupying the major component of the polymer, which has a high molecular weight. The hydrophobic regions are interrupted by small hydrophilic spacers, and the N- and C-termini of the chains are also highly hydrophilic. The hydrophobic domains of the H-chain contain a repetitive hexapeptide sequence of Gly-Ala-Gly-Ala-Gly-Ser and repeats of Gly-Ala/Ser/Tyr dipeptides, which can form stable anti-parallel-sheet

crystallites. The amino acid sequence of the L-chain is non-repetitive, so the L-chain is more hydrophilic and relatively elastic. The hydrophilic (Tyr, Ser) and hydrophobic (Gly, Ala) chain segments in silk fibroin molecules are arranged alternatively such that allows self-assembling of silk fibroin molecules.

5 Provided herein are methods for producing pure and highly scalable silk fibroin-protein fragment mixture solutions that may be used across multiple industries for a variety of applications. Without wishing to be bound by any particular theory, it is believed that these methods are equally applicable to fragmentation of any SPF described herein, including without limitation recombinant silk proteins, and
10 fragmentation of silk-like or fibroin-like proteins.

 As used herein, the term “fibroin” includes silk worm fibroin and insect or spider silk protein. In an embodiment, fibroin is obtained from *Bombyx mori*. Raw silk from *Bombyx mori* is composed of two primary proteins: silk fibroin (approximately 75%) and sericin (approximately 25%). Silk fibroin is a fibrous
15 protein with a semi-crystalline structure that provides stiffness and strength. As used herein, the term “silk fibroin” means the fibers of the cocoon of *Bombyx mori* having a weight average molecular weight of about 370,000 Da. Conversion of these insoluble silk fibroin fibrils into water-soluble silk fibroin protein fragments requires the addition of a concentrated neutral salt (e.g., 8-10 M lithium bromide), which
20 interferes with inter- and intramolecular ionic and hydrogen bonding that would otherwise render the fibroin protein insoluble in water. Methods of making silk fibroin protein fragments, and/or compositions thereof, are known and are described for example in U.S. Patents Nos. 9,187,538, 9,511,012, 9,517,191, 9,522,107, 9,522,108, 9,545,369, and 10,166,177.

25 The raw silk cocoons from the silkworm *Bombyx mori* was cut into pieces. The pieces silk cocoons were processed in an aqueous solution of Na₂CO₃ at about 100 °C for about 60 minutes to remove sericin (degumming). The volume of the water used equals about 0.4 x raw silk weight and the amount of Na₂CO₃ is about 0.848 x the weight of the raw silk cocoon pieces. The resulting degummed silk cocoon pieces
30 were rinsed with deionized water three times at about 60 °C (20 minutes per rinse). The volume of rinse water for each cycle was 0.2 L x the weight of the raw silk cocoon pieces. The excess water from the degummed silk cocoon pieces was removed. After the DI water washing step, the wet degummed silk cocoon pieces were dried at room temperature. The degummed silk cocoon pieces were mixed with a

LiBr solution, and the mixture was heated to about 100 °C. The warmed mixture was placed in a dry oven and was heated at about 100 °C for about 60 minutes to achieve complete dissolution of the native silk protein. The resulting silk fibroin solution was filtered and dialyzed using Tangential Flow Filtration (TFF) and a 10 kDa membrane against deionized water for 72 hours. The resulting silk fibroin aqueous solution has a concentration of about 8.5 wt. %. Then, 8.5 % silk solution was diluted with water to result in a 1.0 % w/v silk solution. TFF can then be used to further concentrate the pure silk solution to a concentration of 20.0 % w/w silk to water.

Dialyzing the silk through a series of water changes is a manual and time intensive process, which could be accelerated by changing certain parameters, for example diluting the silk solution prior to dialysis. The dialysis process could be scaled for manufacturing by using semi-automated equipment, for example a tangential flow filtration system.

In some embodiments, the silk solutions are prepared under various preparation condition parameters such as: 90 °C 30 min, 90 °C 60 min, 100 °C 30 min, and 100 °C 60 min. Briefly, 9.3 M LiBr was prepared and allowed to sit at room temperature for at least 30 minutes. 5 mL of LiBr solution was added to 1.25 g of silk and placed in the 60 °C oven. Samples from each set were removed at 4, 6, 8, 12, 24, 168 and 192 hours.

In some embodiments, the silk solutions are prepared under various preparation condition parameters such as: 90 °C 30 min, 90 °C 60 min, 100 °C 30 min, and 100 °C 60 min. Briefly, 9.3 M LiBr solution was heated to one of four temperatures: 60 °C, 80 °C, 100 °C or boiling. 5 mL of hot LiBr solution was added to 1.25 g of silk and placed in the 60 °C oven. Samples from each set were removed at 1, 4 and 6 hours.

In some embodiments, the silk solutions are prepared under various preparation condition parameters such as: Four different silk extraction combinations were used: 90 °C 30 min, 90 °C 60 min, 100 °C 30 min, and 100 °C 60 min. Briefly, 9.3 M LiBr solution was heated to one of four temperatures: 60 °C, 80 °C, 100 °C or boiling. 5 mL of hot LiBr solution was added to 1.25 g of silk and placed in the oven at the same temperature of the LiBr. Samples from each set were removed at 1, 4 and 6 hours. 1 mL of each sample was added to 7.5 mL of 9.3 M LiBr and refrigerated for viscosity testing.

In some embodiments, SPF are obtained by dissolving raw unscoured, partially scoured, or scoured silkworm fibers with a neutral lithium bromide salt. The raw silkworm silks are processed under selected temperature and other conditions in order to remove any sericin and achieve the desired weight average molecular weight (M_w) and polydispersity (PD) of the fragment mixture. Selection of process parameters may be altered to achieve distinct final silk protein fragment characteristics depending upon the intended use. The resulting final fragment solution is silk fibroin protein fragments and water with parts per million (ppm) to non-detectable levels of process contaminants, levels acceptable in the pharmaceutical, medical and consumer eye care markets. The concentration, size and polydispersity of SPF may further be altered depending upon the desired use and performance requirements.

Methods of making silk protein fragments used in the compositions of the present disclosure are demonstrated in U.S. Patent Application Publication Nos. 2015/00933340, 2015/0094269, 2016/0193130, 2016/0022560, 2016/0022561, 2016/0022562, 2016/0022563, and 2016/0222579, 2016/0281294, and U.S. Patent Nos. 9,187,538, 9,522,107, 9,517,191, 9,522,108, 9,511,012, and 9,545,369, the entirety of which are incorporated herein by reference. However, an exemplary method is demonstrated in Fig. 70, which is a flow chart showing various embodiments for producing pure silk fibroin-based protein fragments (SPFs) of the present disclosure. It should be understood that not all of the steps illustrated are necessarily required to fabricate all silk solutions of the present disclosure. As illustrated in Fig. 70, step A, cocoons (heat-treated or non-heat-treated), silk fibers, silk powder or spider silk can be used as the silk source. If starting from raw silk cocoons from *Bombyx mori*, the cocoons can be cut into small pieces, for example pieces of approximately equal size, step B1. The raw silk is then extracted and rinsed to remove any sericin, step C1a. This results in substantially sericin free raw silk. In an embodiment, water is heated to a temperature between 84 °C and 100 °C (ideally boiling) and then Na₂CO₃ (sodium carbonate) is added to the boiling water until the Na₂CO₃ is completely dissolved. The raw silk is added to the boiling water/ Na₂CO₃ (100 °C) and submerged for approximately 15 - 90 minutes, where boiling for a longer time results in smaller silk protein fragments. In an embodiment, the water volume equals about 0.4 x raw silk weight and the Na₂CO₃ volume equals about 0.848 x raw silk weight. In an embodiment, the water volume equals 0.1 x raw silk weight

and the Na_2CO_3 volume is maintained at 2.12 g/L. This is demonstrated in Fig. 72 and Fig. 73: silk mass (x-axis) was varied in the same volume of extraction solution (i.e., the same volume of water and concentration of Na_2CO_3) achieving sericin removal (substantially sericin free) as demonstrated by an overall silk mass loss of 26 to 31 percent (y-axis).

Subsequently, the water dissolved Na_2CO_3 solution is drained and excess water/ Na_2CO_3 is removed from the silk fibroin fibers (e.g., ring out the fibroin extract by hand, spin cycle using a machine, etc.). The resulting silk fibroin extract is rinsed with warm to hot water to remove any remaining adsorbed sericin or contaminate, typically at a temperature range of about 40 °C to about 80 °C, changing the volume of water at least once (repeated for as many times as required). The resulting silk fibroin extract is a substantially sericin-depleted silk fibroin. In an embodiment, the resulting silk fibroin extract is rinsed with water at a temperature of about 60 °C. In an embodiment, the volume of rinse water for each cycle equals 0.1 L to 0.2 L x raw silk weight. It may be advantageous to agitate, turn or circulate the rinse water to maximize the rinse effect. After rinsing, excess water is removed from the extracted silk fibroin fibers (e.g., ring out fibroin extract by hand or using a machine). Alternatively, methods known to one skilled in the art such as pressure, temperature, or other reagents or combinations thereof may be used for the purpose of sericin extraction. Alternatively, the silk gland (100% sericin free silk protein) can be removed directly from a worm. This would result in liquid silk protein, without any alteration of the protein structure, free of sericin.

The extracted fibroin fibers are then allowed to dry completely. Once dry, the extracted silk fibroin is dissolved using a solvent added to the silk fibroin at a temperature between ambient and boiling, step C1b. In an embodiment, the solvent is a solution of Lithium bromide (LiBr) (boiling for LiBr is 140 °C). Alternatively, the extracted fibroin fibers are not dried but wet and placed in the solvent; solvent concentration can then be varied to achieve similar concentrations as to when adding dried silk to the solvent. The final concentration of LiBr solvent can range from 0.1 M to 9.3 M. Table D is a table summarizing the Molecular Weights of silk dissolved from different concentrations of Lithium Bromide (LiBr) and from different extraction and dissolution sizes. Complete dissolution of the extracted fibroin fibers can be achieved by varying the treatment time and temperature along with the concentration of dissolving solvent. Other solvents may be used including, but not

limited to, phosphate phosphoric acid, calcium nitrate, calcium chloride solution or other concentrated aqueous solutions of inorganic salts. To ensure complete dissolution, the silk fibers should be fully immersed within the already heated solvent solution and then maintained at a temperature ranging from about 60 °C to about 140 °C for 1-168 hrs. In an embodiment, the silk fibers should be fully immersed within the solvent solution and then placed into a dry oven at a temperature of about 100 °C for about 1 hour.

Table D: Molecular Weights of silk dissolved from different concentrations of LiBr and from different extraction and dissolution sizes

Sample	LiBr (M)	Avg MW	PD
STI 1(TFF-10-0019)	9.3	15727	2.033
STI 2(TFF-10-0033)	9.3	24587	2.3669
STI 3(TFF-10-0034)	9.3	25273	2.338
STI 9.3 M avg		21862	2.25
STI 1(TFF-10-0031)	~7.5	29645	3.0868
STI 2(TFF-10-0030)	~7.5	26856	2.9748
STI 7.5M avg		28250.5	3.0308
* TFF-10-0019 from 2 25 g extraction / 35 g dissolution			
* TFF-10-0034 from 100 g extraction/ 17-35 g dissolution			
* TFF-10-0033 from 100 g extraction/ 100 g dissolution			

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The temperature at which the silk fibroin extract is added to the LiBr solution (or vice versa) has an effect on the time required to completely dissolve the fibroin and on the resulting molecular weight and polydispersity of the final SPF mixture solution. In an embodiment, silk solvent solution concentration is less than or equal to 20% w/v. In addition, agitation during introduction or dissolution may be used to facilitate dissolution at varying temperatures and concentrations. The temperature of the LiBr solution will provide control over the silk protein fragment mixture molecular weight and polydispersity created. In an embodiment, a higher temperature will more quickly dissolve the silk offering enhanced process scalability and mass

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production of silk solution. In an embodiment, using a LiBr solution heated to a temperature between 80 °C - 140 °C reduces the time required in an oven in order to achieve full dissolution. Varying time and temperature at or above 60 °C of the dissolution solvent will alter and control the MW and polydispersity of the SPF mixture solutions formed from the original molecular weight of the native silk fibroin protein.

Alternatively, whole cocoons may be placed directly into a solvent, such as LiBr, bypassing extraction, step B2. This requires subsequent filtration of silk worm particles from the silk and solvent solution and sericin removal using methods known in the art for separating hydrophobic and hydrophilic proteins such as a column separation and/or chromatography, ion exchange, chemical precipitation with salt and/or pH, and or enzymatic digestion and filtration or extraction, all methods are common examples and without limitation for standard protein separation methods, step C2. Non-heat treated cocoons with the silkworm removed, may alternatively be placed into a solvent such as LiBr, bypassing extraction. The methods described above may be used for sericin separation, with the advantage that non-heat treated cocoons will contain significantly less worm debris.

Dialysis may be used to remove the dissolution solvent from the resulting dissolved fibroin protein fragment solution by dialyzing the solution against a volume of water, step E1. Pre-filtration prior to dialysis is helpful to remove any debris (i.e., silk worm remnants) from the silk and LiBr solution, step D. In one example, a 3 µm or 5 µm filter is used with a flow-rate of 200-300 mL/min to filter a 0.1% to 1.0% silk-LiBr solution prior to dialysis and potential concentration if desired. A method disclosed herein, as described above, is to use time and/or temperature to decrease the concentration from 9.3 M LiBr to a range from 0.1 M to 9.3 M to facilitate filtration and downstream dialysis, particularly when considering creating a scalable process method. Alternatively, without the use of additional time or temperature, a 9.3 M LiBr-silk protein fragment solution may be diluted with water to facilitate debris filtration and dialysis. The result of dissolution at the desired time and temperature filtration is a translucent particle-free room temperature shelf-stable silk protein fragment-LiBr solution of a known MW and polydispersity. It is advantageous to change the dialysis water regularly until the solvent has been removed (e.g., change water after 1 hour, 4 hours, and then every 12 hours for a total of 6 water changes). The total number of water volume changes may be varied based on the resulting concentration of solvent

used for silk protein dissolution and fragmentation. After dialysis, the final silk solution maybe further filtered to remove any remaining debris (i.e., silk worm remnants).

Alternatively, Tangential Flow Filtration (TFF), which is a rapid and efficient method for the separation and purification of biomolecules, may be used to remove the solvent from the resulting dissolved fibroin solution, step E2. TFF offers a highly pure aqueous silk protein fragment solution and enables scalability of the process in order to produce large volumes of the solution in a controlled and repeatable manner. The silk and LiBr solution may be diluted prior to TFF (20% down to 0.1% silk in either water or LiBr). Pre-filtration as described above prior to TFF processing may maintain filter efficiency and potentially avoids the creation of silk gel boundary layers on the filter's surface as the result of the presence of debris particles. Pre-filtration prior to TFF is also helpful to remove any remaining debris (i.e., silk worm remnants) from the silk and LiBr solution that may cause spontaneous or long-term gelation of the resulting water only solution, step D. TFF, recirculating or single pass, may be used for the creation of water-silk protein fragment solutions ranging from 0.1% silk to 30.0% silk (more preferably, 0.1% - 6.0% silk). Different cutoff size TFF membranes may be required based upon the desired concentration, molecular weight and polydispersity of the silk protein fragment mixture in solution. Membranes ranging from 1-100 kDa may be necessary for varying molecular weight silk solutions created for example by varying the length of extraction boil time or the time and temperate in dissolution solvent (e.g., LiBr). In an embodiment, a TFF 5 or 10 kDa membrane is used to purify the silk protein fragment mixture solution and to create the final desired silk-to-water ratio. As well, TFF single pass, TFF, and other methods known in the art, such as a falling film evaporator, may be used to concentrate the solution following removal of the dissolution solvent (e.g., LiBr) (with resulting desired concentration ranging from 0.1% to 30% silk). This can be used as an alternative to standard HFIP concentration methods known in the art to create a water-based solution. A larger pore membrane could also be utilized to filter out small silk protein fragments and to create a solution of higher molecular weight silk with and/or without tighter polydispersity values.

Table C is a table summarizing Molecular Weights for some embodiments of silk protein solutions of the present disclosure. Silk protein solution processing conditions were as follows: 100 °C extraction for 20 min, room temperature rinse,

LiBr in 60 °C oven for 4-6 hours. TFF processing conditions for water-soluble films were as follows: 100 °C extraction for 60 min, 60 °C rinse, 100 °C LiBr in 100 °C oven for 60 min. Figs. 93-104 further demonstrate manipulation of extraction time, LiBr dissolution conditions, and TFF processing and resultant example molecular weights and polydispersities. These examples are not intended to be limiting, but rather to demonstrate the potential of specifying parameters for specific molecular weight silk fragment solutions.

Table C: Molecular Weights of silk protein solutions of the present disclosure

Sample ID	Sample Description	Mn	Mw	Polydispersity (PD) (Mw/Mn)
A	TFF 5kDa	14,497	33,874	2.3366
B	TFF 10 kDa	14,542	33,455	2.3006
C	TFF 10 kDa	14,972	34,026	2.2726
D	Silk protein solution in water	12,055	26,531	2.2008

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assay for LiBr and Na₂CO₃ detection was performed using an HPLC system equipped with evaporative light scattering detector (ELSD). The calculation was performed by linear regression of the resulting peak areas for the analyte plotted against concentration. More than one sample of a number of formulations of the present disclosure was used for sample preparation and analysis. Generally, four samples of different formulations were weighed directly in a 10 mL volumetric flask. The samples were suspended in 5 mL of 20 mM ammonium formate (pH 3.0) and kept at 2-8 °C for 2 hours with occasional shaking to extract analytes from the film. After 2 hours the solution was diluted with 20 mM ammonium formate (pH 3.0). The sample solution from the volumetric flask was transferred into HPLC vials and injected into the HPLC-ELSD system for the estimation of sodium carbonate and lithium bromide.

The analytical method developed for the quantitation of Na₂CO₃ and LiBr in silk protein formulations was found to be linear in the range 10 - 165 µg/mL, with RSD for injection precision as 2% and 1% for area and 0.38% and 0.19% for retention time for sodium carbonate and lithium bromide respectively. The analytical method can be applied for the quantitative determination of sodium carbonate and lithium bromide in silk protein formulations.

The final silk protein fragment solution is pure silk protein fragments and water with PPM to undetectable levels of particulate debris and/or process

contaminants, including LiBr and Na₂CO₃. Tables A and B are tables summarizing LiBr and Na₂CO₃ concentrations in solutions of the present disclosure. In Table A, the processing conditions included 100 °C extraction for 60 min, 60 °C rinse, 100 °C LiBr in 100 °C oven for 60 min. TFF conditions including pressure differential and number of dia-filtration volumes were varied. In Table B, the processing conditions included 100 °C boil for 60 min, 60 °C rinse, LiBr in 60 °C oven for 4-6 hours.

Table A: Lithium Bromide and Sodium Carbonate Concentration in Silk Protein Solution

Sample ID	Sample Description	Average Concentration of Na ₂ CO ₃ (ppm)	Average Concentration of LiBr (ppm)
A	TFF 5kDa	32.13	90.85
B	TFF 10 kDa	42.91	107
C	TFF 10 kDa	49.06	78.55
D	STI 1(TFF-10-0019)	2.17	129.07
E	STI 2(TFF-10-0033)	2.63	196.2
F	STI 3(TFF-10-0034)	4.18	248.93

10 **Table B:** Lithium Bromide and Sodium Carbonate content in Silk Protein Solution

Sample ID	Sample Weight (mg)	Concentration
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	Solution Volume Equivalent to (X) Films		Na ₂ CO ₃	LiBr
1	6	0.171	ND	ND
2	8	0.228	ND	ND
3	10	0.285	ND	ND
4	12	0.342	ND	ND
5	Neat	-	ND	ND

*ND=None Detected

Either the silk fragment-water solutions, the lyophilized silk protein fragment mixture, or any other compositions including SPFs, can be sterilized following standard methods in the art not limited to filtration, heat, radiation or e-beam. It is anticipated that the silk protein fragment mixture, because of its shorter protein polymer length, will withstand sterilization better than intact silk protein solutions described in the art. Additionally, silk articles created from the SPF mixtures described herein may be sterilized as appropriate to application.

Fig. 71 is a flow chart showing various parameters that can be modified during the process of producing a silk protein fragment solution of the present disclosure during the extraction and the dissolution steps. Select method parameters may be altered to achieve distinct final solution characteristics depending upon the intended use, e.g., molecular weight and polydispersity. It should be understood that not all of the steps illustrated are necessarily required to fabricate all silk solutions of the present disclosure.

In an embodiment, a process for producing a silk protein fragment solution of the present disclosure includes forming pieces of silk cocoons from the Bombyx mori silk worm; extracting the pieces at about 100 °C in a solution of water and Na₂CO₃ for about 60 minutes, wherein a volume of the water equals about 0.4 x raw silk weight and the amount of Na₂CO₃ is about 0.848 x the weight of the pieces to form a silk

fibroin extract; triple rinsing the silk fibroin extract at about 60 °C for about 20 minutes per rinse in a volume of rinse water, wherein the rinse water for each cycle equals about 0.2 L x the weight of the pieces; removing excess water from the silk fibroin extract; drying the silk fibroin extract; dissolving the dry silk fibroin extract in a LiBr solution, wherein the LiBr solution is first heated to about 100 °C to create a silk and LiBr solution and maintained; placing the silk and LiBr solution in a dry oven at about 100 °C for about 60 minutes to achieve complete dissolution and further fragmentation of the native silk protein structure into mixture with desired molecular weight and polydispersity; filtering the solution to remove any remaining debris from the silkworm; diluting the solution with water to result in a 1% silk solution; and removing solvent from the solution using Tangential Flow Filtration (TFF). In an embodiment, a 10 kDa membrane is utilized to purify the silk solution and create the final desired silk-to-water ratio. TFF can then be used to further concentrate the pure silk solution to a concentration of 2% silk to water.

Each process step from raw cocoons to dialysis is scalable to increase efficiency in manufacturing. Whole cocoons are currently purchased as the raw material, but pre-cleaned cocoons or non-heat treated cocoons, where worm removal leaves minimal debris, have also been used. Cutting and cleaning the cocoons is a manual process, however for scalability this process could be made less labor intensive by, for example, using an automated machine in combination with compressed air to remove the worm and any particulates, or using a cutting mill to cut the cocoons into smaller pieces. The extraction step, currently performed in small batches, could be completed in a larger vessel, for example an industrial washing machine where temperatures at or in between 60 °C to 100 °C can be maintained. The rinsing step could also be completed in the industrial washing machine, eliminating the manual rinse cycles. Dissolution of the silk in LiBr solution could occur in a vessel other than a convection oven, for example a stirred tank reactor. Dialyzing the silk through a series of water changes is a manual and time intensive process, which could be accelerated by changing certain parameters, for example diluting the silk solution prior to dialysis. The dialysis process could be scaled for manufacturing by using semi-automated equipment, for example a tangential flow filtration system.

Without wishing to be bound by any particular theory, varying extraction (i.e., time and temperature), LiBr (i.e., temperature of LiBr solution when added to silk fibroin extract or vice versa) and dissolution (i.e., time and temperature) parameters

results in solvent and silk solutions with different viscosities, homogeneities, and colors. While also not wishing to be bound by any particular theory, increasing the temperature for extraction, lengthening the extraction time, using a higher temperature LiBr solution at emersion and over time when dissolving the silk and increasing the time at temperature (e.g., in an oven as shown here, or an alternative heat source) all resulted in less viscous and more homogeneous solvent and silk solutions. While almost all parameters resulted in a viable silk solution, methods that allow complete dissolution to be achieved in fewer than 4 to 6 hours are preferred for process scalability.

10 In an embodiment, solutions of silk fibroin protein fragments having a weight average selected from between about 6 kDa to about 17 kDa are prepared according to following steps: degumming a silk source by adding the silk source to a boiling (100 °C) aqueous solution of sodium carbonate for a treatment time of between about 30 minutes to about 60 minutes; removing sericin from the solution to produce a silk
15 fibroin extract comprising non- detectable levels of sericin; draining the solution from the silk fibroin extract; dissolving the silk fibroin extract in a solution of lithium bromide having a starting temperature upon placement of the silk fibroin extract in the lithium bromide solution that ranges from about 60 °C to about 140 °C; maintaining the solution of silk fibroin-lithium bromide in an oven having a temperature of about
20 140 °C for a period of at most 1 hour; removing the lithium bromide from the silk fibroin extract; and producing an aqueous solution of silk protein fragments, the aqueous solution comprising: fragments having a weight average molecular weight selected from between about 6 kDa to about 17 kDa, and a polydispersity of between 1 and about 5, or between about 1.5 and about 3.0. The method may further comprise
25 drying the silk fibroin extract prior to the dissolving step. The aqueous solution of silk fibroin protein fragments may comprise lithium bromide residuals of less than 300 ppm as measured using a high-performance liquid chromatography lithium bromide assay. The aqueous solution of silk fibroin protein fragments may comprise sodium carbonate residuals of less than 100 ppm as measured using a high-performance liquid
30 chromatography sodium carbonate assay. The aqueous solution of silk fibroin protein fragments may be lyophilized. In some embodiments, the silk fibroin protein fragment solution may be further processed into various forms including gel, powder, and nanofiber.

In an embodiment, solutions of silk fibroin protein fragments having a weight average molecular weight selected from between about 17 kDa to about 39 kDa are prepared according to the following steps: adding a silk source to a boiling (100 °C) aqueous solution of sodium carbonate for a treatment time of between about 30 minutes to about 60 minutes so as to result in degumming; removing sericin from the solution to produce a silk fibroin extract comprising non-detectable levels of sericin; draining the solution from the silk fibroin extract; dissolving the silk fibroin extract in a solution of lithium bromide having a starting temperature upon placement of the silk fibroin extract in the lithium bromide solution that ranges from about 80 °C to about 140 °C; maintaining the solution of silk fibroin-lithium bromide in a dry oven having a temperature in the range between about 60 °C to about 100 °C for a period of at most 1 hour; removing the lithium bromide from the silk fibroin extract; and producing an aqueous solution of silk fibroin protein fragments, wherein the aqueous solution of silk fibroin protein fragments comprises lithium bromide residuals of between about 10 ppm and about 300 ppm, wherein the aqueous solution of silk protein fragments comprises sodium carbonate residuals of between about 10 ppm and about 100 ppm, wherein the aqueous solution of silk fibroin protein fragments comprises fragments having a weight average molecular weight selected from between about 17 kDa to about 39 kDa, and a polydispersity of between 1 and about 5, or between about 1.5 and about 3.0. The method may further comprise drying the silk fibroin extract prior to the dissolving step. The aqueous solution of silk fibroin protein fragments may comprise lithium bromide residuals of less than 300 ppm as measured using a high- performance liquid chromatography lithium bromide assay. The aqueous solution of silk fibroin protein fragments may comprise sodium carbonate residuals of less than 100 ppm as measured using a high-performance liquid chromatography sodium carbonate assay.

In some embodiments, a method for preparing an aqueous solution of silk fibroin protein fragments having an average weight average molecular weight selected from between about 6 kDa to about 17 kDa includes the steps of: degumming a silk source by adding the silk source to a boiling (100 °C) aqueous solution of sodium carbonate for a treatment time of between about 30 minutes to about 60 minutes; removing sericin from the solution to produce a silk fibroin extract comprising non-detectable levels of sericin; draining the solution from the silk fibroin extract; dissolving the silk fibroin extract in a solution of lithium bromide having a starting

temperature upon placement of the silk fibroin extract in the lithium bromide solution that ranges from about 60 °C to about 140 °C; maintaining the solution of silk fibroin-lithium bromide in an oven having a temperature of about 140 °C for a period of at least 1 hour; removing the lithium bromide from the silk fibroin extract; and

5 producing an aqueous solution of silk protein fragments, the aqueous solution comprising: fragments having an average weight average molecular weight selected from between about 6 kDa to about 17 kDa, and a polydispersity of between 1 and about 5, or between about 1.5 and about 3.0. The method may further comprise drying the silk fibroin extract prior to the dissolving step. The aqueous solution of pure silk

10 fibroin protein fragments may comprise lithium bromide residuals of less than 300 ppm as measured using a high-performance liquid chromatography lithium bromide assay. The aqueous solution of pure silk fibroin protein fragments may comprise sodium carbonate residuals of less than 100 ppm as measured using a high-performance liquid chromatography sodium carbonate assay. The method may further

15 comprise adding a therapeutic agent to the aqueous solution of pure silk fibroin protein fragments. The method may further comprise adding a molecule selected from one of an antioxidant or an enzyme to the aqueous solution of pure silk fibroin protein fragments. The method may further comprise adding a vitamin to the aqueous solution of pure silk fibroin protein fragments. The vitamin may be vitamin C or a

20 derivative thereof. The aqueous solution of pure silk fibroin protein fragments may be lyophilized. The method may further comprise adding an alpha hydroxy acid to the aqueous solution of pure silk fibroin protein fragments. The alpha hydroxy acid may be selected from the group consisting of glycolic acid, lactic acid, tartaric acid and citric acid. The method may further comprise adding hyaluronic acid or its salt form

25 at a concentration of about 0.5 % to about 10.0 % to the aqueous solution of pure silk fibroin protein fragments. The method may further comprise adding at least one of zinc oxide or titanium dioxide. A film may be fabricated from the aqueous solution of pure silk fibroin protein fragments produced by this method. The film may comprise from about 1.0 wt. % to about 50.0 wt. % of vitamin C or a derivative thereof. The

30 film may have a water content ranging from about 2.0 wt. % to about 20.0 wt. %. The film may comprise from about 30.0 wt. % to about 99.5 wt. % of pure silk fibroin protein fragments. A gel may be fabricated from the aqueous solution of pure silk fibroin protein fragments produced by this method. The gel may comprise from about

0.5 wt. % to about 20.0 wt. % of vitamin C or a derivative thereof. The gel may have a silk content of at least 2 % and a vitamin content of at least 20 %.

In some embodiments, a method for preparing an aqueous solution of silk fibroin protein fragments having an average weight average molecular weight selected from between about 17 kDa to about 39 kDa includes the steps of: adding a silk source to a boiling (100 °C) aqueous solution of sodium carbonate for a treatment time of between about 30 minutes to about 60 minutes so as to result in degumming; removing sericin from the solution to produce a silk fibroin extract comprising non-detectable levels of sericin; draining the solution from the silk fibroin extract; dissolving the silk fibroin extract in a solution of lithium bromide having a starting temperature upon placement of the silk fibroin extract in the lithium bromide solution that ranges from about 80 °C to about 140 °C; maintaining the solution of silk fibroin-lithium bromide in a dry oven having a temperature in the range between about 60 °C to about 100 °C for a period of at least 1 hour; removing the lithium bromide from the silk fibroin extract; and producing an aqueous solution of pure silk fibroin protein fragments, wherein the aqueous solution of pure silk fibroin protein fragments comprises lithium bromide residuals of between about 10 ppm and about 300 ppm, wherein the aqueous solution of silk protein fragments comprises sodium carbonate residuals of between about 10 ppm and about 100 ppm, wherein the aqueous solution of pure silk fibroin protein fragments comprises fragments having an average weight average molecular weight selected from between about 17 kDa to about 39 kDa, and a polydispersity of between 1 and about 5, or between about 1.5 and about 3.0. The method may further comprise drying the silk fibroin extract prior to the dissolving step. The aqueous solution of pure silk fibroin protein fragments may comprise lithium bromide residuals of less than 300 ppm as measured using a high-performance liquid chromatography lithium bromide assay. The aqueous solution of pure silk fibroin protein fragments may comprise sodium carbonate residuals of less than 100 ppm as measured using a high-performance liquid chromatography sodium carbonate assay. The method may further comprise adding a therapeutic agent to the aqueous solution of pure silk fibroin protein fragments. The method may further comprise adding a molecule selected from one of an antioxidant or an enzyme to the aqueous solution of pure silk fibroin protein fragments. The method may further comprise adding a vitamin to the aqueous solution of pure silk fibroin protein fragments. The vitamin may be vitamin C or a derivative thereof. The aqueous solution of pure silk

fibroin protein fragments may be lyophilized. The method may further comprise adding an alpha hydroxy acid to the aqueous solution of pure silk fibroin protein fragments. The alpha hydroxy acid may be selected from the group consisting of glycolic acid, lactic acid, tartaric acid and citric acid. The method may further
5 comprise adding hyaluronic acid or its salt form at a concentration of about 0.5% to about 10.0% to the aqueous solution of pure silk fibroin protein fragments. The method may further comprise adding at least one of zinc oxide or titanium dioxide. A film may be fabricated from the aqueous solution of pure silk fibroin protein fragments produced by this method. The film may comprise from about 1.0 wt. % to
10 about 50.0 wt. % of vitamin C or a derivative thereof. The film may have a water content ranging from about 2.0 wt. % to about 20.0 wt. %. The film may comprise from about 30.0 wt. % to about 99.5 wt. % of pure silk fibroin protein fragments. A gel may be fabricated from the aqueous solution of pure silk fibroin protein fragments produced by this method. The gel may comprise from about 0.5 wt. % to about 20.0
15 wt. % of vitamin C or a derivative thereof. The gel may have a silk content of at least 2% and a vitamin content of at least 20%.

In an embodiment, solutions of silk fibroin protein fragments having a weight average molecular weight selected from between about 39 kDa to about 80 kDa are prepared according to the following steps: adding a silk source to a boiling (100 °C)
20 aqueous solution of sodium carbonate for a treatment time of about 30 minutes so as to result in degumming; removing sericin from the solution to produce a silk fibroin extract comprising non-detectable levels of sericin; draining the solution from the silk fibroin extract; dissolving the silk fibroin extract in a solution of lithium bromide having a starting temperature upon placement of the silk fibroin extract in the lithium
25 bromide solution that ranges from about 80 °C to about 140 °C; maintaining the solution of silk fibroin-lithium bromide in a dry oven having a temperature in the range between about 60 °C to about 100 °C for a period of at most 1 hour; removing the lithium bromide from the silk fibroin extract; and producing an aqueous solution of silk fibroin protein fragments, wherein the aqueous solution of silk fibroin protein
30 fragments comprises lithium bromide residuals of between about 10 ppm and about 300 ppm, sodium carbonate residuals of between about 10 ppm and about 100 ppm, fragments having a weight average molecular weight selected from between about 39 kDa to about 80 kDa, and a polydispersity of between 1 and about 5, or between about 1.5 and about 3.0. The method may further comprise drying the silk fibroin extract

prior to the dissolving step. The aqueous solution of silk fibroin protein fragments may comprise lithium bromide residuals of less than 300 ppm as measured using a high-performance liquid chromatography lithium bromide assay. The aqueous solution of silk fibroin protein fragments may comprise sodium carbonate residuals of less than 100 ppm as measured using a high-performance liquid chromatography sodium carbonate assay. In some embodiments, the method may further comprise adding an active agent (e.g., therapeutic agent) to the aqueous solution of pure silk fibroin protein fragments. The method may further comprise adding an active agent selected from one of an antioxidant or an enzyme to the aqueous solution of pure silk fibroin protein fragments. The method may further comprise adding a vitamin to the aqueous solution of pure silk fibroin protein fragments. The vitamin may be vitamin C or a derivative thereof. The aqueous solution of pure silk fibroin protein fragments may be lyophilized. The method may further comprise adding an alpha-hydroxy acid to the aqueous solution of pure silk fibroin protein fragments. The alpha hydroxy acid may be selected from the group consisting of glycolic acid, lactic acid, tartaric acid and citric acid. The method may further comprise adding hyaluronic acid or its salt form at a concentration of about 0.5% to about 10.0% to the aqueous solution of pure silk fibroin protein fragments. A film may be fabricated from the aqueous solution of pure silk fibroin protein fragments produced by this method. The film may comprise from about 1.0 wt. % to about 50.0 wt. % of vitamin C or a derivative thereof. The film may have a water content ranging from about 2.0 wt. % to about 20.0 wt. %. The film may comprise from about 30.0 wt. % to about 99.5 wt. % of pure silk fibroin protein fragments. A gel may be fabricated from the aqueous solution of pure silk fibroin protein fragments produced by this method. The gel may comprise from about 0.5 wt. % to about 20.0 wt. % of vitamin C or a derivative thereof. The gel may have a silk content of at least 2 wt. % and a vitamin content of at least 20 wt. %.

Molecular weight of the silk protein fragments may be controlled based upon the specific parameters utilized during the extraction step, including extraction time and temperature; specific parameters utilized during the dissolution step, including the LiBr temperature at the time of submersion of the silk in to the lithium bromide and time that the solution is maintained at specific temperatures; and specific parameters utilized during the filtration step. By controlling process parameters using the disclosed methods, it is possible to create SPF mixture solutions with polydispersity equal to or lower than 2.5 at a variety of different molecular weight ranging from 1

kDa to 250 kDa, 5 kDa to 200 kDa, 5 kDa to 150 kDa, 10 kDa to 150 kDa, or 10 kDa to 80 kDa. By altering process parameters to achieve silk solutions with different molecular weights, a range of fragment mixture end products, with desired polydispersity of equal to or less than 2.5 may be targeted based upon the desired performance requirements. For example, a lower molecular weight silk film containing a drug may have a faster release rate compared to a higher molecular weight SPF preparation. Additionally, SPF mixture solutions with a polydispersity of greater than 2.5 can be achieved. Further, two solutions with different average molecular weights and polydispersities can be mixed to create combination solutions. Alternatively, a liquid silk gland (100% sericin free silk protein) that has been removed directly from a worm could be used in combination with any of the SPF mixture solutions of the present disclosure. Molecular weight of the pure silk fibroin-based protein fragment composition was determined using High Pressure Liquid Chromatography (HPLC) with a Refractive Index Detector (RID). Polydispersity was calculated using Cirrus GPC Online GPC/SEC Software Version 3.3 (Agilent).

Differences in the processing parameters can result in regenerated silk fibroins that vary in molecular weight, and peptide chain size distribution (polydispersity, PD). This, in turn, influences the regenerated silk fibroin performance, including mechanical strength, water solubility etc.

Parameters were varied during the processing of raw silk cocoons into the silk solution. Varying these parameters affected the MW of the resulting silk solution. Parameters manipulated included (i) time and temperature of extraction, (ii) temperature of LiBr, (iii) temperature of dissolution oven, and (iv) dissolution time. Molecular weight was determined with mass spec as shown in Figs. 74-90.

Experiments were carried out to determine the effect of varying the extraction time. Figs. 74-90 are graphs showing these results and **Tables A-G** summarize the results. Below is a summary:

- A sericin extraction time of 30 minutes resulted in larger molecular weight than a sericin extraction time of 60 minutes
- Molecular weight decreases with time in the oven
- 140 °C LiBr and oven resulted in the low end of the confidence interval to be below a molecular weight of 9500 Da
- 30 min extraction at the 1 hour and 4 hour time points have undigested silk

- 30 min extraction at the 1 hour time point resulted in a significantly high molecular weight with the low end of the confidence interval being 35,000 Da
 - The range of molecular weight reached for the high end of the confidence interval was 18000 to 216000 Da (important for offering solutions with specified upper
- 5 limit).

Table A. The effect of extraction time (30 min vs 60 min) on molecular weight of silk processed under the conditions of 100 °C Extraction Temperature, 100 °C Lithium Bromide (LiBr) and 100 °C Oven Dissolution (Oven/Dissolution Time was varied).						
Boil Time	Oven Time	Average Mw	Std dev	Confidence Interval		PD
30	1	57247	12780	35093	93387	1.63
60	1	31520	1387	11633	85407	2.71
30	4	40973	2632	14268	117658	2.87
60	4	25082	1248	10520	59803	2.38
30	6	25604	1405	10252	63943	2.50
60	6	20980	1262	10073	43695	2.08

Table B. The effect of extraction time (30 min vs 60 min) on molecular weight of silk processed under the conditions of 100 °C Extraction Temperature, boiling Lithium Bromide (LiBr) and 60 °C Oven Dissolution for 4 hr.						
Sample	Boil Time	Average Mw	SStd dev	Confidence Interval		PD
30 min, 4 hr	30	49656	4580	17306	142478	2.87
60 min, 4 hr	60	30042	1536	11183	80705	2.69

Table C. The effect of extraction time (30 min vs 60 min) on molecular weight of silk processed under the conditions of 100 °C Extraction Temperature, 60 °C Lithium Bromide (LiBr) and 60 °C Oven Dissolution (Oven/Dissolution Time was varied).						
Sample	Boil Time	Oven Time	Average Mw	SStd dev	Confidence Interval	PD

30 min, 1 hr	30	1	58436		22201	153809	2.63
60 min, 1 hr	60	1	31700		11931	84224	2.66
30 min, 4 hr	30	4	61956.5	13337	21463	178847	2.89
60 min, 4 hr	60	4	25578.5	2446	9979	65564	2.56

Table D. The effect of extraction time (30 min vs 60 min) on molecular weight of silk processed under the conditions of 100 °C Extraction Temperature, 80 °C Lithium Bromide (LiBr) and 80 °C Oven Dissolution for 6 hr.

Sample	Boil Time	Average Mw	Std dev	Confidence Interval		PD
30 min, 6 hr	30	63510		18693	215775	3.40
60 min, 6 hr	60	25164	238	9637	65706	2.61

Table E. The effect of extraction time (30 min vs 60 min) on molecular weight of silk processed under the conditions of 100 °C Extraction Temperature, 80 °C Lithium Bromide (LiBr) and 60 °C Oven Dissolution (Oven/Dissolution Time was varied).

Sample	Boil Time	Oven Time	Average Mw	Std dev	Confidence Interval		PD
30 min, 4 hr	30	4	59202	14028	19073	183760	3.10
60 min, 4 hr	60	4	26312.5	637	10266	67442	2.56
30 min, 6 hr	30	6	46824		18076	121293	2.59
60 min, 6 hr	60	6	26353		10168	68302	2.59

Table F. The effect of extraction time (30 min vs 60 min) on molecular weight of silk processed under the conditions of 100 °C Extraction Temperature, 140 °C Lithium Bromide (LiBr) and 140 °C Oven Dissolution (Oven/Dissolution Time was varied).

Sample	Boil Time	Oven Time	Average Mw	Std dev	Confidence Interval		PD
30 min, 4 hr	30	4	9024.5	1102	4493	18127	2.00865
60 min, 4 hr	60	4	15548		6954	34762	2.2358

30 min, 6 hr	30	6	13021		5987	28319	2.1749
60 min, 6 hr	60	6	10888		5364	22100	2.0298
Table G. The effect of extraction time (30 min vs 60 min) on molecular weight of silk processed under the conditions of 100 °C Extraction Temperature, 140 °C Lithium Bromide (LiBr) and 140 °C Oven Dissolution (Oven/Dissolution Time was varied).							
Sample	Boil Time	Oven Time	Average Mw	Standard deviation	Confidence Interval		PD
30 min, 4 hr	30	4	9024.5	1102	4493	18127	2.00865
60 min, 4 hr	60	4	15548		6954	34762	2.2358
30 min, 6 hr	30	6	13021		5987	28319	2.1749
60 min, 6 hr	60	6	10888		5364	22100	2.0298

Experiments were carried out to determine the effect of varying the extraction temperature. Fig. 74 is a graph showing these results and **Table H** summarizes the results. Below is a summary:

- 5 – Sericin extraction at 90 °C resulted in higher MW than sericin extraction at 100 °C extraction
- Both 90 °C and 100 °C show decreasing MW over time in the oven.

Table H. The effect of extraction temperature (90 °C vs. 100 °C) on molecular weight of silk processed under the conditions of 60 min. Extraction Temperature, 100 °C Lithium Bromide (LiBr) and 100 °C Oven Dissolution (Oven/Dissolution Time was varied).							
Sample	Boil Time	Oven Time	Average Mw	Std dev	Confidence Interval		PD
90 °C, 4 hr	60	4	37308	4204	13368	104119	2.79
100 °C, 4 hr	60	4	25082	1248	10520	59804	2.38
90 °C, 6 hr	60	6	34224	1135	12717	92100	2.69
100 °C, 6 hr	60	6	20980	1262	10073	43694	2.08

- Experiments were carried out to determine the effect of varying the Lithium Bromide (LiBr) temperature when added to silk. Figs. 82-83 are graphs showing these results and **Tables I-J** summarize the results. Below is a summary:
- 10 – No impact on molecular weight or confidence interval (all CI ~10500-6500 Da)

- Studies illustrated that the temperature of LiBr-silk dissolution, as LiBr is added and begins dissolving, rapidly drops below the original LiBr temperature due to the majority of the mass being silk at room temperature

Table I. The effect of Lithium Bromide (LiBr) temperature on molecular weight of silk processed under the conditions of 60 min. Extraction Time., 100 °C Extraction Temperature and 60 °C Oven Dissolution (Oven/Dissolution Time was varied).

Sample	LiBr Temp °C)	Oven Time	Average Mw	Std dev	Confidence Interval		PD
60 °C LiBr, 1 hr	60	1	31700		11931	84223	2.66
100 °C LiBr, 1 hr	100	1	27907	200	10735	72552	2.60
RT LiBr, 4 hr	RT	4	29217	1082	10789	79119	2.71
60 °C LiBr, 4 hr	60	4	25578	2445	9978	65564	2.56
80 °C LiBr, 4 hr	80	4	26312	637	10265	67441	2.56
100 °C LiBr, 4 hr	100	4	27681	1729	11279	67931	2.45
Boil LiBr, 4 hr	Boil	4	30042	1535	11183	80704	2.69
RT LiBr, 6 hr	RT	6	26543	1893	10783	65332	2.46
80 °C LiBr, 6 hr	80	6	26353		10167	68301	2.59
100 °C LiBr, 6 hr	100	6	27150	916	11020	66889	2.46

5

Table J. The effect of Lithium Bromide (LiBr) temperature on molecular weight of silk processed under the conditions of 30 min. Extraction Time, 100 °C Extraction Temperature and 60 °C Oven Dissolution (Oven/Dissolution Time was varied).

Sample	LiBr Temp °C	Oven Time	Average Mw	Std dev	Confidence Interval		PD
60 °C LiBr,	0	4	61956	13336	21463	178847	2.89

4 hr							
80 °C LiBr, 4 hr	80	4	59202	14027	19073	183760	3.10
100 °C LiBr, 4 hr	100	4	47853		19757	115899	2.42
80 °C LiBr, 6 hr	80	6	46824		18075	121292	2.59
100 °C LiBr, 6 hr	100	6	55421	8991	19152	160366	2.89

Experiments were carried out to determine the effect of oven/dissolution temperature.

Figs. 84-88 are graphs showing these results and **Tables K-O** summarize the results.

Below is a summary:

- 5 – Oven temperature has less of an effect on 60 min extracted silk than 30 min extracted silk. Without wishing to be bound by theory, it is believed that the 30 min silk is less degraded during extraction and therefore the oven temperature has more of an effect on the larger MW, less degraded portion of the silk.
- 10 – For 60 °C vs. 140 °C oven the 30 min extracted silk showed a very significant effect of lower MW at higher oven temp, while 60 min extracted silk had an effect but much less
- The 140 °C oven resulted in a low end in the confidence interval at ~6000 Da.

Table K. The effect of oven/dissolution temperature on molecular weight of silk processed under the conditions of 100 °C Extraction Temperature, 30 min. Extraction Time, and 100 °C Lithium Bromide (LiBr) (Oven/Dissolution Time was varied).

Boil Time	Oven Temp (°C)	Oven Time	Average Mw	Std dev	Confidence Interval		PD
30	60	4	47853		19758	115900	2.42
30	100	4	40973	2632	14268	117658	2.87
30	60	6	55421	8992	19153	160366	2.89
30	100	6	25604	1405	10252	63943	2.50

15

Table L. The effect of oven/dissolution temperature on molecular weight of silk processed under the conditions of 100 °C Extraction Temperature, 60 min. Extraction Time, and 100 °C Lithium Bromide (LiBr) (Oven/Dissolution Time was varied).							
Boil Time (minutes)	Oven Temp (°C)	Oven Time	Average Mw	Std dev	Confidence Interval		PD
60	60	1	27908	200	10735	72552	2.60
60	100	1	31520	1387	11633	85407	2.71
60	60	4	27681	1730	11279	72552	2.62
60	100	4	25082	1248	10520	59803	2.38
60	60	6	27150	916	11020	66889	2.46
60	100	6	20980	1262	10073	43695	2.08

Table M. The effect of oven/dissolution temperature on molecular weight of silk processed under the conditions of 100 °C Extraction Temperature, 60 min. Extraction Time, and 140 °C Lithium Bromide (LiBr) (Oven/Dissolution Time was varied).							
Boil Time (minutes)	Oven Temp(°C)	Oven Time	Average Mw	Std dev	Confidence Interval		PD
60	60	4	30042	1536	11183	80705	2.69
60	140	4	15548		7255	33322	2.14

5

Table N. The effect of oven/dissolution temperature on molecular weight of silk processed under the conditions of 100 °C Extraction Temperature, 30 min. Extraction Time, and 140 °C Lithium Bromide (LiBr) (Oven/Dissolution Time was varied).							
Boil Time (minutes)	Oven Temp (°C)	Oven Time	Average Mw	Std dev	Confidence Interval		PD
30	60	4	49656	4580	17306	142478	2.87

30	140	4	9025	1102	4493	18127	2.01
30	60	6	59383	11640	17641	199889	3.37
30	140	6	13021		5987	28319	2.17

Table O. The effect of oven/dissolution temperature on molecular weight of silk processed under the conditions of 100 °C Extraction Temperature, 60 min. Extraction Time, and 80 °C Lithium Bromide (LiBr) (Oven/Dissolution Time was varied).							
Boil Time (minutes)	Oven Temp (°C)	Oven Time	Average Mw	Std dev	Confidence Interval		PD
60	60	4	26313	637	10266	67442	2.56
60	80	4	30308	4293	12279	74806	2.47
60	60	6	26353		10168	68302	2.59
60	80	6	25164	238	9637	65706	2.61

In an embodiment, the methods disclosed herein result in a solution with characteristics that can be controlled during manufacturing, including, but not limited to: MW – may be varied by changing extraction and/or dissolution time and temp (e.g., LiBr temperature), pressure, and filtration (e.g., size exclusion chromatography); Structure – removal or cleavage of heavy or light chain of the fibroin protein polymer; Purity – hot water rinse temperature for improved sericin removal or filter capability for improved particulate removal that adversely affects shelf stability of the silk fragment protein mixture solution; Color – the color of the solution can be controlled with, for example, LiBr temp and time; Viscosity; Clarity; and Stability of solution. The resultant pH of the solution is typically about 7 and can be altered using an acid or base as appropriate to storage requirements.

The raw silk cocoons from the silkworm *Bombyx mori* was cut into pieces. The pieces of raw silk cocoons were boiled in an aqueous solution of Na₂CO₃ (about 100 °C) for a period of time between about 30 minutes to about 60 minutes to remove sericin (degumming). The volume of the water used equals about 0.4 x raw silk weight and the amount of Na₂CO₃ is about 0.848 x the weight of the raw silk cocoon pieces. The resulting degummed silk cocoon pieces were rinsed with deionized water three times at about 60 °C (20 minutes per rinse). The volume of rinse water for each

cycle was 0.2 L x the weight of the raw silk cocoon pieces. The excess water from the degummed silk cocoon pieces was removed. After the DI water washing step, the wet degummed silk cocoon pieces were dried at room temperature. The degummed silk cocoon pieces were mixed with a LiBr solution, and the mixture was heated to about 100 °C. The warmed mixture was placed in a dry oven and was heated at a temperature ranging from about 60 °C to about 140 °C for about 60 minutes to achieve complete dissolution of the native silk protein. The resulting solution was allowed to cool to room temperature and then was dialyzed to remove LiBr salts using a 3,500 Da MWCO membrane. Multiple exchanges were performed in DI water until Br⁻ ions were less than 1 ppm as determined in the hydrolyzed fibroin solution read on an Oakton Bromide (Br⁻) double-junction ion-selective electrode.

The resulting silk fibroin aqueous solution has a concentration of about 8.0 % w/v containing pure silk fibroin protein fragments having an average weight average molecular weight selected from between about 6 kDa to about 16 kDa, about 17 kDa to about 39 kDa, and about 39 kDa to about 80 kDa and a polydispersity of between about 1.5 and about 3.0. The 8.0 % w/v was diluted with DI water to provide a 1.0 % w/v, 2.0 % w/v, 3.0 % w/v, 4.0 % w/v, 5.0 % w/v by the coating solution.

A variety of % silk concentrations have been produced through the use of Tangential Flow Filtration (TFF). In all cases a 1 % silk solution was used as the input feed. A range of 750-18,000 mL of 1% silk solution was used as the starting volume. Solution is diafiltered in the TFF to remove lithium bromide. Once below a specified level of residual LiBr, solution undergoes ultrafiltration to increase the concentration through removal of water. See examples below.

Six (6) silk solutions were utilized in standard silk structures with the following results:

Solution #1 is a silk concentration of 5.9 wt. %, average MW of 19.8 kDa and 2.2 PDI (made with a 60 min boil extraction, 100 °C LiBr dissolution for 1 hour).

Solution #2 is a silk concentration of 6.4 wt. % (made with a 30 min boil extraction, 60 °C LiBr dissolution for 4 hrs).

Solution #3 is a silk concentration of 6.17 wt. % (made with a 30 min boil extraction 100 °C LiBr dissolution for 1 hour).

Solution #4 is a silk concentration of 7.30 wt. %: A 7.30 % silk solution was produced beginning with 30 minute extraction batches of 100 g silk cocoons per batch. Extracted silk fibers were then dissolved using 100 °C 9.3 M LiBr in a 100 °C

oven for 1 hour. 100 g of silk fibers were dissolved per batch to create 20% silk in LiBr. Dissolved silk in LiBr was then diluted to 1% silk and filtered through a 5 μ m filter to remove large debris. 15,500 mL of 1 %, filtered silk solution was used as the starting volume/diafiltration volume for TFF. Once LiBr was removed, the solution
5 was ultrafiltered to a volume around 1300 mL. 1262 mL of 7.30 % silk was then collected. Water was added to the feed to help remove the remaining solution and 547 mL of 3.91 % silk was then collected.

Solution #5 is a silk concentration of 6.44 wt. %: A 6.44 wt. % silk solution was produced beginning with 60 minute extraction batches of a mix of 25, 33, 50, 75
10 and 100 g silk cocoons per batch. Extracted silk fibers were then dissolved using 100 $^{\circ}$ C 9.3 M LiBr in a 100 $^{\circ}$ C oven for 1 hour. 35, 42, 50 and 71 g per batch of silk fibers were dissolved to create 20 % silk in LiBr and combined. Dissolved silk in LiBr was then diluted to 1 % silk and filtered through a 5 μ m filter to remove large debris. 17,000 mL of 1 %, filtered silk solution was used as the starting volume/diafiltration
15 volume for TFF. Once LiBr was removed, the solution was ultrafiltered to a volume around 3000 mL. 1490 mL of 6.44 % silk was then collected. Water was added to the feed to help remove the remaining solution and 1454 mL of 4.88 % silk was then collected.

Solution #6 is a silk concentration of 2.70 wt. %: A 2.70 % silk solution was
20 produced beginning with 60-minute extraction batches of 25 g silk cocoons per batch. Extracted silk fibers were then dissolved using 100 $^{\circ}$ C 9.3 M LiBr in a 100 $^{\circ}$ C oven for 1 hour. 35.48 g of silk fibers were dissolved per batch to create 20 % silk in LiBr. Dissolved silk in LiBr was then diluted to 1% silk and filtered through a 5 μ m filter to remove large debris. 1000 mL of 1%, filtered silk solution was used as the starting
25 volume/diafiltration volume for TFF. Once LiBr was removed, the solution was ultrafiltered to a volume around 300 mL. 312 mL of 2.7 % silk was then collected.

The preparation of silk fibroin solutions with higher molecular weights is given in **Table O**.

Table O. Preparation and properties of silk fibroin solutions.

Sample Name	Extraction Time (mins)	Extraction Temp ($^{\circ}$ C)	LiBr Temp ($^{\circ}$ C)	Oven/Sol'n Temp	Average weight average molecular	Average polydispersity
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					weight (kDa)	
Group A TFF	60	100	100	100 °C oven	34.7	2.94
Group A DIS	60	100	100	100 °C oven	44.7	3.17
Group B TFF	60	100	100	100 °C sol'n	41.6	3.07
Group B DIS	60	100	100	100 °C sol'n	44.0	3.12
Group D DIS	30	90	60	60 °C sol'n	129.7	2.56
Group D FIL	30	90	60	60 °C sol'n	144.2	2.73
Group E DIS	15	100	RT	60 °C sol'n	108.8	2.78
Group E FIL	15	100	RT	60 °C sol'n	94.8	2.62

Silk aqueous coating composition for application to fabrics are given in
Tables P and Q below.

Table P. Silk Solution Characteristics					
	Molecular Weight:	57 kDa			
	Polydispersity:	1.6			
	% Silk	5.0%	3.0%	1.0%	0.5%
Process Parameters					
	Extraction				
	Boil Time:	30 minutes			
	Boil Temperature:	100 °C			
	Rinse Temperature:	60 °C			
	Dissolution				
	LiBr Temperature:	100			
	Oven Temperature:	100 °C			
	Oven Time:	60 minutes			

Table Q. Silk Solution Characteristics					
	Molecular Weight:	25 kDa			
	Polydispersity:	2.4			

	% Silk	5.0%	3.0%	1.0%	0.5%
Process Parameters					
	Extraction				
	Boil Time:	60 minutes			
	Boil Temperature:	100 °C			
	Rinse Temperature:	60 °C			
	Dissolution				
	LiBr Temperature:	100 °C			
	Oven Temperature:	100 °C			
	Oven Time:	60 minutes			

Three (3) silk solutions were utilized in film making with the following results:

- 5 Solution #1 is a silk concentration of 5.9 %, average MW of 19.8 kDa and 2.2 PD (made with a 60 min boil extraction, 100 °C LiBr dissolution for 1 hr).

Solution #2 is a silk concentration of 6.4 % (made with a 30 min boil extraction, 60 °C LiBr dissolution for 4 hrs).

Solution #3 is a silk concentration of 6.17 % (made with a 30 min boil extraction, 100 °C LiBr dissolution for 1 hour).

- 10 Films were made in accordance with Rockwood et al. (Nature Protocols; Vol. 6; No. 10; published on-line Sep. 22, 2011; doi:10.1038/nprot.2011.379). 4 mL of 1% or 2% (wt/vol) aqueous silk solution was added into 100 mm Petri dish (Volume of silk can be varied for thicker or thinner films and is not critical) and allowed to dry overnight uncovered. The bottom of a vacuum desiccator was filled with water. Dry
15 films were placed in the desiccator and vacuum applied, allowing the films to water anneal for 4 hours prior to removal from the dish. Films cast from solution #1 did not result in a structurally continuous film; the film was cracked in several pieces. These pieces of film dissolved in water in spite of the water annealing treatment.

- 20 Silk solutions of various molecular weights and/or combinations of molecular weights can be optimized for gel applications. The following provides an example of

this process but it not intended to be limiting in application or formulation. Three (3) silk solutions were utilized in gel making with the following results:

Solution #1 is a silk concentration of 5.9 %, average MW of 19.8 kDa and 2.2 PD (made with a 60 min boil extraction, 100 °C LiBr dissolution for 1 hr).

5 Solution #2 is a silk concentration of 6.4 % (made with a 30 min boil extraction, 60 °C LiBr dissolution for 4 hrs).

Solution #3 is a silk concentration of 6.17 % (made with a 30 min boil extraction, 100 °C LiBr dissolution for 1 hour).

“Egel” is an electrogelation process as described in Rockwood of al. Briefly,
10 10 ml of aqueous silk solution is added to a 50 ml conical tube and a pair of platinum wire electrodes immersed into the silk solution. A 20 volt potential was applied to the platinum electrodes for 5 minutes, the power supply turned off and the gel collected. Solution #1 did not form an EGEL over the 5 minutes of applied electric current.

Solutions #2 and #3 were gelled in accordance with the published horseradish
15 peroxidase (HRP) protocol. Behavior seemed typical of published solutions.

Materials and Methods: the following equipment and material are used in determination of Silk Molecular weight: Agilent 1100 with chemstation software ver. 10.01; Refractive Index Detector (RID); analytical balance; volumetric flasks (1000 mL, 10 mL and 5 mL); HPLC grade water; ACS grade sodium chloride; ACS grade
20 sodium phosphate dibasic heptahydrate; phosphoric acid; dextran MW Standards- Nominal Molecular Weights of 5 kDa, 11.6 kDa, 23.8 kDa, 48.6 kDa, and 148 kDa; 50 mL PET or polypropylene disposable centrifuge tubes; graduated pipettes; amber glass HPLC vials with Teflon caps; Phenomenex PolySep GFC P-4000 column (size: 7.8 mm x 300 mm).

25 Procedural Steps:

A) Preparation of 1 L Mobile Phase (0.1 M Sodium Chloride solution in 0.0125 M Sodium phosphate buffer)

Take a 250 mL clean and dry beaker, place it on the balance and tare the weight. Add about 3.3509 g of sodium phosphate dibasic heptahydrate to the beaker.
30 Note down the exact weight of sodium phosphate dibasic weighed. Dissolve the weighed sodium phosphate by adding 100 mL of HPLC water into the beaker. Take care not to spill any of the content of the beaker. Transfer the solution carefully into a clean and dry 1000 mL volumetric flask. Rinse the beaker and transfer the rinse into

the volumetric flask. Repeat the rinse 4-5 times. In a separate clean and dry 250 mL beaker weigh exactly about 5.8440 g of sodium chloride. Dissolve the weighed sodium chloride in 50 mL of water and transfer the solution to the sodium phosphate solution in the volumetric flask. Rinse the beaker and transfer the rinse into the

5 volumetric flask. Adjust the pH of the solution to 7.0 ± 0.2 with phosphoric acid. Make up the volume in volumetric flask with HPLC water to 1000 mL and shake it vigorously to homogeneously mix the solution. Filter the solution through 0.45 μm polyamide membrane filter. Transfer the solution to a clean and dry solvent bottle and label the bottle. The volume of the solution can be varied to the requirement by

10 correspondingly varying the amount of sodium phosphate dibasic heptahydrate and sodium chloride.

B) Preparation of Dextran Molecular Weight Standard solutions

At least five different molecular weight standards are used for each batch of samples that are run so that the expected value of the sample to be tested is bracketed

15 by the value of the standard used. Label six 20 mL scintillation glass vials respective to the molecular weight standards. Weigh accurately about 5 mg of each of dextran molecular weight standards and record the weights. Dissolve the dextran molecular weight standards in 5 mL of mobile phase to make a 1 mg/mL standard solution.

C) Preparation of Sample Solutions

20 When preparing sample solutions, if there are limitations on how much sample is available, the preparations may be scaled as long as the ratios are maintained. Depending on sample type and silk protein content in sample weigh enough sample in a 50 mL disposable centrifuge tube on an analytical balance to make a 1 mg/mL sample solution for analysis. Dissolve the sample in equivalent volume of mobile

25 phase make a 1 mg/mL solution. Tightly cap the tubes and mix the samples (in solution). Leave the sample solution for 30 minutes at room temperature. Gently mix the sample solution again for 1 minute and centrifuge at 4000 RPM for 10 minutes.

D) HPLC analysis of the samples

Transfer 1.0 mL of all the standards and sample solutions into individual

30 HPLC vials. Inject the molecular weight standards (one injection each) and each sample in duplicate. Analyze all the standards and sample solutions using the following HPLC conditions:

Column	PolySep GFC P-4000 (7.8 x 300 mm)
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Column Temperature	25 °C
Detector	Refractive Index Detector (Temperature @ 35 °C)
Injection Volume	25.0 µL
Mobile Phase	0.1 M Sodium Chloride solution in 0.0125 M sodium phosphate buffer
Flow Rate	1.0 mL/min
Run Time	20.0 min

Data analysis and calculations – Calculation of Average Molecular Weight using Cirrus Software

- 5 Upload the chromatography data files of the standards and the analytical samples into Cirrus SEC data collection and molecular weight analysis software. Calculate the weight average molecular weight (M_w), number average molecular weight (M_n), peak average molecular weight (M_p), and polydispersity for each injection of the sample.

Spider Silk Fragments

- 10 Spider silks are natural polymers that consist of three domains: a repetitive middle core domain that dominates the protein chain, and non-repetitive N-terminal and C-terminal domains. The large core domain is organized in a block copolymer-like arrangement, in which two basic sequences, crystalline [poly(A) or poly(GA)] and less crystalline (GGX or GPGXX (SEQ ID NO: 6)) polypeptides alternate.
- 15 Dragline silk is the protein complex composed of major ampullate dragline silk protein 1 (MaSp1) and major ampullate dragline silk protein 2 (*MaSp2*). Both silks are approximately 3500 amino acid long. *MaSp1* can be found in the fibre core and the periphery, whereas *MaSp2* forms clusters in certain core areas. The large central domains of *MaSp1* and *MaSp2* are organized in block copolymer-like arrangements,
- 20 in which two basic sequences, crystalline [poly(A) or poly(GA)] and less crystalline (GGX or GPGXX (SEQ ID NO: 6)) polypeptides alternate in core domain. Specific secondary structures have been assigned to poly(A)/(GA), GGX and GPGXX (SEQ ID NO: 6) motifs including β -sheet, α -helix and β -spiral respectively. The primary sequence, composition and secondary structural elements of the repetitive core

domain are responsible for mechanical properties of spider silks; whereas, non-repetitive N- and C-terminal domains are essential for the storage of liquid silk dope in a lumen and fibre formation in a spinning duct.

The main difference between *MaSp1* and *MaSp2* is the presence of proline (P) residues accounting for 15% of the total amino acid content in *MaSp2*, whereas *MaSp1* is proline-free. By calculating the number of proline residues in *N. clavipes* dragline silk, it is possible to estimate the presence of the two proteins in fibres; 81% *MaSp1* and 19% *MaSp2*. Different spiders have different ratios of *MaSp1* and *MaSp2*. For example, a dragline silk fibre from the orb weaver *Argiope aurantia* contains 41% *MaSp1* and 59% *MaSp2*. Such changes in the ratios of major ampullate silks can dictate the performance of the silk fibre.

At least seven different types of silk proteins are known for one orb-weaver species of spider. Silks differ in primary sequence, physical properties and functions. For example, dragline silks used to build frames, radii and lifelines are known for outstanding mechanical properties including strength, toughness and elasticity. On an equal weight basis, spider silk has a higher toughness than steel and Kevlar. Flageliform silk found in capture spirals has extensibility of up to 500%. Minor ampullate silk, which is found in auxiliary spirals of the orb-web and in prey wrapping, possesses high toughness and strength almost similar to major ampullate silks, but does not supercontract in water.

Spider silks are known for their high tensile strength and toughness. The recombinant silk proteins also confer advantageous properties to cosmetic or dermatological compositions, in particular to be able to improve the hydrating or softening action, good film forming property and low surface density. Diverse and unique biomechanical properties together with biocompatibility and a slow rate of degradation make spider silks excellent candidates as biomaterials for tissue engineering, guided tissue repair and drug delivery, for cosmetic products (e.g. nail and hair strengthener, skin care products), and industrial materials (e.g. nanowires, nanofibers, surface coatings).

In an embodiment, a silk protein may include a polypeptide derived from natural spider silk proteins. The polypeptide is not limited particularly as long as it is derived from natural spider silk proteins, and examples of the polypeptide include natural spider silk proteins and recombinant spider silk proteins such as variants, analogs, derivatives or the like of the natural spider silk proteins. In terms of excellent

tenacity, the polypeptide may be derived from major dragline silk proteins produced in major ampullate glands of spiders. Examples of the major dragline silk proteins include major ampullate spidroin MaSp1 and MaSp2 from *Nephila clavipes*, and ADF3 and ADF4 from *Araneus diadematus*, etc. Examples of the polypeptide derived from major dragline silk proteins include variants, analogs, derivatives or the like of the major dragline silk proteins. Further, the polypeptide may be derived from flagelliform silk proteins produced in flagelliform glands of spiders. Examples of the flagelliform silk proteins include flagelliform silk proteins derived from *Nephila clavipes*, etc.

Examples of the polypeptide derived from major dragline silk proteins include a polypeptide containing two or more units of an amino acid sequence represented by the formula 1: REP1-REP2 (1), preferably a polypeptide containing five or more units thereof, and more preferably a polypeptide containing ten or more units thereof. Alternatively, the polypeptide derived from major dragline silk proteins may be a polypeptide that contains units of the amino acid sequence represented by the formula 1: REP1-REP2 (1) and that has, at a C-terminal, an amino acid sequence represented by any of SEQ ID NOS: 52 to 54, which is also described in U.S. Patent No. 9,051,453, which is incorporated by reference herein in its entirety, or an amino acid sequence having a homology of 90% or more with the amino acid sequence represented by any of SEQ ID NOS: 52 to 54, which is also described in U.S. Patent No. 9,051,453, which is incorporated by reference herein in its entirety. In the polypeptide derived from major dragline silk proteins, units of the amino acid sequence represented by the formula 1: REP1-REP2 (1) may be the same or may be different from each other. In the case of producing a recombinant protein using a microbe such as *Escherichia coli* as a host, the molecular weight of the polypeptide derived from major dragline silk proteins is 500 kDa or less, or 300 kDa or less, or 200 kDa or less, in terms of productivity.

In the formula (1), the REP1 indicates polyalanine. In the REP1, the number of alanine residues arranged in succession is preferably 2 or more, more preferably 3 or more, further preferably 4 or more, and particularly preferably 5 or more. Further, in the REP1, the number of alanine residues arranged in succession is preferably 20 or less, more preferably 16 or less, further preferably 12 or less, and particularly preferably 10 or less. In the formula (1), the REP2 is an amino acid sequence composed of 10 to 200 amino acid residues. The total number of glycine, serine,

glutamine and alanine residues contained in the amino acid sequence is 40% or more, preferably 60% or more, and more preferably 70% or more with respect to the total number of amino acid residues contained therein.

5 In the major dragline silk, the REP1 corresponds to a crystal region in a fiber where a crystal β sheet is formed, and the REP2 corresponds to an amorphous region in a fiber where most of the parts lack regular configurations and that has more flexibility. Further, the [REP1-REP2] corresponds to a repetitious region (repetitive sequence) composed of the crystal region and the amorphous region, which is a characteristic sequence of dragline silk proteins.

10 Recombinant Silk Fragments

In some embodiments, the recombinant silk protein refers to recombinant spider silk polypeptides, recombinant insect silk polypeptides, or recombinant mussel silk polypeptides. In some embodiments, the recombinant silk protein fragment disclosed herein include recombinant spider silk polypeptides of *Araneidae* or *Araneoids*, or recombinant insect silk polypeptides of *Bombyx mori*. In some
15 embodiments, the recombinant silk protein fragment disclosed herein include recombinant spider silk polypeptides of *Araneidae* or *Araneoids*. In some embodiments, the recombinant silk protein fragment disclosed herein include block copolymer having repetitive units derived from natural spider silk polypeptides of *Araneidae* or *Araneoids*. In some
20 embodiments, the recombinant silk protein fragment disclosed herein include block copolymer having synthetic repetitive units derived from spider silk polypeptides of *Araneidae* or *Araneoids* and non-repetitive units derived from natural repetitive units of spider silk polypeptides of *Araneidae* or *Araneoids*.

25 Recent advances in genetic engineering have provided a route to produce various types of recombinant silk proteins. Recombinant DNA technology has been used to provide a more practical source of silk proteins. As used herein “recombinant silk protein” refers to synthetic proteins produced heterologously in prokaryotic or eukaryotic expression systems using genetic engineering methods.

30 Various methods for synthesizing recombinant silk peptides are known and have been described by Ausubel et al., Current Protocols in Molecular Biology § 8 (John Wiley & Sons 1987, (1990)), incorporated herein by reference. A gram-negative, rod-shaped bacterium *E. coli* is a well-established host for industrial scale

production of proteins. Therefore, the majority of recombinant silks have been produced in *E. coli*. *E. coli* which is easy to manipulate, has a short generation time, is relatively low cost and can be scaled up for larger amounts protein production.

The recombinant silk proteins can be produced by transformed prokaryotic or eukaryotic systems containing the cDNA coding for a silk protein, for a fragment of this protein or for an analog of such a protein. The recombinant DNA approach enables the production of recombinant silks with programmed sequences, secondary structures, architectures and precise molecular weight. There are four main steps in the process: (i) design and assembly of synthetic silk-like genes into genetic ‘cassettes’, (ii) insertion of this segment into a DNA recombinant vector, (iii) transformation of this recombinant DNA molecule into a host cell and (iv) expression and purification of the selected clones.

The term “recombinant vectors”, as used herein, includes any vectors known to the skilled person including plasmid vectors, cosmid vectors, phage vectors such as lambda phage, viral vectors such as adenoviral or baculoviral vectors, or artificial chromosome vectors such as bacterial artificial chromosomes (BAC), yeast artificial chromosomes (YAC), or P1 artificial chromosomes (PAC). Said vectors include expression as well as cloning vectors. Expression vectors comprise plasmids as well as viral vectors and generally contain a desired coding sequence and appropriate DNA sequences necessary for the expression of the operably linked coding sequence in a particular host organism (e.g., bacteria, yeast, or plant) or in in vitro expression systems. Cloning vectors are generally used to engineer and amplify a certain desired DNA fragment and may lack functional sequences needed for expression of the desired DNA fragments.

The prokaryotic systems include Gram-negative bacteria or Gram-positive bacteria. The prokaryotic expression vectors can include an origin of replication which can be recognized by the host organism, a homologous or heterologous promoter which is functional in the said host, the DNA sequence coding for the spider silk protein, for a fragment of this protein or for an analogous protein. Nonlimiting examples of prokaryotic expression organisms are *Escherichia coli*, *Bacillus subtilis*, *Bacillus megaterium*, *Corynebacterium glutamicum*, *Anabaena*, *Caulobacter*, *Gluconobacter*, *Rhodobacter*, *Pseudomonas*, *Paracoccus*, *Bacillus* (e.g. *Bacillus subtilis*) *Brevibacterium*, *Corynebacterium*, *Rhizobium* (*Sinorhizobium*),

Flavobacterium, Klebsiella, Enterobacter, Lactobacillus, Lactococcus, Methylobacterium, Propionibacterium, Staphylococcus or Streptomyces cells.

The eukaryotic systems include yeasts and insect, mammalian or plant cells. In this case, the expression vectors can include a yeast plasmid origin of replication or an autonomous replication sequence, a promoter, a DNA sequence coding for a spider silk protein, for a fragment or for an analogous protein, a polyadenylation sequence, a transcription termination site and, lastly, a selection gene. Nonlimiting examples of eukaryotic expression organisms include yeasts, such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *basidiosporogenous*, *ascosporogenous*, filamentous fungi, such as *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus nidulans*, *Trichoderma reesei*, *Acremonium chrysogenum*, *Candida*, *Hansenula*, *Kluyveromyces*, *Saccharomyces* (e.g. *Saccharomyces cerevisiae*), *Schizosaccharomyces*, *Pichia* (e.g. *Pichia pastoris*) or *Yarrowia cells* etc., mammalian cells, such as HeLa cells, COS cells, CHO cells etc., insect cells, such as Sf9 cells, MEL cells, etc., “insect host cells” such as *Spodoptera frugiperda* or *Trichoplusia ni* cells. *SF9 cells*, *SF-21 cells* or *High-Five cells*, wherein SF-9 and SF-21 are ovarian cells from *Spodoptera frugiperda*, and High-Five cells are egg cells from *Trichoplusia ni*, “plant host cells”, such as tobacco, potato or pea cells.

A variety of heterologous host systems have been explored to produce different types of recombinant silks. Recombinant partial spidroins as well as engineered silks have been cloned and expressed in bacteria (*Escherichia coli*), yeast (*Pichia pastoris*), insects (*silkworm larvae*), plants (tobacco, soybean, potato, Arabidopsis), mammalian cell lines (BHT/hamster) and transgenic animals (mice, goats). Most of the silk proteins are produced with an N- or C-terminal His-tags to make purification simple and produce enough amounts of the protein.

In some embodiments, the host suitable for expressing the recombinant spider silk protein using heterogeneous system may include transgenic animals and plants. In some embodiments, the host suitable for expressing the recombinant spider silk protein using heterogeneous system comprises bacteria, yeasts, mammalian cell lines. In some embodiments, the host suitable for expressing the recombinant spider silk protein using heterogeneous system comprises *E. coli*. In some embodiments, the host suitable for expressing the recombinant spider silk protein using heterogeneous system comprises transgenic *B. mori* silkworm generated using genome editing technologies (e.g. CRISPR).

The recombinant silk protein in this disclosure comprises synthetic proteins which are based on repeat units of natural silk proteins. Besides the synthetic repetitive silk protein sequences, these can additionally comprise one or more natural nonrepetitive silk protein sequences.

5 In some embodiments, “recombinant silk protein” refers to recombinant silkworm silk protein or fragments thereof. The recombinant production of silk fibroin and silk sericin has been reported. A variety of hosts are used for the production including *E. coli*, *Saccharomyces cerevisiae*, *Pseudomonas sp.*, *Rhodopseudomonas sp.*, *Bacillus sp.*, and *Streptomyces*. See EP 0230702, which is incorporated by reference
10 herein by its entirety.

 Provided herein also include design and biological-synthesis of silk fibroin protein-like multiblock polymer comprising GAGAGX (SEQ ID NO: 1) hexapeptide (X is A, Y, V or S) derived from the repetitive domain of *B. mori* silk heavy chain (H chain)

15 In some embodiments, this disclosure provides silk protein-like multiblock polymers derived from the repetitive domain of *B. mori* silk heavy chain (H chain) comprising the GAGAGS (SEQ ID NO: 2) hexapeptide repeating units. The GAGAGS (SEQ ID NO: 2) hexapeptide is the core unit of H-chain and plays an important role in the formation of crystalline domains. The silk protein-like
20 multiblock polymers containing the GAGAGS (SEQ ID NO: 2) hexapeptide repeating units spontaneously aggregate into β -sheet structures, similar to natural silk fibroin protein, where in the silk protein-like multiblock polymers having any weight average molecular weight described herein.

 In some embodiments, this disclosure provides silk-peptide like multiblock
25 copolymers composed of the GAGAGS (SEQ ID NO: 2) hexapeptide repetitive fragment derived from H chain of *B. mori* silk heavy chain and mammalian elastin VPGVG (SEQ ID NO: 3) motif produced by *E. coli*. In some embodiments, this disclosure provides fusion silk fibroin proteins composed of the GAGAGS (SEQ ID NO: 2) hexapeptide repetitive fragment derived from H chain of *B. mori* silk heavy
30 chain and GVGVP (SEQ ID NO: 4) produced by *E. coli*, where in the silk protein-like multiblock polymers having any weight average molecular weight described herein.

 In some embodiments, this disclosure provides *B. mori* silkworm recombinant proteins composed of the (GAGAGS)₁₆ (SEQ ID NO: 55) repetitive fragment. In some embodiments, this disclosure provides recombinant proteins composed of the

[illegible]

10 In some embodiments, “recombinant silk protein” refers to recombinant spider
silk protein or fragments thereof. The productions of recombinant spider silk proteins
based on a partial cDNA clone have been reported. The recombinant spider silk
proteins produced as such comprise a portion of the repetitive sequence derived from
a dragline spider silk protein, *Spidroin 1*, from the spider *Nephila clavipes*. see Xu et
15 al. (Proc. Natl. Acad. Sci. U.S.A., 87:7120–7124 (1990). cDNA clone encoding a
portion of the repeating sequence of a second fibroin protein, *Spidroin 2*, from
dragline silk of *Nephila clavipes* and the recombinant synthesis thereof is described in
J. Biol. Chem., 1992, volume 267, pp. 19320–19324. The recombinant synthesis of
spider silk proteins including protein fragments and variants of *Nephila clavipes* from
20 transformed *E. coli* is described in U.S. Pat. Nos. 5,728,810 and 5,989,894. cDNA
clones encoding minor ampullate spider silk proteins and the expression thereof is
described in U.S. Pat. Nos. 5,733,771 and 5,756,677. cDNA clone encoding the
flagelliform silk protein from an orb-web spinning spider is described in U.S. Pat. No.
5,994,099. U.S. Pat. No. 6,268,169 describes the recombinant synthesis of spider silk
25 like proteins derived from the repeating peptide sequence found in the natural spider
dragline of *Nephila clavipes* by *E. coli*, *Bacillus subtilis*, and *Pichia pastoris*
recombinant expression systems. WO 03/020916 describes the cDNA clone encoding
and recombinant production of spider spider silk proteins having repetitive sequences
derived from the major ampullate glands of *Nephila madagascariensis*, *Nephila*
30 *senegalensis*, *Tetragnatha kauaiensis*, *Tetragnatha versicolor*, *Argiope aurantia*,
Argiope trifasciata, *Gasteracantha mammosa*, and *Latrodectus geometricus*, the
flagelliform glands of *Argiope trifasciata*, the ampullate glands of *Dolomedes*
tenebrosus, two sets of silk glands from *Plectreurys tristis*, and the silk glands of the

mygalomorph Euagrus chisoseus. Each of the above reference is incorporated herein by reference in its entirety.

In some embodiments, the recombinant spider silk protein is a hybrid protein of a spider silk protein and an insect silk protein, a spider silk protein and collagen, a spider silk protein and resilin, or a spider silk protein and keratin. The spider silk repetitive unit comprises or consists of an amino acid sequence of a region that comprises or consists of at least one peptide motif that repetitively occurs within a naturally occurring major ampullate gland polypeptide, such as a dragline spider silk polypeptide, a minor ampullate gland polypeptide, a flagelliform polypeptide, an aggregate spider silk polypeptide, an aciniform spider silk polypeptide or a pyriform spider silk polypeptide.

In some embodiments, the recombinant spider silk protein in this disclosure comprises synthetic spider silk proteins derived from repetitive units of natural spider silk proteins, consensus sequence, and optionally one or more natural non-repetitive spider silk protein sequences. The repeated units of natural spider silk polypeptide may include dragline spider silk polypeptides or flagelliform spider silk polypeptides of *Araneidae* or *Araneoids*.

As used herein, the spider silk “repetitive unit” comprises or consists of at least one peptide motif that repetitively occurs within a naturally occurring major ampullate gland polypeptide, such as a dragline spider silk polypeptide, a minor ampullate gland polypeptide, a flagelliform polypeptide, an aggregate spider silk polypeptide, an aciniform spider silk polypeptide or a pyriform spider silk polypeptide. A “repetitive unit” refers to a region which corresponds in amino acid sequence to a region that comprises or consists of at least one peptide motif (e.g. AAAAAA (SEQ ID NO: 20)) or GPGQQ (SEQ ID NO: 15)) that repetitively occurs within a naturally occurring silk polypeptide (e.g. MaSpI, ADF-3, ADF-4, or Flag) (i.e. identical amino acid sequence) or to an amino acid sequence substantially similar thereto (i.e. variational amino acid sequence). A “repetitive unit” having an amino acid sequence which is “substantially similar” to a corresponding amino acid sequence within a naturally occurring silk polypeptide (i.e. wild-type repetitive unit) is also similar with respect to its properties, e.g. a silk protein comprising the “substantially similar repetitive unit” is still insoluble and retains its insolubility. A “repetitive unit” having an amino acid sequence which is “identical” to the amino acid sequence of a naturally occurring silk polypeptide, for example, can be a portion of a

silk polypeptide corresponding to one or more peptide motifs of MaSpI (SEQ ID NO: 48), MaSpII (SEQ ID NO: 49), ADF-3 (SEQ ID NO: 50) and/or ADF-4 (SEQ ID NO: 51). A “repetitive unit” having an amino acid sequence which is “substantially similar” to the amino acid sequence of a naturally occurring silk polypeptide, for example, can be a portion of a silk polypeptide corresponding to one or more peptide motifs of MaSpI (SEQ ID NO: 48), MaSpII (SEQ ID NO: 49), ADF-3 (SEQ ID NO: 50) and/or ADF-4 (SEQ ID NO: 51) but having one or more amino acid substitution at specific amino acid positions.

As used herein, the term “consensus peptide sequence” refers to an amino acid sequence which contains amino acids which frequently occur in a certain position (e.g. “G”) and wherein, other amino acids which are not further determined are replaced by the place holder “X”. In some embodiments, the consensus sequence is at least one of (i) GPGXX (SEQ ID NO: 6), wherein X is an amino acid selected from A, S, G, Y, P and Q; (ii) GGX, wherein X is an amino acid selected from Y, P, R, S, A, T, N and Q, preferably Y, P and Q; (iii) A_x, wherein x is an integer from 5 to 10.

The consensus peptide sequences GPGXX (SEQ ID NO: 6) and GGX, i.e. glycine rich motifs, provide flexibility to the silk polypeptide and thus, to the thread formed from the silk protein containing said motifs. In detail, the iterated GPGXX (SEQ ID NO: 6) motif forms turn spiral structures, which imparts elasticity to the silk polypeptide. Major ampullate and flagelliform silks both have a GPGXX (SEQ ID NO: 6) motif. The iterated GGX motif is associated with a helical structure having three amino acids per turn and is found in most spider silks. The GGX motif may provide additional elastic properties to the silk. The iterated polyalanine A_x (peptide) motif forms a crystalline β -sheet structure that provides strength to the silk polypeptide, as described for example in WO 03/057727.

In some embodiments, the recombinant spider silk protein in this disclosure comprises two identical repetitive units each comprising at least one, preferably one, amino acid sequence selected from the group consisting of: GGRPSDTYG (SEQ ID NO: 7) and GGRPSSSYG (SEQ ID NO: 8) derived from Resilin. Resilin is an elastomeric protein found in most arthropods that provides low stiffness and high strength.

As used herein, “non-repetitive units” refers to an amino acid sequence which is “substantially similar” to a corresponding non-repetitive (carboxy terminal) amino acid sequence within a naturally occurring dragline polypeptide (i.e. wild-type non-

repetitive (carboxy terminal) unit), preferably within ADF-3 (SEQ ID NO: 50), ADF-4 (SEQ ID NO: 51), NR3 (SEQ ID NO: 62), NR4 (SEQ ID NO: 63) of the spider *Araneus diadematus*, which is also described in U.S. Pat. No. 9,217,017, which is incorporated by reference herein in its entirety, C16 peptide (spider silk protein eADF4, molecular weight of 47.7 kDa, AMSilk) comprising the 16 repeats of the sequence GSSAAAAAASGPGGYGPENQGPSGPGGYGPGGP (SEQ ID NO: 9), an amino acid sequence adapted from the natural sequence of ADF4 from *A. diadematus*. Non-repetitive ADF-4 and variants thereof display efficient assembly behavior.

10 Among the synthetic spider silk proteins, the recombinant silk protein in this disclosure comprises in some embodiments the C16-protein having the polypeptide sequence SEQ ID NO: 64, which is also described in U.S. Patent No. 8,288,512, which is incorporated by reference herein in its entirety. Besides the polypeptide sequence shown in SEQ ID NO: 64, particularly functional equivalents, functional
15 derivatives and salts of this sequence are also included.

As used herein, “functional equivalents” refers to mutant which, in at least one sequence position of the abovementioned amino acid sequences, have an amino acid other than that specifically mentioned.

20 In some embodiments, the recombinant spider silk protein in this disclosure comprises, in an effective amount, at least one natural or recombinant silk protein including spider silk protein, corresponding to Spidroin major 1 described by Xu et al., PNAS, USA, 87, 7120, (1990), Spidroin major 2 described by Hinman and Lewis, J. Biol. Chem., 267, 19320, (1992), recombinant spider silk protein as described in U.S. Patent Application No. 2016/0222174 and U.S. Patent Nos. 9,051,453,
25 9,617,315, 9,689,089, 8,173,772, 8,642,734, 8,367,803, 8,097,583, 8,030,024, 7,754,851, 7,148,039, 7,060,260, or alternatively the minor Spidroins described in patent application WO 95/25165. Each of the above-cited references is incorporated herein by reference in its entirety. Additional recombinant spider silk proteins suitable for the recombinant RSPF of this disclosure include ADF3 and ADF4 from the
30 “Major Ampullate” gland of *Araneus diadematus*.

Recombinant silk is also described in other patents and patent applications, incorporated by reference herein: US 2004590196, US 7,754,851, US 2007654470, US 7,951,908, US 2010785960, US 8,034,897, US 20090263430, US 2008226854, US 20090123967, US 2005712095, US 2007991037, US 20090162896, US

200885266, US 8,372,436, US 2007989907, US 2009267596, US 2010319542, US
 2009265344, US 2012684607, US 2004583227, US 8,030,024, US 2006643569, US
 7,868,146, US 2007991916, US 8,097,583, US 2006643200, US 8,729,238, US
 8,877,903, US 20190062557, US 20160280960, US 20110201783, US 2008991916,
 5 US 2011986662, US 2012697729, US 20150328363, US 9,034,816, US
 20130172478, US 9,217,017, US 20170202995, US 8,721,991, US 2008227498, US
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 2010809102, US 8,367,803, US 2010664902, US 7,569,660, US 1999138833, US
 10 2000591632, US 20120065126, US 20100278882, US 2008161352, US
 20100015070, US 2009513709, US 20090194317, US 2004559286, US 200589551,
 US 2008187824, US 20050266242, US 20050227322, and US 20044418.

Recombinant silk is also described in other patents and patent applications,
 incorporated by reference herein: US 20190062557, US 20150284565, US
 15 20130225476, US 20130172478, US 20130136779, US 20130109762, US
 20120252294, US 20110230911, US 20110201783, US 20100298877, US
 10,478,520, US 10,253,213, US 10,072,152, US 9,233,067, US 9,217,017, US
 9,034,816, US 8,877,903, US 8,729,238, US 8,721,991, US 8,097,583, US 8,034,897,
 US 8,030,024, US 7,951,908, US 7,868,146, and US 7,754,851.

20 In some embodiments, the recombinant spider silk protein in this disclosure
 comprises or consists of 2 to 80 repetitive units, each independently selected from
 GPGXX (SEQ ID NO: 6), GGX and A_x as defined herein.

In some embodiments, the recombinant spider silk protein in this disclosure
 comprises or consists of repetitive units each independently selected from selected
 25 from the group consisting of GPGAS (SEQ ID NO: 10), GPGSG (SEQ ID NO: 11),
 GPGGY (SEQ ID NO: 12), GPGGP (SEQ ID NO: 13), GPGGA (SEQ ID NO: 14),
 GPGQQ (SEQ ID NO: 15), GPGGG (SEQ ID NO: 16), GPGQG (SEQ ID NO: 17),
 GPGGS (SEQ ID NO: 18), GGY, GGP, GGA, GGR, GGS, GGT, GGN, GGQ,
 AAAAA (SEQ ID NO: 19), AAAAAA (SEQ ID NO: 20), AAAAAAA (SEQ ID NO:
 30 21), AAAAAAAA (SEQ ID NO: 22), AAAAAAAA (SEQ ID NO: 23),
 AAAAAAAA (SEQ ID NO: 24), GGRPSDTYG (SEQ ID NO: 7) and
 GGRPSSSYG (SEQ ID NO: 8), (i) GPYGPASAAAAAGGYGPGSGQQ (SEQ ID
 NO: 25), (ii) GSSAAAAAASGPGGYGPENQGPGSGPGGYGPGGP (SEQ ID NO:
 9), (iii) GPGQQGPGQQGPGQQGPGQQ (SEQ ID NO: 26): (iv)

GPGGAGGPYPGGAGGPYPGGAGGPY (SEQ ID NO: 27), (v)
 GGTTHIEDLDITIDGADGPITISEELTI (SEQ ID NO: 28), (vi)
 PGSSAAAAAASGPGQGQGQGQGGRPSDTYG (SEQ ID NO: 29), (vii)
 SAAAAAAGPGGGNGGRPSDTYGAPGGGNGGRPSSSYG (SEQ ID NO: 30),
 5 (viii) GGAGGAGGAGGSGGAGGS (SEQ ID NO: 31), (ix)
 GPGGAGPGGYGPGGSGPGGYGPGGSGPGGY (SEQ ID NO: 32), (x)
 GPYGPASAAAAAGGYGPGCGQQ (SEQ ID NO: 33), (xi)
 GPYGPASAAAAAGGYGPGKGQQ (SEQ ID NO: 34), (xii)
 GSSAAAAAASGPGGYGPENQGPCPGGYGPGGP (SEQ ID NO: 35), (xiii)
 10 GSSAAAAAASGPGGYGPKNQGPSGPGGYGPGGP (SEQ ID NO: 36), (xiv)
 GSSAAAAAASGPGGYGPKNQGPCPGGYGPGGP (SEQ ID NO: 37), or
 variants thereof as described in U.S. Pat. No. 8,877,903, for example, a synthetic
 spider peptide having sequential order of GPAS (SEQ ID NO: 10), GGY, GPGSG
 (SEQ ID NO: 11) in the peptide chain, or sequential order of AAAAAAA (SEQ ID
 15 NO: 22), GPGGY (SEQ ID NO: 12), GPGGP (SEQ ID NO: 13) in the peptide chain,
 sequential order of AAAAAAA (SEQ ID NO: 22), GPGQG (SEQ ID NO: 17),
 GGR in the peptide chain.

In some embodiments, this disclosure provides silk protein-like multiblock
 peptides that imitate the repeating units of amino acids derived from natural spider
 20 silk proteins such as *Spidroin major* 1 domain, *Spidroin major* 2 domain or *Spidroin*
minor 1 domain and the profile of variation between the repeating units without
 modifying their three-dimensional conformation, wherein these silk protein-like
 multiblock peptides comprise a repeating unit of amino acids corresponding to one of
 the sequences (I), (II), (III) and/or (IV) below.

25 $[(XGG)_w(XGA)(GXG)_x(AGA)_y(G)_zAG]_p$ (SEQ ID NO: 38) Formula (I) in
 which: X corresponds to tyrosine or to glutamine, w is an integer equal to 2 or 3, x is
 an integer from 1 to 3, y is an integer from 5 to 7, z is an integer equal to 1 or 2, and p
 is an integer and having any weight average molecular weight described herein,
 and/or

30 $[(GPG_2YGPGQ_2)_a(X')_2S(A)_b]_p$ (SEQ ID NO: 39) Formula (II) in which: X'
 corresponds to the amino acid sequence GPS or GPG, a is equal to 2 or 3, b is an
 integer from 7 to 10, and p is an integer and having any weight average molecular
 weight described herein, and/or

$[(GR)(GA)_l(A)_m(GGX)_n(GA)_l(A)_m]_p$ (SEQ ID NO: 40) Formula (III) and/or $[(GGX'')(GA)_m(A)_l]_p$ (SEQ ID NO: 41) Formula (IV) in which: X'' corresponds to tyrosine, glutamine or alanine, l is an integer from 1 to 6, m is an integer from 0 to 4, n is an integer from 1 to 4, and p is an integer.

5 In some embodiments, the recombinant spider silk protein or an analog of a spider silk protein comprising an amino acid repeating unit of sequence (V):

$[(Xaa\ Gly\ Gly)_w(Xaa\ Gly\ Ala)(Gly\ Xaa\ Gly)_x(Ala\ Gly\ Ala)_y(Gly)_zAla\ Gly]_p$ Formula (V), wherein Xaa is tyrosine or glutamine, w is an integer equal to 2 or 3, x is an integer from 1 to 3, y is an integer from 5 to 7, z is an integer equal to 1 or 2, and
10 p is an integer.

In some embodiments, the recombinant spider silk protein in this disclosure is selected from the group consisting of ADF-3 or variants thereof, ADF-4 or variants thereof, MaSpI or variants thereof, MaSpII or variants thereof as described in U.S. Pat. No. 9,217,017.

15 In some embodiments, this disclosure provides water soluble recombinant spider silk proteins produced in mammalian cells. The solubility of the spider silk proteins produced in mammalian cells was attributed to the presence of the COOH-terminus in these proteins, which makes them more hydrophilic. These COOH-terminal amino acids are absent in spider silk proteins expressed in microbial hosts.

20 In some embodiments, the recombinant spider silk protein in this disclosure comprises water soluble recombinant spider silk protein C16 modified with an amino or carboxyl terminal selected from the amino acid sequences consisting of: GCGGGGGG (SEQ ID NO: 42), GKGGGGGG (SEQ ID NO: 43), GCGGSGGGSGGGG (SEQ ID NO: 44), GKGGGGGGSGGGG (SEQ ID NO: 45),
25 and GCGGGGGSGGGG (SEQ ID NO: 46). In some embodiments, the recombinant spider silk protein in this disclosure comprises C₁₆NR₄, C₃₂NR₄, C₁₆, C₃₂, NR₄C₁₆NR₄, NR₄C₃₂NR₄, NR₃C₁₆NR₃, or NR₃C₃₂NR₃ such that the molecular weight of the protein ranges as described herein.

30 In some embodiments, the recombinant spider silk protein in this disclosure comprises recombinant spider silk protein having a synthetic repetitive peptide segments and an amino acid sequence adapted from the natural sequence of ADF4 from *A. diadematus* as described in U.S. Pat. No. 8,877,903. In some embodiments, the RSPF in this disclosure comprises the recombinant spider silk proteins having repeating peptide units derived from natural spider silk proteins such as *Spidroin*

major 1 domain, *Spidroin major* 2 domain or *Spidroin minor* 1 domain, wherein the repeating peptide sequence is

GSSAAAAAASGPGQGQGQGQGGRPSDTYG (SEQ ID NO: 47) or

SAAAAAAGPGGGNGGRPSDTYGAPGGGNGGRPSSSYG (SEQ ID NO: 30),

5 as described in U.S. Pat. No. 8,367,803, which is incorporated by reference herein in its entirety.

In some embodiments, this disclosure provides recombinant spider proteins composed of the GPGGAGPGGYGPGGSGPGGYGPGGSGPGGY (SEQ ID NO: 32) repetitive fragment and having a molecular weight as described herein.

10 As used herein, the term “recombinant silk” refers to recombinant spider and/or silkworm silk protein or fragments thereof. In an embodiment, the spider silk protein is selected from the group consisting of swathing silk (Achniform gland silk), egg sac silk (Cylindriform gland silk), egg case silk (Tubuliform silk), non-sticky dragline silk (Ampullate gland silk), attaching thread silk (Pyriform gland silk), sticky
15 silk core fibers (Flagelliform gland silk), and sticky silk outer fibers (Aggregate gland silk). For example, recombinant spider silk protein, as described herein, includes the proteins described in U.S. Patent Application No. 2016/0222174 and U.S. Patent Nos. 9,051,453, 9,617,315, 9,689,089, 8,173,772, and 8,642,734.

Some organisms make multiple silk fibers with unique sequences, structural
20 elements, and mechanical properties. For example, orb weaving spiders have six unique types of glands that produce different silk polypeptide sequences that are polymerized into fibers tailored to fit an environmental or lifecycle niche. The fibers are named for the gland they originate from and the polypeptides are labeled with the gland abbreviation (e.g. “Ma”) and “Sp” for spidroin (short for spider fibroin). In orb
25 weavers, these types include Major Ampullate (MaSp, also called dragline), Minor Ampullate (MiSp), Flagelliform (Flag), Aciniform (AcSp), Tubuliform (TuSp), and Pyriform (PySp). This combination of polypeptide sequences across fiber types, domains, and variation amongst different genus and species of organisms leads to a vast array of potential properties that can be harnessed by commercial production of
30 the recombinant fibers. To date, the vast majority of the work with recombinant silks has focused on the Major Ampullate Spidroins (MaSp).

Aciniform (AcSp) silks tend to have high toughness, a result of moderately high strength coupled with moderately high extensibility. AcSp silks are characterized by large block (“ensemble repeat”) sizes that often incorporate motifs of poly serine

and GPX. Tubuliform (TuSp or Cylindrical) silks tend to have large diameters, with modest strength and high extensibility. TuSp silks are characterized by their poly serine and poly threonine content, and short tracts of poly alanine. Major Ampullate (MaSp) silks tend to have high strength and modest extensibility. MaSp silks can be one of two subtypes: MaSp1 and MaSp2. MaSp1 silks are generally less extensible than MaSp2 silks, and are characterized by poly alanine, GX, and GGX motifs. MaSp2 silks are characterized by poly alanine, GGX, and GPX motifs. Minor Ampullate (MiSp) silks tend to have modest strength and modest extensibility. MiSp silks are characterized by GGX, GA, and poly A motifs, and often contain spacer elements of approximately 100 amino acids. Flagelliform (Flag) silks tend to have very high extensibility and modest strength. Flag silks are usually characterized by GPG, GGX, and short spacer motifs.

Silk polypeptides are characteristically composed of a repeat domain (REP) flanked by non-repetitive regions (e.g., C-terminal and N-terminal domains). In an embodiment, both the C-terminal and N-terminal domains are between 75-350 amino acids in length. The repeat domain exhibits a hierarchical architecture. The repeat domain comprises a series of blocks (also called repeat units). The blocks are repeated, sometimes perfectly and sometimes imperfectly (making up a quasi-repeat domain), throughout the silk repeat domain. The length and composition of blocks varies among different silk types and across different species. Table 1 of U.S. Published Application No. 2016/0222174, the entirety of which is incorporated herein, lists examples of block sequences from selected species and silk types, with further examples presented in Rising, A. et al., Spider silk proteins: recent advances in recombinant production, structure-function relationships and biomedical applications, *Cell Mol. Life Sci.*, 68:2, pg 169-184 (2011); and Gatesy, J. et al., Extreme diversity, conservation, and convergence of spider silk fibroin sequences, *Science*, 291:5513, pg. 2603-2605 (2001). In some cases, blocks may be arranged in a regular pattern, forming larger macro-repeats that appear multiple times (usually 2-8) in the repeat domain of the silk sequence. Repeated blocks inside a repeat domain or macro-repeat, and repeated macro-repeats within the repeat domain, may be separated by spacing elements.

The construction of certain spider silk block copolymer polypeptides from the blocks and/or macro-repeat domains, according to certain embodiments of the disclosure, is illustrated in U.S. Published Patent Application No. 2016/0222174.

The recombinant block copolymer polypeptides based on spider silk sequences produced by gene expression in a recombinant prokaryotic or eukaryotic system can be purified according to methods known in the art. In a preferred embodiment, a commercially available expression/secretion system can be used, 5 whereby the recombinant polypeptide is expressed and thereafter secreted from the host cell, to be easily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant block copolymer polypeptide from cell lysates (remains of cells following disruption of cellular integrity) derived from prokaryotic or eukaryotic cells in which a polypeptide 10 was expressed. Methods for generation of such cell lysates are known to those of skill in the art. In some embodiments, recombinant block copolymer polypeptides are isolated from cell culture supernatant.

Recombinant block copolymer polypeptide may be purified by affinity separation, such as by immunological interaction with antibodies that bind 15 specifically to the recombinant polypeptide or nickel columns for isolation of recombinant polypeptides tagged with 6-8 histidine residues at their N-terminus or C-terminus. Alternative tags may comprise the FLAG epitope or the hemagglutinin epitope. Such methods are commonly used by skilled practitioners.

A solution of such polypeptides (i.e., recombinant silk protein) may then be 20 prepared and used as described herein.

In another embodiment, recombinant silk protein may be prepared according to the methods described in U.S. Patent No. 8,642,734, the entirety of which is incorporated herein, and used as described herein.

In an embodiment, a recombinant spider silk protein is provided. The spider 25 silk protein typically consists of from 170 to 760 amino acid residues, such as from 170 to 600 amino acid residues, preferably from 280 to 600 amino acid residues, such as from 300 to 400 amino acid residues, more preferably from 340 to 380 amino acid residues. The small size is advantageous because longer spider silk proteins tend to form amorphous aggregates, which require use of harsh solvents for solubilization and 30 polymerization. The recombinant spider silk protein may contain more than 760 residues, in particular in cases where the spider silk protein contains more than two fragments derived from the N-terminal part of a spider silk protein. The spider silk protein comprises an N-terminal fragment consisting of at least one fragment (NT) derived from the corresponding part of a spider silk protein, and a repetitive fragment

(REP) derived from the corresponding internal fragment of a spider silk protein.

Optionally, the spider silk protein comprises a C-terminal fragment (CT) derived from the corresponding fragment of a spider silk protein. The spider silk protein comprises typically a single fragment (NT) derived from the N-terminal part of a spider silk

5 protein, but in preferred embodiments, the N-terminal fragment include at least two, such as two fragments (NT) derived from the N-terminal part of a spider silk protein. Thus, the spidroin can schematically be represented by the formula NT_m -REP, and alternatively NT_m -REP-CT, where m is an integer that is 1 or higher, such as 2 or higher, preferably in the ranges of 1-2, 1-4, 1-6, 2-4 or 2-6. Preferred spidroins can
10 schematically be represented by the formulas NT_2 -REP or NT-REP, and alternatively NT_2 -REP-CT or NT-REP-CT. The protein fragments are covalently coupled, typically via a peptide bond. In one embodiment, the spider silk protein consists of the NT fragment(s) coupled to the REP fragment, which REP fragment is optionally coupled to the CT fragment.

15 In one embodiment, the first step of the method of producing polymers of an isolated spider silk protein involves expression of a polynucleic acid molecule which encodes the spider silk protein in a suitable host, such as *Escherichia coli*. The thus obtained protein is isolated using standard procedures. Optionally, lipopolysaccharides and other pyrogens are actively removed at this stage.

20 In the second step of the method of producing polymers of an isolated spider silk protein, a solution of the spider silk protein in a liquid medium is provided. By the terms “soluble” and “in solution” is meant that the protein is not visibly aggregated and does not precipitate from the solvent at 60,000×g. The liquid medium can be any suitable medium, such as an aqueous medium, preferably a physiological
25 medium, typically a buffered aqueous medium, such as a 10-50 mM Tris-HCl buffer or phosphate buffer. The liquid medium has a pH of 6.4 or higher and/or an ion composition that prevents polymerization of the spider silk protein. That is, the liquid medium has either a pH of 6.4 or higher or an ion composition that prevents polymerization of the spider silk protein, or both.

30 Ion compositions that prevent polymerization of the spider silk protein can readily be prepared by the skilled person utilizing the methods disclosed herein. A preferred ion composition that prevents polymerization of the spider silk protein has an ionic strength of more than 300 mM. Specific examples of ion compositions that prevent polymerization of the spider silk protein include above 300 mM NaCl, 100

mM phosphate and combinations of these ions having desired preventive effect on the polymerization of the spider silk protein, e.g. a combination of 10 mM phosphate and 300 mM NaCl.

5 The presence of an NT fragment improves the stability of the solution and prevents polymer formation under these conditions. This can be advantageous when immediate polymerization may be undesirable, e.g. during protein purification, in preparation of large batches, or when other conditions need to be optimized. It is preferred that the pH of the liquid medium is adjusted to 6.7 or higher, such as 7.0 or higher, or even 8.0 or higher, such as up to 10.5, to achieve high solubility of the spider silk protein. It can also be advantageous that the pH of the liquid medium is
10 adjusted to the range of 6.4-6.8, which provides sufficient solubility of the spider silk protein but facilitates subsequent pH adjustment to 6.3 or lower.

In the third step, the properties of the liquid medium are adjusted to a pH of 6.3 or lower and ion composition that allows polymerization. That is, if the liquid
15 medium wherein the spider silk protein is dissolved has a pH of 6.4 or higher, the pH is decreased to 6.3 or lower. The skilled person is well aware of various ways of achieving this, typically involving addition of a strong or weak acid. If the liquid medium wherein the spider silk protein is dissolved has an ion composition that prevents polymerization, the ion composition is changed so as to allow
20 polymerization. The skilled person is well aware of various ways of achieving this, e.g. dilution, dialysis or gel filtration. If required, this step involves both decreasing the pH of the liquid medium to 6.3 or lower and changing the ion composition so as to allow polymerization. It is preferred that the pH of the liquid medium is adjusted to 6.2 or lower, such as 6.0 or lower. In particular, it may be advantageous from a
25 practical point of view to limit the pH drop from 6.4 or 6.4-6.8 in the preceding step to 6.3 or 6.0-6.3, e.g. 6.2 in this step. In a preferred embodiment, the pH of the liquid medium of this step is 3 or higher, such as 4.2 or higher. The resulting pH range, e.g. 4.2-6.3 promotes rapid polymerization,

In the fourth step, the spider silk protein is allowed to polymerize in the liquid
30 medium having pH of 6.3 or lower and an ion composition that allows polymerization of the spider silk protein. Although the presence of the NT fragment improves solubility of the spider silk protein at a pH of 6.4 or higher and/or an ion composition that prevents polymerization of the spider silk protein, it accelerates polymer formation at a pH of 6.3 or lower when the ion composition allows polymerization of

the spider silk protein. The resulting polymers are preferably solid and macroscopic, and they are formed in the liquid medium having a pH of 6.3 or lower and an ion composition that allows polymerization of the spider silk protein. In a preferred embodiment, the pH of the liquid medium of this step is 3 or higher, such as 4.2 or
5 higher. The resulting pH range, e.g. 4.2-6.3 promotes rapid polymerization. Resulting polymer may be provided at the molecular weights described herein and prepared as a solution form that may be used as necessary for article coatings.

Ion compositions that allow polymerization of the spider silk protein can readily be prepared by the skilled person utilizing the methods disclosed herein. A
10 preferred ion composition that allows polymerization of the spider silk protein has an ionic strength of less than 300 mM. Specific examples of ion compositions that allow polymerization of the spider silk protein include 150 mM NaCl, 10 mM phosphate, 20 mM phosphate and combinations of these ions lacking preventive effect on the polymerization of the spider silk protein, e.g. a combination of 10 mM phosphate or
15 20 mM phosphate and 150 mM NaCl. It is preferred that the ionic strength of this liquid medium is adjusted to the range of 1-250 mM.

Without desiring to be limited to any specific theory, it is envisaged that the NT fragments have oppositely charged poles, and that environmental changes in pH affects the charge balance on the surface of the protein followed by polymerization,
20 whereas salt inhibits the same event.

At neutral pH, the energetic cost of burying the excess negative charge of the acidic pole may be expected to prevent polymerization. However, as the dimer approaches its isoelectric point at lower pH, attractive electrostatic forces will eventually become dominant, explaining the observed salt and pH-dependent
25 polymerization behavior of NT and NT-containing minispidroins. It is proposed that, in some embodiments, pH-induced NT polymerization, and increased efficiency of fiber assembly of NT-minispidroins, are due to surface electrostatic potential changes, and that clustering of acidic residues at one pole of NT shifts its charge balance such that the polymerization transition occurs at pH values of 6.3 or lower.

30 In a fifth step, the resulting, preferably solid spider silk protein polymers are isolated from said liquid medium. Optionally, this step involves actively removing lipopolysaccharides and other pyrogens from the spidroin polymers.

Without desiring to be limited to any specific theory, it has been observed that formation of spidroin polymers progresses via formation of water-soluble spidroin

dimers. The present disclosure thus also provides a method of producing dimers of an isolated spider silk protein, wherein the first two method steps are as described above. The spider silk proteins are present as dimers in a liquid medium at a pH of 6.4 or higher and/or an ion composition that prevents polymerization of said spider silk protein. The third step involves isolating the dimers obtained in the second step, and optionally removal of lipopolysaccharides and other pyrogens. In a preferred embodiment, the spider silk protein polymer of the disclosure consists of polymerized protein dimers. The present disclosure thus provides a novel use of a spider silk protein, preferably those disclosed herein, for producing dimers of the spider silk protein.

According to another aspect, the disclosure provides a polymer of a spider silk protein as disclosed herein. In an embodiment, the polymer of this protein is obtainable by any one of the methods therefor according to the disclosure. Thus, the disclosure provides various uses of recombinant spider silk protein, preferably those disclosed herein, for producing polymers of the spider silk protein as recombinant silk based coatings. According to one embodiment, the present disclosure provides a novel use of a dimer of a spider silk protein, preferably those disclosed herein, for producing polymers of the isolated spider silk protein as recombinant silk based coatings. In these uses, it is preferred that the polymers are produced in a liquid medium having a pH of 6.3 or lower and an ion composition that allows polymerization of said spider silk protein. In an embodiment, the pH of the liquid medium is 3 or higher, such as 4.2 or higher. The resulting pH range, e.g. 4.2-6.3 promotes rapid polymerization,

Using the method(s) of the present disclosure, it is possible to control the polymerization process, and this allows for optimization of parameters for obtaining silk polymers with desirable properties and shapes.

In an embodiment, the recombinant silk proteins described herein, include those described in U.S. patent No. 8,642,734, the entirety of which is incorporated by reference.

In another embodiment, the recombinant silk proteins described herein may be prepared according to the methods described in U.S. Patent No. 9,051,453, the entirety of which is incorporated herein by reference.

An amino acid sequence represented by SEQ ID NO: 52, which is also described in U.S. Patent No. 9,051,453, is identical to an amino acid sequence that is composed of 50 amino acid residues of an amino acid sequence of ADF3 at the C-

terminal (NCBI Accession No.: AAC47010, GI: 1263287). An amino acid sequence represented by SEQ ID NO: 53, which is also described in U.S. Patent No. 9,051,453, is identical to an amino acid sequence represented by SEQ ID NO: 52, which is also described in U.S. Patent No. 9,051,453, from which 20 residues have been removed from the C-terminal. An amino acid sequence represented by SEQ ID NO: 54, which is also described in U.S. Patent No. 9,051,453, is identical to an amino acid sequence represented by SEQ ID NO: 52 from which 29 residues have been removed from the C-terminal.

An example of the polypeptide that contains units of the amino acid sequence represented by the formula 1: REP1-REP2 (1) and that has, at a C-terminal, an amino acid sequence represented by any of SEQ ID NOS: 52 to 54 or an amino acid sequence having a homology of 90% or more with the amino acid sequence represented by any of SEQ ID NOS: 52 to 54, which are also described in U.S. Patent No. 9,051,453, is a polypeptide having an amino acid sequence represented by SEQ ID NO: 65, which is also described in U.S. Patent No. 9,051,453, which is incorporated by reference herein in its entirety. The polypeptide having the amino acid sequence represented by SEQ ID NO: 65, which is also described in U.S. Patent No. 9,051,453, is obtained by the following mutation: in an amino acid sequence of ADF3 (NCBI Accession No.: AAC47010, GI: 1263287) to the N-terminal of which has been added an amino acid sequence (SEQ ID NO: 66, which is also described in U.S. Patent No. 9,051,453) composed of a start codon, His 10 tags and an HRV3C Protease (Human rhinovirus 3C Protease) recognition site, 1st to 13th repetitive regions are about doubled and the translation ends at the 1154th amino acid residue. In the polypeptide having the amino acid sequence represented by SEQ ID NO: 65, which is also described in U.S. Patent No. 9,051,453, the C-terminal sequence is identical to the amino acid sequence represented by SEQ ID NO: 54.

Further, the polypeptide that contains units of the amino acid sequence represented by the formula 1: REP1-REP2 (1) and that has, at a C-terminal, an amino acid sequence represented by any of SEQ ID NOS: 52 to 54, which are also described in U.S. Patent No. 9,051,453, or an amino acid sequence having a homology of 90% or more with the amino acid sequence represented by any of SEQ ID NOS: 52 to 54, which are also described in U.S. Patent No. 9,051,453, may be a protein that has an amino acid sequence represented by SEQ ID NO: 65, which is also described in U.S. Patent No. 9,051,453, in which one or a plurality of amino acids have been

substituted, deleted, inserted and/or added and that has a repetitious region composed of a crystal region and an amorphous region.

Further, an example of the polypeptide containing two or more units of the amino acid sequence represented by the formula 1: REP1-REP2 (1) is a recombinant protein derived from ADF4 having an amino acid sequence represented by SEQ ID NO: 67, which is also described in U.S. Patent No. 9,051,453, which is incorporated by reference herein in its entirety. The amino acid sequence represented by SEQ ID NO: 67, which is also described in U.S. Patent No. 9,051,453, is an amino acid sequence obtained by adding the amino acid sequence (SEQ ID NO: 66, which is also described in U.S. Patent No. 9,051,453) composed of a start codon, His 10 tags and an HRV3C Protease (Human rhinovirus 3C Protease) recognition site, to the N-terminal of a partial amino acid sequence of ADF4 obtained from the NCBI database (NCBI Accession No.: AAC47011, GI: 1263289). Further, the polypeptide containing two or more units of the amino acid sequence represented by the formula 1: REP1-REP2 (1) may be a polypeptide that has an amino acid sequence represented by SEQ ID NO: 67, which is also described in U.S. Patent No. 9,051,453, in which one or a plurality of amino acids have been substituted, deleted, inserted and/or added and that has a repetitious region composed of a crystal region and an amorphous region. Further, an example of the polypeptide containing two or more units of the amino acid sequence represented by the formula 1: REP1-REP2 (1) is a recombinant protein derived from MaSp2 that has an amino acid sequence represented by SEQ ID NO: 68, which is also described in of U.S. Patent No. 9,051,453, which is incorporated by reference here in its entirety. The amino acid sequence represented by SEQ ID NO: 68, which is also described in of U.S. Patent No. 9,051,453, is an amino acid sequence obtained by adding the amino acid sequence (SEQ ID NO: 66, which is also described in of U.S. Patent No. 9,051,453,) composed of a start codon, His 10 tags and an HRV3C Protease (Human rhinovirus 3C Protease) recognition site, to the N-terminal of a partial sequence of MaSp2 obtained from the NCBI web database (NCBI Accession No.: AAT75313, GI: 50363147). Furthermore, the polypeptide containing two or more units of the amino acid sequence represented by the formula 1: REP1-REP2 (1) may be a polypeptide that has an amino acid sequence represented by SEQ ID NO: 68, which is also described in of U.S. Patent No. 9,051,453, in which one or a plurality of amino acids have been substituted, deleted, inserted and/or added and that has a repetitious region composed of a crystal region and an amorphous region.

Examples of the polypeptide derived from flagelliform silk proteins include a polypeptide containing 10 or more units of an amino acid sequence represented by the formula 2: REP3 (2), preferably a polypeptide containing 20 or more units thereof, and more preferably a polypeptide containing 30 or more units thereof. In the case of
5 producing a recombinant protein using a microbe such as *Escherichia coli* as a host, the molecular weight of the polypeptide derived from flagelliform silk proteins is preferably 500 kDa or less, more preferably 300 kDa or less, and further preferably 200 kDa or less, in terms of productivity.

In the formula (2), the REP 3 indicates an amino acid sequence composed of
10 Gly-Pro-Gly-Gly-X (SEQ ID NO: 69), where X indicates an amino acid selected from the group consisting of Ala, Ser, Tyr and Val.

A major characteristic of the spider silk is that the flagelliform silk does not have a crystal region, but has a repetitious region composed of an amorphous region. Since the major dragline silk and the like have a repetitious region composed of a
15 crystal region and an amorphous region, they are expected to have both high stress and stretchability. Meanwhile, as to the flagelliform silk, although the stress is inferior to that of the major dragline silk, the stretchability is high. The reason for this is considered to be that most of the flagelliform silk is composed of amorphous regions.

An example of the polypeptide containing 10 or more units of the amino acid
20 sequence represented by the formula 2: REP3 (2) is a recombinant protein derived from flagelliform silk proteins having an amino acid sequence represented by SEQ ID NO: 70, which is also described in U.S. Patent No. 9,051,453, which is incorporated by reference herein in its entirety. The amino acid sequence represented by SEQ ID NO: 70, which is also described in U.S. Patent No. 9,051,453, is an amino acid
25 sequence obtained by combining a partial sequence of flagelliform silk protein of *Nephila clavipes* obtained from the NCBI database (NCBI Accession No.: AAF36090, GI: 7106224), specifically, an amino acid sequence thereof from the 1220th residue to the 1659th residue from the N-terminal that corresponds to repetitive sections and motifs (referred to as a PR1 sequence), with a partial sequence of
30 flagelliform silk protein of *Nephila clavipes* obtained from the NCBI database (NCBI Accession No.: AAC38847, GI: 2833649), specifically, a C-terminal amino acid sequence thereof from the 816th residue to the 907th residue from the C-terminal, and thereafter adding the amino acid sequence (SEQ ID NO: 66, which is also described in U.S. Patent No. 9,051,453,) composed of a start codon, His 10 tags and an HRV3C

Protease recognition site, to the N-terminal of the combined sequence. Further, the polypeptide containing 10 or more units of the amino acid sequence represented by the formula 2: REP3 (2) may be a polypeptide that has an amino acid sequence represented by SEQ ID NO: 70, which is also described in U.S. Patent No. 9,051,453, in which one or a plurality of amino acids have been substituted, deleted, inserted and/or added and that has a repetitious region composed of an amorphous region.

The polypeptide can be produced using a host that has been transformed by an expression vector containing a gene encoding a polypeptide. A method for producing a gene is not limited particularly, and it may be produced by amplifying a gene encoding a natural spider silk protein from a cell derived from spiders by a polymerase chain reaction (PCR), etc., and cloning it, or may be synthesized chemically. Also, a method for chemically synthesizing a gene is not limited particularly, and it can be synthesized as follows, for example: based on information of amino acid sequences of natural spider silk proteins obtained from the NCBI web database, etc., oligonucleotides that have been synthesized automatically with AKTA oligopilot plus 10/100 (GE Healthcare Japan Corporation) are linked by PCR, etc. At this time, in order to facilitate the purification and observation of protein, it is possible to synthesize a gene that encodes a protein having an amino acid sequence of the above-described amino acid sequence to the N-terminal of which has been added an amino acid sequence composed of a start codon and His 10 tags.

Examples of the expression vector include a plasmid, a phage, a virus, and the like that can express protein based on a DNA sequence. The plasmid-type expression vector is not limited particularly as long as it allows a target gene to be expressed in a host cell and it can amplify itself. For example, in the case of using *Escherichia coli* Rosetta (DE3) as a host, a pET22b(+) plasmid vector, a pCold plasmid vector, and the like can be used. Among these, in terms of productivity of protein, it is preferable to use the pET22b(+) plasmid vector. Examples of the host include animal cells, plant cells, microbes, etc.

The polypeptide used in the present disclosure is preferably a polypeptide derived from ADF3, which is one of two principal dragline silk proteins of *Araneus diadematus*. This polypeptide has advantages of basically having high strength-elongation and toughness and of being synthesized easily.

Accordingly, the recombinant silk protein (e.g., the recombinant spider silk-based protein) used in accordance with the embodiments, articles, and/or methods

described herein, may include one or more recombinant silk proteins described above or recited in U.S. Patent Nos. 8,173,772, 8,278,416, 8,618,255, 8,642,734, 8,691,581, 8,729,235, 9,115,204, 9,157,070, 9,309,299, 9,644,012, 9,708,376, 9,051,453, 9,617,315, 9,968,682, 9,689,089, 9,732,125, 9,856,308, 9,926,348, 10,065,997, 5 10,316,069, and 10,329,332; and U.S. Patent Publication Nos. 2009/0226969, 2011/0281273, 2012/0041177, 2013/0065278, 2013/0115698, 2013/0316376, 2014/0058066, 2014/0079674, 2014/0245923, 2015/0087046, 2015/0119554, 2015/0141618, 2015/0291673, 2015/0291674, 2015/0239587, 2015/0344542, 2015/0361144, 2015/0374833, 2015/0376247, 2016/0024464, 2017/0066804, 10 2017/0066805, 2015/0293076, 2016/0222174, 2017/0283474, 2017/0088675, 2019/0135880, 2015/0329587, 2019/0040109, 2019/0135881, 2019/0177363, 2019/0225646, 2019/0233481, 2019/0031842, 2018/0355120, 2019/0186050, 2019/0002644, 2020/0031887, 2018/0273590, 2019/094403, 2019/0031843, 2018/0251501, 2017/0066805, 2018/0127553, 2019/0329526, 2020/0031886, 15 2018/0080147, 2019/0352349, 2020/0043085, 2019/0144819, 2019/0228449, 2019/0340666, 2020/0000091, 2019/0194710, 2019/0151505, 2018/0265555, 2019/0352330, 2019/0248847, and 2019/0378191, the entirety of which are incorporated herein by reference.

Silk Fibroin-like Protein Fragments

20 The recombinant silk protein in this disclosure comprises synthetic proteins which are based on repeat units of natural silk proteins. Besides the synthetic repetitive silk protein sequences, these can additionally comprise one or more natural nonrepetitive silk protein sequences. As used herein, "silk fibroin-like protein fragments" refer to protein fragments having a molecular weight and polydispersity as 25 defined herein, and a certain degree of homology to a protein selected from native silk protein, fibroin heavy chain, fibroin light chain, or any protein comprising one or more GAGAGS (SEQ ID NO: 2) hexa amino acid repeating units. In some embodiments, a degree of homology is selected from about 99%, about 98%, about 97%, about 96%, about 95%, about 94%, about 93%, about 92%, about 91%, about 30 90%, about 89%, about 88%, about 87%, about 86%, about 85%, about 84%, about 83%, about 82%, about 81%, about 80%, about 79%, about 78%, about 77%, about 76%, about 75%, or less than 75%.

As described herein, a protein such as native silk protein, fibroin heavy chain, fibroin light chain, or any protein comprising one or more GAGAGS (SEQ ID NO: 2) hexa amino acid repeating units includes between about 9% and about 45% glycine, or about 9% glycine, or about 10% glycine, about 43% glycine, about 44% glycine, about 45% glycine, or about 46% glycine. As described herein, a protein such as native silk protein, fibroin heavy chain, fibroin light chain, or any protein comprising one or more GAGAGS (SEQ ID NO: 2) hexa amino acid repeating units includes between about 13% and about 30% alanine, or about 13% alanine, or about 28% alanine, or about 29% alanine, or about 30% alanine, or about 31% alanine. As described herein, a protein such as native silk protein, fibroin heavy chain, fibroin light chain, or any protein comprising one or more GAGAGS (SEQ ID NO: 2) hexa amino acid repeating units includes between 9% and about 12% serine, or about 9% serine, or about 10% serine, or about 11% serine, or about 12% serine.

In some embodiments, a silk fibroin-like protein described herein includes about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20%, about 21%, about 22%, about 23 %, about 24%, about 25%, about 26%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%, about 39%, about 40%, about 41%, about 42%, about 43%, about 44%, about 45%, about 46%, about 47%, about 48%, about 49%, about 50%, about 51%, about 52%, about 53%, about 54%, or about 55% glycine. In some embodiments, a silk fibroin-like protein described herein includes about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 26%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%, or about 39% alanine. In some embodiments, a silk fibroin-like protein described herein includes about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20%, about 21%, or about 22% serine. In some embodiments, a silk fibroin-like protein described herein may include independently any amino acid known to be included in natural fibroin. In some embodiments, a silk fibroin-like protein described herein may exclude independently any amino acid known to be included in natural fibroin. In some embodiments, on

average 2 out of 6 amino acids, 3 out of 6 amino acids, or 4 out of 6 amino acids in a silk fibroin-like protein described herein is glycine. In some embodiments, on average 1 out of 6 amino acids, 2 out of 6 amino acids, or 3 out of 6 amino acids in a silk fibroin-like protein described herein is alanine. In some embodiments, on average
5 none out of 6 amino acids, 1 out of 6 amino acids, or 2 out of 6 amino acids in a silk fibroin-like protein described herein is serine.

Sericin or Sericin Fragments

The main body of the raw silk is silk fibroin fiber, and the silk fibroin fiber is coated with an adhesive substance silk sericin. Sericin is a colloidal silk protein that
10 covers the surface of the silk thread and is composed of bulky amino acids rich in chemical reactivity such as serine, threonine, and aspartic acid, in addition to glycine and alanine. In the various processes of producing silk from raw silk, sericin is important in controlling the solubility of silk and producing high quality silk. Moreover, it plays an extremely important role as an adhesion functional protein.
15 When silk fiber is used as a clothing material, most of the silk sericin covering the silk thread is removed and discarded, so sericin is a valuable unused resource.

In some embodiments, the silk protein fragments described herein include sericin or sericin fragments. Methods of preparing sericin or sericin fragments and their applications in various fields are known and are described herein , and are also
20 described, for example, in U.S. Patents Nos. 7,115,388, 7,157,273, and 9,187,538, all of which are incorporated by reference herein in their entireties.

In some embodiments, sericin removed from the raw silk cocoons, such as in a degumming step, can be collected and used in the methods described herein. Sericin can also be reconstituted from a powder, and used within the compositions and
25 methods of the disclosure.

Other Properties of SPF

Compositions of the present disclosure are “biocompatible” or otherwise exhibit “biocompatibility” meaning that the compositions are compatible with living tissue or a living system by not being toxic, injurious, or physiologically reactive and
30 not causing immunological rejection or an inflammatory response. Such biocompatibility can be evidenced by participants topically applying compositions of the present disclosure on their skin for an extended period of time. In an embodiment,

the extended period of time is about 3 days. In an embodiment, the extended period of time is about 7 days. In an embodiment, the extended period of time is about 14 days. In an embodiment, the extended period of time is about 21 days. In an embodiment, the extended period of time is about 30 days. In an embodiment, the extended period of time is selected from the group consisting of about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, about 12 months, and indefinitely. For example, in some embodiments, the coatings described herein are biocompatible coatings.

10 In some embodiments, compositions described herein, which may be biocompatible compositions (e.g., biocompatible coatings that include silk), may be evaluated and comply with International Standard ISO 10993-1, titled the “Biological evaluation of medical devices – Part 1: Evaluation and testing within a risk management process.” In some embodiments, compositions described herein, which
15 may be biocompatible compositions, may be evaluated under ISO 106993-1 for one or more of cytotoxicity, sensitization, hemocompatibility, pyrogenicity, implantation, genotoxicity, carcinogenicity, reproductive and developmental toxicity, and degradation.

Compositions of the present disclosure are “hypoallergenic” meaning that they
20 are relatively unlikely to cause an allergic reaction. Such hypoallergenicity can be evidenced by participants topically applying compositions of the present disclosure on their skin for an extended period of time. In an embodiment, the extended period of time is about 3 days. In an embodiment, the extended period of time is about 7 days. In an embodiment, the extended period of time is about 14 days. In an embodiment,
25 the extended period of time is about 21 days. In an embodiment, the extended period of time is about 30 days. In an embodiment, the extended period of time is selected from the group consisting of about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, about 12 months, and indefinitely.

30 In an embodiment, the stability of a composition of the present disclosure is about 1 day. In an embodiment, the stability of a composition of the present disclosure is about 2 days. In an embodiment, the stability of a composition of the present disclosure is about 3 days. In an embodiment, the stability of a composition of the present disclosure is about 4 days. In an embodiment, the stability of a composition of

the present disclosure is about 5 days. In an embodiment, the stability of a composition of the present disclosure is about 6 days. In an embodiment, the stability of a composition of the present disclosure is about 7 days. In an embodiment, the stability of a composition of the present disclosure is about 8 days. In an embodiment, the stability of a composition of the present disclosure is about 9 days. In an embodiment, the stability of a composition of the present disclosure is about 10 days.

In an embodiment, the stability of a composition of the present disclosure is about 11 days, about 12 days, about 13 days, about 14 days, about 15 days, about 16 days, about 17 days, about 18 days, about 19 days, about 20 days, about 21 days, about 22 days, about 23 days, about 24 days, about 25 days, about 26 days, about 27 days, about 28 days, about 29 days, or about 30 days.

In an embodiment, the stability of a composition of the present disclosure is 10 days to 6 months. In an embodiment, the stability of a composition of the present disclosure is 6 months to 12 months. In an embodiment, the stability of a composition of the present disclosure is 12 months to 18 months. In an embodiment, the stability of a composition of the present disclosure is 18 months to 24 months. In an embodiment, the stability of a composition of the present disclosure is 24 months to 30 months. In an embodiment, the stability of a composition of the present disclosure is 30 months to 36 months. In an embodiment, the stability of a composition of the present disclosure is 36 months to 48 months. In an embodiment, the stability of a composition of the present disclosure is 48 months to 60 months.

In an embodiment, a SPF composition of the present disclosure is not soluble in an aqueous solution due to the crystallinity of the protein. In an embodiment, a SPF composition of the present disclosure is soluble in an aqueous solution. In an embodiment, the SPF of a composition of the present disclosure include a crystalline portion of about two-thirds and an amorphous region of about one-third. In an embodiment, the SPF of a composition of the present disclosure include a crystalline portion of about one-half and an amorphous region of about one-half. In an embodiment, the SPF of a composition of the present disclosure include a 99% crystalline portion and a 1% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 95% crystalline portion and a 5% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 90% crystalline portion and a 10% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 85%

crystalline portion and a 15% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 80% crystalline portion and a 20% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 75% crystalline portion and a 25% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 70% crystalline portion and a 30% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 65% crystalline portion and a 35% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 60% crystalline portion and a 40% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 50% crystalline portion and a 50% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 40% crystalline portion and a 60% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 35% crystalline portion and a 65% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 30% crystalline portion and a 70% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 25% crystalline portion and a 75% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 20% crystalline portion and a 80% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 15% crystalline portion and a 85% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 10% crystalline portion and a 90% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 5% crystalline portion and a 90% amorphous region. In an embodiment, the SPF of a composition of the present disclosure include a 1% crystalline portion and a 99% amorphous region.

As used herein, the term “substantially free of inorganic residuals” means that the composition exhibits residuals of 0.1 % (w/w) or less. In an embodiment, substantially free of inorganic residuals refers to a composition that exhibits residuals of 0.05% (w/w) or less. In an embodiment, substantially free of inorganic residuals refers to a composition that exhibits residuals of 0.01 % (w/w) or less. In an embodiment, the amount of inorganic residuals is between 0 ppm (“non-detectable” or “ND”) and 1000 ppm. In an embodiment, the amount of inorganic residuals is ND to about 500 ppm. In an embodiment, the amount of inorganic residuals is ND to about

400 ppm. In an embodiment, the amount of inorganic residuals is ND to about 300 ppm. In an embodiment, the amount of inorganic residuals is ND to about 200 ppm. In an embodiment, the amount of inorganic residuals is ND to about 100 ppm. In an embodiment, the amount of inorganic residuals is between 10 ppm and 1000 ppm.

5 As used herein, the term “substantially free of organic residuals” means that the composition exhibits residuals of 0.1 % (w/w) or less, in an embodiment, substantially free of organic residuals refers to a composition that exhibits residuals of 0.05% (w/w) or less. In an embodiment, substantially free of organic residuals refers to a composition that exhibits residuals of 0.01% (w/w) or less. In an embodiment, the
10 amount of organic residuals is between 0 ppm (“non-detectable” or “ND”) and 1000 ppm. In an embodiment, the amount of organic residuals is ND to about 500 ppm. In an embodiment, the amount of organic residuals is ND to about 400 ppm. In an embodiment, the amount of organic residuals is ND to about 300 ppm. In an embodiment, the amount of organic residuals is ND to about 200 ppm. In an
15 embodiment, the amount of organic residuals is ND to about 100 ppm. In an embodiment, the amount of organic residuals is between 10 ppm and 1000 ppm.

 Compositions of the present disclosure exhibit “biocompatibility” meaning that the compositions are compatible with living tissue or a living system by not being toxic, injurious, or physiologically reactive and not causing immunological rejection.
20 Such biocompatibility can be evidenced by participants topically applying compositions of the present disclosure on their skin for an extended period of time. In an embodiment, the extended period of time is about 3 days. In an embodiment, the extended period of time is about 7 days, in an embodiment, the extended period of time is about 14 days, in an embodiment, the extended period of time is about 21
25 days. In an embodiment, the extended period of time is about 30 days. In an embodiment, the extended period of time is selected from the group consisting of about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, about 12 months, and indefinitely.

30 Compositions of the present disclosure are “hypoallergenic” meaning that they are relatively unlikely to cause an allergic reaction. Such hypoallergenicity can be evidenced by participants topically applying compositions of the present disclosure on their skin for an extended period of time. In an embodiment, the extended period of time is about 3 days. In an embodiment, the extended period of time is about 7 days.

In an embodiment, the extended period of time is about 14 days. In an embodiment, the extended period of time is about 21 days. In an embodiment, the extended period of time is about 30 days. In an embodiment, the extended period of time is selected from the group consisting of about 1 month, about 2 months, about 3 months, about 4
5 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, about 12 months, and indefinitely.

Following are non-limiting examples of suitable ranges for various parameters in and for preparation of the silk solutions of the present disclosure. The silk solutions of the present disclosure may include one or more, but not necessarily all, of these
10 parameters and may be prepared using various combinations of ranges of such parameters.

In an embodiment, the percent SPF in the solution is less than 30.0 wt. %. In an embodiment, the percent SPF in the solution is less than 25.0 wt. %. In an embodiment, the percent SPF in the solution is less than 20.0 wt. %. In an
15 embodiment, the percent SPF in the solution is less than 19.0 wt. %. In an embodiment, the percent SPF in the solution is less than 18.0 wt. %. In an embodiment, the percent SPF in the solution is less than 17.0 wt. %. In an embodiment, the percent SPF in the solution is less than 16.0 wt. %. In an embodiment, the percent SPF in the solution is less than 15.0 wt. %. In an
20 embodiment, the percent SPF in the solution is less than 14.0 wt. %. In an embodiment, the percent SPF in the solution is less than 13.0 wt. %. In an embodiment, the percent SPF in the solution is less than 12.0 wt. %. In an embodiment, the percent SPF in the solution is less than 11.0 wt. %. In an embodiment, the percent SPF in the solution is less than 10.0 wt. %. In an
25 embodiment, the percent SPF in the solution is less than 9.0 wt. %. In an embodiment, the percent SPF in the solution is less than 8.0 wt. %. In an embodiment, the percent SPF in the solution is less than 7.0 wt. %. In an embodiment, the percent SPF in the solution is less than 6.0 wt. %. In an embodiment, the percent SPF in the solution is less than 5.0 wt. %. In an embodiment, the percent SPF in the solution is less than 4.0
30 wt. %. In an embodiment, the percent SPF in the solution is less than 3.0 wt. %. In an embodiment, the percent SPF in the solution is less than 2.0 wt. %. In an embodiment, the percent SPF in the solution is less than 1.0 wt. %. In an embodiment, the percent SPF in the solution is less than 0.9 wt. %. In an embodiment, the percent SPF in the solution is less than 0.8 wt. %. In an embodiment, the percent SPF in the solution is

less than 0.7 wt. %. In an embodiment, the percent SPF in the solution is less than 0.6 wt. %. In an embodiment, the percent SPF in the solution is less than 0.5 wt. %. In an embodiment, the percent SPF in the solution is less than 0.4 wt. %. In an embodiment, the percent SPF in the solution is less than 0.3 wt. %. In an embodiment, the percent
5 SPF in the solution is less than 0.2 wt. %. In an embodiment, the percent SPF in the solution is less than 0.1 wt. %.

In an embodiment, the percent SPF in the solution is greater than 0.1 wt. %. In an embodiment, the percent SPF in the solution is greater than 0.2 wt. %. In an embodiment, the percent SPF in the solution is greater than 0.3 wt. %. In an
10 embodiment, the percent SPF in the solution is greater than 0.4 wt. %. In an embodiment, the percent SPF in the solution is greater than 0.5 wt. %. In an embodiment, the percent SPF in the solution is greater than 0.6 wt. %. In an embodiment, the percent SPF in the solution is greater than 0.7 wt. %. In an embodiment, the percent SPF in the solution is greater than 0.8 wt. %. In an
15 embodiment, the percent SPF in the solution is greater than 0.9 wt. %. In an embodiment, the percent SPF in the solution is greater than 1.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 2.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 3.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 4.0 wt. %. In an
20 embodiment, the percent SPF in the solution is greater than 5.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 6.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 7.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 8.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 9.0 wt. %. In an
25 embodiment, the percent SPF in the solution is greater than 10.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 11.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 12.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 13.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 14.0 wt. %. In an
30 embodiment, the percent SPF in the solution is greater than 15.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 16.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 17.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 18.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 19.0 wt. %. In an

embodiment, the percent SPF in the solution is greater than 20.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 25.0 wt. %.

[illegible]

embodiment, the percent SPF in the solution ranges from about 1.0 wt. % to about 3.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 1.0 wt. % to about 2.5 wt. %. In an embodiment, the percent SPF in the solution ranges from about 1.0 wt. % to about 2.4 wt. %. In an embodiment, the percent SPF in the solution
5 ranges from about 1.0 wt. % to about 2.0 wt. %.

In an embodiment, the percent SPF in the solution ranges from about 20.0 wt. % to about 30.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 10.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 1.0 wt. % to about 10.0 wt. %. In an embodiment, the
10 percent SPF in the solution ranges from about 2 wt. % to about 10.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 6.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 6.0 wt. % to about 10.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 6.0 wt. % to about 8.0 wt. %. In an embodiment, the percent SPF in the solution
15 ranges from about 6.0 wt. % to about 9.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 10.0 wt. % to about 20.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 11.0 wt. % to about 19.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 12.0 wt. % to about 18.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 13.0
20 wt. % to about 17.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 14.0 wt. % to about 16.0 wt. %. In an embodiment, the percent SPF in the solution is about 1.0 wt. %. In an embodiment, the percent SPF in the solution is about 0.5 wt. %. In an embodiment, the percent SPF in the solution is about 1.5 wt. %. In an embodiment, the percent SPF in the solution is about 2.0 wt.%. In an
25 embodiment, the percent SPF in the solution is about 2.4 wt. %. In an embodiment, the percent SPF in the solution is 3.0 wt. %. In an embodiment, the percent SPF in the solution is 3.5 wt. %. In an embodiment, the percent SPF in the solution is about 4.0 wt. %. In an embodiment, the percent SPF in the solution is about 4.5 wt. %. In an embodiment, the percent SPF in the solution is about 5.0 wt. %. In an embodiment,
30 the percent SPF in the solution is about 5.5 wt. %. In an embodiment the percent SPF in the solution is about 6.0 wt. %. In an embodiment, the percent SPF in the solution is about 6.5 wt. %. In an embodiment, the percent SPF in the solution is about 7.0 wt. %. In an embodiment, the percent SPF in the solution is about 7.5 wt. %. In an embodiment, the percent SPF in the solution is about 8.0 wt. %. In an embodiment,

the percent SPF in the solution is about 8.5 wt. %. In an embodiment, the percent SPF in the solution is about 9.0 wt. %. In an embodiment, the percent SPF in the solution is about 9.5 wt. %. In an embodiment, the percent SPF in the solution is about 10.0 wt. %.

5 In an embodiment, the percent sericin in the solution is non-detectable to 25.0 wt. %. In an embodiment, the percent sericin in the solution is non-detectable to 5.0 wt. %. In an embodiment, the percent sericin in the solution is 1.0 wt. %. In an embodiment, the percent sericin in the solution is 2.0 wt. %. In an embodiment, the percent sericin in the solution is 3.0 wt. %. In an embodiment, the percent sericin in the solution is 4.0 wt. %. In an embodiment, the percent sericin in the solution is 5.0 wt. %. In an embodiment, the percent sericin in the solution is 10.0 wt. %. In an embodiment, the percent sericin in the solution is 25.0 wt. %.

 In some embodiments, the silk fibroin protein fragments of the present disclosure are shelf stable (they will not slowly or spontaneously gel when stored in an aqueous solution and there is no aggregation of fragments and therefore no increase in molecular weight over time), from 10 days to 3 years depending on storage conditions, percent SPF, and number of shipments and shipment conditions. Additionally, pH may be altered to extend shelf life and/or support shipping conditions by preventing premature folding and aggregation of the silk. In an embodiment, the stability of the LiBr-silk fragment solution is 0 to 1 year. In an embodiment, the stability of the LiBr-silk fragment solution is 0 to 2 years. In an embodiment, the stability of the LiBr-silk fragment solution is 0 to 3 years. In an embodiment, the stability of the LiBr-silk fragment solution is 0 to 4 years. In an embodiment, the stability of the LiBr-silk fragment solution is 0 to 5 years. In an embodiment, the stability of the LiBr-silk fragment solution is 1 to 2 years. In an embodiment, the stability of the LiBr-silk fragment solution is 1 to 3 years. In an embodiment, the stability of the LiBr-silk fragment solution is 1 to 4 years. In an embodiment, the stability of the LiBr-silk fragment solution is 1 to 5 years. In an embodiment, the stability of the LiBr-silk fragment solution is 2 to 3 years. In an embodiment, the stability of the LiBr-silk fragment solution is 2 to 4 years. In an embodiment, the stability of the LiBr-silk fragment solution is 2 to 5 years. In an embodiment, the stability of the LiBr-silk fragment solution is 3 to 4 years. In an embodiment, the stability of the LiBr-silk fragment solution is 3 to 5 years. In an embodiment, the stability of the LiBr-silk fragment solution is 4 to 5 years.

In an embodiment, the stability of a composition of the present disclosure is 10 days to 6 months. In an embodiment, the stability of a composition of the present disclosure is 6 months to 12 months. In an embodiment, the stability of a composition of the present disclosure is 12 months to 18 months. In an embodiment, the stability of a composition of the present disclosure is 18 months to 24 months. In an embodiment, the stability of a composition of the present disclosure is 24 months to 30 months. In an embodiment, the stability of a composition of the present disclosure is 30 months to 36 months. In an embodiment, the stability of a composition of the present disclosure is 36 months to 48 months. In an embodiment, the stability of a composition of the present disclosure is 48 months to 60 months.

In an embodiment, a composition of the present disclosure having SPF has non-detectable levels of LiBr residuals. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is between 10 ppm and 1000 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is between 10 ppm and 300 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is less than 25 ppm. In an embodiment, the amount of the Li Br residuals in a composition of the present disclosure is less than 50 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is less than 75 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is less than 100 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is less than 200 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is less than 300 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is less than 400 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is less than 500 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is less than 600 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is less than 700 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is less than 800 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is less than 900 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is less than 1000 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is non-

detectable to 500 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is non-detectable to 450 ppm. In an embodiment, the amount of the LiBr residue in a composition of the present disclosure is non-detectable to 400 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is non-detectable to 350 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is non-detectable to 300 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is non-detectable to 250 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is non-detectable to 200 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is non-detectable to 150 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is non-detectable to 100 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is 100 ppm to 200 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is 200 ppm to 300 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is 300 ppm to 400 ppm. In an embodiment, the amount of the LiBr residuals in a composition of the present disclosure is 400 ppm to 500 ppm.

In an embodiment, a composition of the present disclosure having SPF, has non-detectable levels of Na_2CO_3 residuals. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is less than 100 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is less than 200 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is less than 300 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is less than 400 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is less than 500 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is less than 600 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is less than 700 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is less than 800 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is less than 900 ppm. In an

embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is less than 1000 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is non-detectable to 500 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is non-detectable to 450 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is non-detectable to 400 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is non-detectable to 350 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is non-detectable to 300 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is non-detectable to 250 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is non-detectable to 200 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is non-detectable to 150 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is non-detectable to 100 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is 100 ppm to 200 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is 200 ppm to 300 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is 300 ppm to 400 ppm. In an embodiment, the amount of the Na_2CO_3 residuals in a composition of the present disclosure is 400 ppm to 500 ppm.

A unique feature of the SPF compositions of the present disclosure are shelf stability (they will not slowly or spontaneously gel when stored in an aqueous solution and there is no aggregation of fragments and therefore no increase in molecular weight over time), from 10 days to 3 years depending on storage conditions, percent silk, and number of shipments and shipment conditions. Additionally pH may be altered to extend shelf-life and/or support shipping conditions by preventing premature folding and aggregation of the silk. In an embodiment, a SPF solution composition of the present disclosure has a shelf stability for up to 2 weeks at room temperature (RT). In an embodiment, a SPF solution composition of the present disclosure has a shelf stability for up to 4 weeks at RT. In an embodiment, a SPF solution composition of the present disclosure has a shelf stability for up to 6 weeks at RT. In an embodiment, a SPF solution composition of the present disclosure has a shelf stability for up to 8 weeks at RT. In an embodiment,

a SPF solution composition of the present disclosure has a shelf stability for up to 10 weeks at RT. In an embodiment, a SPF solution composition of the present disclosure has a shelf stability for up to 12 weeks at RT. In an embodiment, a SPF solution composition of the present disclosure has a shelf stability ranging from about 4 weeks to about 52 weeks at RT.

Table R below shows shelf stability test results for embodiments of SPF compositions of the present disclosure.

Table R. Shelf Stability of SPF Compositions of the Present Disclosure		
% Silk	Temperature	Time to Gelation
2	RT	4 weeks
2	4 °C	> 9 weeks
4	RT	4 weeks
4	4 °C	> 9 weeks
6	RT	2 weeks
6	4 °C	> 9 weeks

In some embodiments, the water solubility of the silk film derived from silk fibroin protein fragments as described herein can be modified by solvent annealing (water annealing or methanol annealing), chemical crosslinking, enzyme crosslinking and heat treatment.

In some embodiments, the process of annealing may involve inducing beta-sheet formation in the silk fibroin protein fragment solutions used as a coating material. Techniques of annealing (e.g., increase crystallinity) or otherwise promoting “molecular packing” of silk fibroin-protein based fragments have been described. In some embodiments, the amorphous silk film is annealed to introduce beta-sheet in the presence of a solvent selected from the group of water or organic solvent. In some embodiments, the amorphous silk film is annealed to introduce beta-sheet in the presence of water (water annealing process). In some embodiments, the amorphous silk fibroin protein fragment film is annealed to introduce beta-sheet in the presence of methanol. In some embodiments, annealing (e.g., the beta sheet formation) is induced by addition of an organic solvent. Suitable organic solvents include, but are not limited to methanol, ethanol, acetone, isopropanol, or combination thereof.

In some embodiments, annealing is carried out by so-called “water-annealing” or “water vapor annealing” in which water vapor is used as an intermediate plasticizing agent or catalyst to promote the packing of beta-sheets. In some embodiments, the process of water annealing may be performed under vacuum.

- 5 Suitable such methods have been described in Jin H-J et al. (2005), Water-stable Silk Films with Reduced Beta-Sheet Content, *Advanced Functional Materials*, 15: 1241-1247; Xiao H. et al. (2011), Regulation of Silk Material Structure by Temperature-Controlled Water Vapor Annealing, *Biomacromolecules*, 12(5): 1686-1696.

The important feature of the water annealing process is to drive the formation
10 of crystalline beta-sheet in the silk fibroin protein fragment peptide chain to allow the silk fibroin self-assembling into a continuous film. In some embodiments, the crystallinity of the silk fibroin protein fragment film is controlled by controlling the temperature of water vapor and duration of the annealing. In some embodiments, the annealing is performed at a temperature ranging from about 65 °C to about 110 °C. In
15 some embodiments, the temperature of the water is maintained at about 80 °C. In some embodiments, annealing is performed at a temperature selected from the group of about 65 °C, about 70 °C, about 75 °C, about 80 °C, about 85 °C, about 90 °C, about 95 °C, about 100 °C, about 105 °C, and about 110 °C.

In some embodiments, the annealing process lasts a period of time selected
20 from the group of about 1 minute to about 40 minutes, about 1 minute to about 50 minutes, about 1 minute to about 60 minutes, about 1 minute to about 70 minutes, about 1 minute to about 80 minutes, about 1 minute to about 90 minutes, about 1 minute to about 100 minutes, about 1 minute to about 110 minutes, about 1 minute to about 120 minutes, about 1 minute to about 130 minutes, about 5 minutes to about
25 40 minutes, about 5 minutes to about 50 minutes, about 5 minutes to about 60 minutes, about 5 minutes to about 70 minutes, about 5 minutes to about 80 minutes, about 5 minutes to about 90 minutes, about 5 minutes to about 100 minutes, about 5 minutes to about 110 minutes, about 5 minutes to about 120 minutes, about 5 minutes to about 130 minutes, about 10 minutes to about 40 minutes, about 10
30 minutes to about 50 minutes, about 10 minutes to about 60 minutes, about 10 minutes to about 70 minutes, about 10 minutes to about 80 minutes, about 10 minutes to about 90 minutes, about 10 minutes to about 100 minutes, about 10 minutes to about 110 minutes, about 10 minutes to about 120 minutes, about 10 minutes to about 130 minutes, about 15 minutes to about 40 minutes, about 15 minutes to about

50 minutes, about 15 minutes to about 60 minutes, about 15 minutes to about 70 minutes, about 15 minutes to about 80 minutes, about 15 minutes to about 90 minutes, about 15 minutes to about 100 minutes, about 15 minutes to about 110 minutes, about 15 minutes to about 120 minutes, about 15 minutes to about 130 minutes, about 20 minutes to about 40 minutes, about 20 minutes to about 50 minutes, about 20 minutes to about 60 minutes, about 20 minutes to about 70 minutes, about 20 minutes to about 80 minutes, about 20 minutes to about 90 minutes, about 20 minutes to about 100 minutes, about 20 minutes to about 110 minutes, about 20 minutes to about 120 minutes, about 20 minutes to about 130 minutes, about 25 minutes to about 40 minutes, about 25 minutes to about 50 minutes, about 25 minutes to about 60 minutes, about 25 minutes to about 70 minutes, about 25 minutes to about 80 minutes, about 25 minutes to about 90 minutes, about 25 minutes to about 100 minutes, about 25 minutes to about 110 minutes, about 25 minutes to about 120 minutes, about 25 minutes to about 130 minutes, about 30 minutes to about 40 minutes, about 30 minutes to about 50 minutes, about 30 minutes to about 60 minutes, about 30 minutes to about 70 minutes, about 30 minutes to about 80 minutes, about 30 minutes to about 90 minutes, about 30 minutes to about 100 minutes, about 30 minutes to about 110 minutes, about 30 minutes to about 120 minutes, about 30 minutes to about 130 minutes, about 35 minutes to about 40 minutes, about 35 minutes to about 50 minutes, about 35 minutes to about 60 minutes, about 35 minutes to about 70 minutes, about 35 minutes to about 80 minutes, about 35 minutes to about 90 minutes, about 35 minutes to about 100 minutes, about 35 minutes to about 110 minutes, about 35 minutes to about 120 minutes, about 35 minutes to about 130 minutes, about 40 minutes to about 50 minutes, about 40 minutes to about 60 minutes, about 40 minutes to about 70 minutes, about 40 minutes to about 80 minutes, about 40 minutes to about 90 minutes, about 40 minutes to about 100 minutes, about 40 minutes to about 110 minutes, about 40 minutes to about 120 minutes, about 40 minutes to about 130 minutes, about 45 minutes to about 50 minutes, about 45 minutes to about 60 minutes, about 45 minutes to about 70 minutes, about 45 minutes to about 80 minutes, about 45 minutes to about 90 minutes, about 45 minutes to about 100 minutes, about 45 minutes to about 110 minutes, about 45 minutes to about 120 minutes, and about 45 minutes to about 130 minutes. In some embodiments, the annealing process lasts a period of time ranging

from about 1 minute to about 60 minutes. In some embodiments, the annealing process lasts a period of time ranging from about 45 minutes to about 60 minutes. The longer water annealing post-processing corresponded an increased crystallinity of silk fibroin protein fragments.

5 In some embodiments, the annealed silk fibroin protein fragment film is immersing the wet silk fibroin protein fragment film in 100 % methanol for 60 minutes at room temperature. The methanol annealing changed the composition of silk fibroin protein fragment film from predominantly amorphous random coil to crystalline antiparallel beta-sheet structure.

10 In some embodiments, the SPF as described herein can be used to prepare SPF microparticles by precipitation with methanol. Alternative flash drying, fluid-bed drying, spray drying or vacuum drying can be applied to remove water from the silk solution. The SPF powder can then be stored and handled without refrigeration or other special handling procedures. In some embodiments, the SPF powders comprise
15 low molecular weight silk fibroin protein fragments. In some embodiments, the SPF powders comprise mid-molecular weight silk fibroin protein fragments. In some embodiments, the SPF powders comprise a mixture of low molecular weight silk fibroin protein fragments and mid-molecular weight silk fibroin protein fragment.

 As used herein, the terms “substantially sericin free” or “substantially devoid
20 of sericin” refer to silk fibers in which a majority of the sericin protein has been removed. In an embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having from about 0.01 wt. % to about 10.0 wt. % sericin. In an embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having about 0.01 wt. % to about 9.0 wt. % sericin. In an embodiment, silk fibroin
25 that is substantially devoid of sericin refers to silk fibroin having from about 0.01 wt. % to about 8.0 wt. % sericin. In an embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having from about 0.01 wt. % to about 7.0 wt. % sericin. In an embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having from about 0.01 wt. % to about 6.0 wt. % sericin. In an
30 embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having from about 0.01 wt. % to about 5.0 wt. % sericin. In an embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having from about 0 wt. % to about 4.0 wt. % sericin. In an embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having from about 0.05 wt. % to about 4.0 wt.

% sericin. In an embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having from about 0.1 wt. % to about 4.0 wt. % sericin. In an embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having from about 0.5 wt. % to about 4.0 wt. % sericin. In an embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having from about 1.0 wt. % to about 4.0 wt. % sericin. In an embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having from about 1.5 wt. % to about 4.0 wt. % sericin. In an embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having from about 2.0 wt. % to about 4.0 wt. % sericin. In an embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having from about 2.5 wt. % to about 4.0 wt. % sericin. In an embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having a sericin content from about 0.01 wt. % to about 0.1 wt. %. In an embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having a sericin content below about 0.1 wt. %. In an embodiment, silk fibroin that is substantially devoid of sericin refers to silk fibroin having a sericin content below about 0.05 wt. %. In an embodiment, when a silk source is added to a boiling (100 °C) aqueous solution of sodium carbonate for a treatment time of between about 30 minutes to about 60 minutes, a degumming loss of about 26.0 wt. % to about 31.0 wt. % is obtained.

Following are non-limiting examples of suitable ranges for various parameters in and for preparation of the silk solutions of the present disclosure. The silk solutions of the present disclosure may include one or more, but not necessarily all, of these parameters and may be prepared using various combinations of ranges of such parameters.

In an embodiment, the percent SPF in the solution is less than 30.0 wt. %. In an embodiment, the percent SPF in the solution is less than 25.0 wt. %. In an embodiment, the percent SPF in the solution is less than 20.0 wt. %. In an embodiment, the percent SPF in the solution is less than 19.0 wt. %. In an embodiment, the percent SPF in the solution is less than 18.0 wt. %. In an embodiment, the percent SPF in the solution is less than 17.0 wt. %. In an embodiment, the percent SPF in the solution is less than 16.0 wt. %. In an embodiment, the percent SPF in the solution is less than 15.0 wt. %. In an embodiment, the percent SPF in the solution is less than 14.0 wt. %. In an embodiment, the percent SPF in the solution is less than 13.0 wt. %. In an

embodiment, the percent SPF in the solution is less than 12.0 wt. %. In an embodiment, the percent SPF in the solution is less than 11.0 wt. %. In an embodiment, the percent SPF in the solution is less than 10.0 wt. %. In an embodiment, the percent SPF in the solution is less than 9.0 wt. %. In an embodiment, the percent SPF in the solution is less than 8.0 wt. %. In an embodiment, the percent SPF in the solution is less than 7.0 wt. %. In an embodiment, the percent SPF in the solution is less than 6.0 wt. %. In an embodiment, the percent SPF in the solution is less than 5.0 wt. %. In an embodiment, the percent SPF in the solution is less than 4.0 wt. %. In an embodiment, the percent SPF in the solution is less than 3.0 wt. %. In an embodiment, the percent SPF in the solution is less than 2.0 wt. %. In an embodiment, the percent SPF in the solution is less than 1.0 wt. %. In an embodiment, the percent SPF in the solution is less than 0.9 wt. %. In an embodiment, the percent SPF in the solution is less than 0.8 wt. %. In an embodiment, the percent SPF in the solution is less than 0.7 wt. %. In an embodiment, the percent SPF in the solution is less than 0.6 wt. %. In an embodiment, the percent SPF in the solution is less than 0.5 wt. %. In an embodiment, the percent SPF in the solution is less than 0.4 wt. %. In an embodiment, the percent SPF in the solution is less than 0.3 wt. %. In an embodiment, the percent SPF in the solution is less than 0.2 wt. %. In an embodiment, the percent SPF in the solution is less than 0.1 wt. %.

1 In an embodiment, the percent SPF in the solution is greater than 0.1 wt. %. In
an embodiment, the percent SPF in the solution is greater than 0.2 wt. %. In an
embodiment, the percent SPF in the solution is greater than 0.3 wt. %. In an
embodiment, the percent SPF in the solution is greater than 0.4 wt. %. In an
embodiment, the percent SPF in the solution is greater than 0.5 wt. %. In an
25 embodiment, the percent SPF in the solution is greater than 0.6 wt. %. In an
embodiment, the percent SPF in the solution is greater than 0.7 wt. %. In an
embodiment, the percent SPF in the solution is greater than 0.8 wt. %. In an
embodiment, the percent SPF in the solution is greater than 0.9 wt. %. In an
embodiment, the percent SPF in the solution is greater than 1.0 wt. %. In an
30 embodiment, the percent SPF in the solution is greater than 2.0 wt. %. In an
embodiment, the percent SPF in the solution is greater than 3.0 wt. %. In an
embodiment, the percent SPF in the solution is greater than 4.0 wt. %. In an
embodiment, the percent SPF in the solution is greater than 5.0 wt. %. In an
embodiment, the percent SPF in the solution is greater than 6.0 wt. %. In an

embodiment, the percent SPF in the solution is greater than 7.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 8.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 9.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 10.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 11.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 12.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 13.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 14.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 15.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 16.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 17.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 18.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 19.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 20.0 wt. %. In an embodiment, the percent SPF in the solution is greater than 25.0 wt. %.

In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 30.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 25.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 20.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 15.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 10.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 9.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 8.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 7.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 6.5 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 6.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 5.5 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 5.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 4.5 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 4.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 3.5 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 3.0

wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 2.5 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 2.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 2.4 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.5 wt. % to about 5.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.5 wt. % to about 4.5 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.5 wt. % to about 4.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.5 wt. % to about 3.5 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.5 wt. % to about 3.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.5 wt. % to about 2.5 wt. %. In an embodiment, the percent SPF in the solution ranges from about 1.0 wt. % to about 4.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 1.0 wt. % to about 3.5 wt. %. In an embodiment, the percent SPF in the solution ranges from about 1.0 wt. % to about 3.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 1.0 wt. % to about 2.5 wt. %. In an embodiment, the percent SPF in the solution ranges from about 1.0 wt. % to about 2.4 wt. %. In an embodiment, the percent SPF in the solution ranges from about 1.0 wt. % to about 2.0 wt. %.

In an embodiment, the percent SPF in the solution ranges from about 20.0 wt. % to about 30.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 10.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 1.0 wt. % to about 10.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 2 wt. % to about 10.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 0.1 wt. % to about 6.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 6.0 wt. % to about 10.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 6.0 wt. % to about 8.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 6.0 wt. % to about 9.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 10.0 wt. % to about 20.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 11.0 wt. % to about 19.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 12.0 wt. % to about 18.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 13.0 wt. % to about 17.0 wt. %. In an embodiment, the percent SPF in the solution ranges from about 14.0 wt. % to about 16.0 wt. %. In an embodiment, the percent SPF in the

solution is about 1.0 wt. %. In an embodiment, the percent SPF in the solution is about 1.5 wt. %. In an embodiment, the percent SPF in the solution is about 2.0 wt. %. In an embodiment, the percent SPF in the solution is about 2.4 wt. %. In an embodiment, the percent SPF in the solution is 3.0 wt. %. In an embodiment, the percent SPF in the solution is 3.5 wt. %. In an embodiment, the percent SPF in the solution is about 4.0 wt. %. In an embodiment, the percent SPF in the solution is about 4.5 wt. %. In an embodiment, the percent SPF in the solution is about 5.0 wt. %. In an embodiment, the percent SPF in the solution is about 5.5 wt. %. In an embodiment, the percent SPF in the solution is about 6.0 wt. %. In an embodiment, the percent SPF in the solution is about 6.5 wt. %. In an embodiment, the percent SPF in the solution is about 7.0 wt. %. In an embodiment, the percent SPF in the solution is about 7.5 wt. %. In an embodiment, the percent SPF in the solution is about 8.0 wt. %. In an embodiment, the percent SPF in the solution is about 8.5 wt. %. In an embodiment, the percent SPF in the solution is about 9.0 wt. %. In an embodiment, the percent SPF in the solution is about 9.5 wt. %. In an embodiment, the percent SPF in the solution is about 10.0 wt. %.

In an embodiment, the percent sericin in the solution is non-detectable to 25.0 wt. %. In an embodiment, the percent sericin in the solution is non-detectable to 5.0 wt. %. In an embodiment, the percent sericin in the solution is 1.0 wt. %. In an embodiment, the percent sericin in the solution is 2.0 wt. %. In an embodiment, the percent sericin in the solution is 3.0 wt. %. In an embodiment, the percent sericin in the solution is 4.0 wt. %. In an embodiment, the percent sericin in the solution is 5.0 wt. %. In an embodiment, the percent sericin in the solution is 10.0 wt. %. In an embodiment, the percent sericin in the solution is 25.0 wt. %.

In some embodiments, the silk fibroin-based protein fragments of the present disclosure are shelf stable (they will not slowly or spontaneously gel when stored in an aqueous solution and there is no aggregation of fragments and therefore no increase in molecular weight over time), from 10 days to 3 years depending on storage conditions, percent SPF, and number of shipments and shipment conditions. Additionally, pH may be altered to extend shelf life and/or support shipping conditions by preventing premature folding and aggregation of the silk. In an embodiment, the stability of the LiBr-silk fragment solution is 0 to 1 year. In an embodiment, the stability of the LiBr-silk fragment solution is 0 to 2 years. In an embodiment, the stability of the LiBr-silk fragment solution is 0 to 3 years. In an

embodiment, the stability of the LiBr-silk fragment solution is 0 to 4 years. In an embodiment, the stability of the LiBr-silk fragment solution is 0 to 5 years. In an embodiment, the stability of the LiBr-silk fragment solution is 1 to 2 years. In an embodiment, the stability of the LiBr-silk fragment solution is 1 to 3 years. In an embodiment, the stability of the LiBr-silk fragment solution is 1 to 4 years. In an embodiment, the stability of the LiBr-silk fragment solution is 1 to 5 years. In an embodiment, the stability of the LiBr-silk fragment solution is 2 to 3 years. In an embodiment, the stability of the LiBr-silk fragment solution is 2 to 4 years. In an embodiment, the stability of the LiBr-silk fragment solution is 2 to 5 years. In an embodiment, the stability of the LiBr-silk fragment solution is 3 to 4 years. In an embodiment, the stability of the LiBr-silk fragment solution is 3 to 5 years. In an embodiment, the stability of the LiBr-silk fragment solution is 4 to 5 years.

In an embodiment, the stability of a composition of the present disclosure is 10 days to 6 months. In an embodiment, the stability of a composition of the present disclosure is 6 months to 12 months. In an embodiment, the stability of a composition of the present disclosure is 12 months to 18 months. In an embodiment, the stability of a composition of the present disclosure is 18 months to 24 months. In an embodiment, the stability of a composition of the present disclosure is 24 months to 30 months. In an embodiment, the stability of a composition of the present disclosure is 30 months to 36 months. In an embodiment, the stability of a composition of the present disclosure is 36 months to 48 months. In an embodiment, the stability of a composition of the present disclosure is 48 months to 60 months.

In an embodiment, a selected property of the SPF coated articles that may be enhanced as compared to non-coated articles may include one or more of dimensional stability to laundering, dimensional stability to dry cleaning, appearance after laundering, appearance after dry cleaning, colorfastness to laundering, colorfastness to dry cleaning, colorfastness to non-chlorine bleach, seam torque/spirality (on knits), colorfastness to crocking, colorfastness to rubbing, colorfastness to water, colorfastness to light, colorfastness to perspiration, colorfastness to chlorinated pool water, colorfastness to sea water, tensile strength, seam slippage, tearing strength, seam breaking strength, abrasion resistance, pilling resistance, stretch recovery, bursting strength, colorfastness to die transfer in storage (labels), colorfastness to ozone, pile retention, bowing and skewing, colorfastness to saliva, snagging resistance, wrinkle resistance (e.g., appearance of apparel, retention of creases in

fabrics, smooth appearance of fabrics), water repellency, water resistance, stain repellent (e.g., water repellency, oil repellency, water/alcohol repellency), vertical wicking, water absorption, dry rate, soil release, air permeability, wicking, antimicrobial properties, ultraviolet protection, resistance to torque, malodor resistant, biocompatibility, wetting time, absorption rate, spreading speed, accumulative one-way transport, flame retardant properties, coloring properties, fabric softening properties, a pH adjusting property, an antifelting property, and overall moisture management capability.

In any of the foregoing embodiments, at least one property of the article is improved, wherein the property that is improved is dimensional stability to laundering, and wherein the property is improved by an amount relative to an uncoated article selected from the group consisting of at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 100%, at least 125%, at least 150%, at least 200%, at least 300%, at least 400%, and at least 500%.

In any of the foregoing embodiments, at least one property of the article is improved, wherein the property that is improved is size retention on laundering, and wherein the property is improved by an amount relative to an uncoated article selected from the group consisting of at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 100%, at least 125%, at least 150%, at least 200%, at least 300%, at least 400%, and at least 500%.

In any of the foregoing embodiments, at least one property of the article is improved, wherein the property that is improved is resistance to shrinkage, and wherein the property is improved by an amount relative to an uncoated article selected from the group consisting of at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 100%, at least 125%, at least 150%, at least 200%, at least 300%, at least 400%, and at least 500%.

Articles Enclosing Solid Silk Protein Fragments

In an embodiment, the disclosure may include a pouch comprising a plurality of holes, wherein the pouch encloses a substantially solid formulation comprising silk fibroin fragments. In an embodiment, the silk fibroin fragments have an average weight average molecular weight described elsewhere herein. In an embodiment, the silk fibroin fragments have a polydispersity described elsewhere herein. In an embodiment, the silk fibroin fragments comprise one or more of low molecular weight silk fibroin fragments and medium molecular weight silk fibroin fragments. In an embodiment, the solid formulation is substantially pure silk fibroin fragments.

In an embodiment, the pouch comprises a polymer selected from the group consisting of: nylon, polyglycolide (PGA), polylactic acid (PLA), poly(lactide-co-glycolide) (PLGA), polycaprolactone (PCL), poly(butylene succinate) (PBS), polybutylene succinate adipate, poly(p-dioxanone) (PPDO), poly(butylene adipate-co-terephthalate) (PBAT), a copolyester of terephthalic acid and lactic acid, a copolyester of terephthalic acid and glycolic acid, a copolyester of terephthalic acid and succinic acid, poly(hydroxybutyrate), poly(hydroxyvalerate), polyhydroxyhexanoate, a poly(hydroxyalkanoate) (PHA), polymethylene adipate/terephthalate, and combinations thereof.

In an embodiment, the pouch comprises nylon. Exemplary types of nylon include, but are not limited to, Nylon 6; Nylon 6,6; Nylon 4,6; Nylon 6,9; Nylon 6,10; Nylon 6,12; Nylon 11; and Nylon 12. In an embodiment, the pouch comprises polylactic acid.

In an embodiment, the pouch is biodegradable. In an embodiment, the pouch is biodegradable by hydrolysis, thermal degradation, photodegradation, oxidation, degradation by microorganisms, or a combination thereof. In another embodiment, the pouch is not biodegradable.

In an embodiment, plurality of holes have a diameter of between about 0.1 μm and about 1,000 μm , between about 10 μm and about 1,000 μm , between about 10 μm and about 900 μm , between about 10 μm and about 800 μm , between about 10 μm and about 700 μm , between about 10 μm and about 600 μm , between about 10 μm and about 500 μm , between about 10 μm and about 400 μm , between about 10 μm and about 300 μm , between about 10 μm and about 200 μm , between about 30 μm and about 180 μm , between about 50 μm and about 160 μm , between about 70 μm

and about 140 μm , between about 90 μm and about 120 μm , or between about 90 μm and about 110 μm . In an embodiment, the plurality of holes have a diameter of about 100 μm . In an embodiment, the holes are evenly distributed throughout the pouch. In another embodiment, the holes are randomly distributed throughout the pouch.

5 In an embodiment, the pouch is rectangular in shape. In an embodiment, the pouch is square in shape. In an embodiment, the edge of each side of the pouch is sealed such that the solid formulation comprising silk fibroin fragments is enclosed within the completely sealed pouch. In an embodiment, the pouch encloses between about between about 0.1 g and about 500 g, between about 0.1 g and about 100 g,
10 between about 0.1 g and about 50 g, 0.1 g and about 20 g, between about 0.1 g and about 15 g, between about 0.1 g and about 10 g, between about 1 g and about 10 g, or between about 1 g and about 5 g of the solid formulation. In one embodiment, the pouch encloses about 3.5 g of the solid formulation.

 In an embodiment, between about 0.01 g and about 1 g, between about 0.01 g
15 and about 0.8 g, between about 0.01 g and about 0.6 g, between about 0.01 g and about 0.4 g, between about 0.01 g and about 0.2 g, or between about 0.05 g and about 0.15 g of the solid formulation is enclosed per cm^2 of the pouch. In one embodiment, about 0.1 g of solid formulation is enclosed per cm^2 of the pouch. Therefore, in one
20 embodiment, a pouch with dimensions of about 6 cm x 6 cm can enclose about 3.5 g of solid formulation.

 In an embodiment, the solid formulation comprises loose formulation fragments or loose formulation particles. In an embodiment, the loose formulation fragments or loose formulation particles have a fragment or particle size of between about 0.1 mm and about 30 mm, about 0.1 mm and about 25 mm, about 0.1 mm and
25 about 20 mm, about 0.1 mm and about 15 mm, about 0.1 mm and about 10 mm, about 0.1 mm and about 5 mm, about 1 mm and about 5 mm, or about 1 mm and about 3 mm. In an embodiment, the loose formulation fragments or loose formulation particles comprise lyophilized silk fibroin fragments. In an embodiment, the lyophilized silk fibroin fragments are frozen.

30 In another embodiment, the solid formulation comprises cryo-pelletized silk fibroin fragments. In an embodiment, the solid formulation comprising cryo-pelletized silk fibroin fragments comprises dry, solid silk fibroin fragments that have been molded into a puck or disc. In an embodiment, the solid formulation is molded using a hand press and a mold to form a disc or puck. In one embodiment, the dry,

solid silk fibroin fragments comprise loose formulation fragments or loose formulation which are molded into a disc or puck. In an embodiment, the loose formulation fragments or loose formulation particles comprise lyophilized silk fibroin fragments. In an embodiment, the lyophilized silk fibroin fragments are frozen.

- 5 In an embodiment, the pouch addresses one or more difficulties of handling solid silk fibroin fragments. In one embodiment, the pouch addresses the difficulty of handling, transferring, and weighing out the loose flakes of dried silk fibroin fragments. In an embodiment, the low density and/or static nature of the solid silk fibroin fragments makes the fragments difficult to handle, transfer, and weigh.
- 10 Therefore, in an embodiment, the pouch addresses one or more difficulties associated with the low density and/or static nature of the solid silk fibroin fragments.

Methods of Reconstituting Silk Protein Fragments Formulations

- In an embodiment, the disclosure may include a method of reconstituting a substantially liquid formulation comprising silk fibroin fragments, the method
- 15 comprising: contacting a pouch enclosing a substantially solid formulation comprising silk fibroin fragments with a solvent, wherein the pouch comprises a plurality of holes. In an embodiment, the contacting dissolves a substantial portion of the solid formulation in the solvent. In an embodiment, the contacting comprises placing the pouch in a vessel and adding the solvent to the vessel.

- 20 The solvent can be any solvent that dissolves at least a portion of the silk fibroin fragments in the solid formulation. In an embodiment, the solvent dissolves a substantial portion of the silk fibroin fragments. In an embodiment, the solvent is a solvent that does not dissolve the pouch. In an embodiment, the solvent is a solvent that does not dissolve one or more impurities in the formulation. In an embodiment,
- 25 the solvent comprises water. In an embodiment, the solvent is water.

- The silk fibroin fragments are described elsewhere herein. In an embodiment, the silk fibroin fragments have an average weight average molecular weight described elsewhere herein. In an embodiment, the silk fibroin fragments have a polydispersity described elsewhere herein. In an embodiment, the silk fibroin fragments comprise
- 30 one or more of low molecular weight silk fibroin fragments and medium molecular weight silk fibroin fragments. In an embodiment, the solid formulation is substantially pure silk fibroin fragments.

The solid formulation comprising silk fibroin fragments is described elsewhere herein. In an embodiment, the solid formulation comprises loose formulation fragments or loose formulation particles described elsewhere herein. In another embodiment, the solid formulation comprises cryo-pelletized silk fibroin fragments described elsewhere herein.

In an embodiment, the solid formulation dissolves in the solvent at room temperature in between about 30 minutes and about 12 hours, between about 30 minutes and about 10 hours, between about 30 minutes and about 8 hours, between about 30 minutes and about 6 hours, between about 30 minutes and about 4 hours, between about 1 hour and about 4 hours, or between about 1.5 hours and about 2.5 hours. In one embodiment, the solid formulation dissolves in the solvent at room temperature in about 2 hours. In an embodiment, the solid formulation dissolves in the solvent at room temperature without any stirring or agitation.

In an embodiment, a 6 cm x 6 cm pouch encloses about 3.5 g of a solid formulation comprising loose formulation fragments or loose formulation particles or comprising cryo-pelletized silk fibroin fragments, wherein the solid formulation dissolves in about 100 mL of water at room temperature in about 2 hours. The dissolution of about 3.5 g of the solid formulation in about 100 mL of water produces a reconstituted substantially liquid silk fibroin fragments formulation comprising about 3% silk fibroin fragments in about 2 hours at room temperature.

The pouch is described elsewhere herein. In an embodiment, the pouch comprises nylon, polylactic acid, or a combination thereof.

In an embodiment, the pouch functions to filter one or more impurities from the solid formulation as the formulation dissolves in the solvent. Therefore, in an embodiment, the pouch eliminates processing steps (e.g. gravity and vacuum filtration, syringe filtration) that would otherwise be used to remove impurities from a solution formed contacting the solid formulation with the solvent. In an embodiment, the pouch functions to filter portions of the solid silk fibroin fragments that did not dissolve in the solvent. Therefore, in an embodiment, one or more impurities and/or a portion of undissolved solid silk fibroin fragments remain in the pouch after the substantially liquid silk fibroin fragments formulation is reconstituted.

In an embodiment, the pouch is discarded after the substantially liquid silk fibroin fragments formulation is reconstituted. In one embodiment, the discarded pouch biodegrades.

Methods of Making a Pouch Enclosing Solid Silk Protein Fragments

In an embodiment, the disclosure may include a method of making a pouch enclosing a substantially solid formulation comprising silk fibroin fragments, the method comprising: folding a rectangular piece of mesh in half; sealing the edges of two sides of the folded mesh, leaving the top of the folded mesh unsealed; loading the formulation into the folded mesh through the unsealed top; and sealing the top edge of the folded mesh to form the pouch.

In an embodiment, one or more edges of the folded mesh are sealed using heat sealing. In an embodiment, each open edge of the folded mesh is sealed using heat sealing. In an embodiment, excess mesh can be removed after the mesh has been completely sealed to form a pouch. In an embodiment, the piece of mesh comprises a polymer described elsewhere herein. In an embodiment, the mesh comprises nylon, polylactic acid, or a combination thereof.

In an embodiment, the size of the rectangular piece of mesh is selected based on the amount of the formulation that is to be enclosed in the pouch. In an embodiment, the pouch encloses between about 0.1 g and about 500 g, between about 0.1 g and about 100 g, between about 0.1 g and about 50 g, between about 0.1 g and about 20 g, between about 0.1 g and about 15 g, between about 0.1 g and about 10 g, between about 1 g and about 10 g, or between about 1 g and about 5 g of the formulation.

In an embodiment, between about 0.01 g and about 1 g, between about 0.01 g and about 0.8 g, between about 0.01 g and about 0.6 g, between about 0.01 g and about 0.4 g, between about 0.01 g and about 0.2 g, or between about 0.05 g and about 0.15 g of the formulation is enclosed per cm² of the pouch. Therefore, in one embodiment, a piece of mesh about 15 cm x 12 cm is used to produce a pouch having dimensions of about 6 cm x 6 cm and enclosing about 3.5 g of the formulation.

The silk fibroin fragments, the solid formulation, and the pouch are described elsewhere herein. In an embodiment, the silk fibroin fragments have an average weight average molecular weight described elsewhere herein. In an embodiment, the silk fibroin fragments have a polydispersity described elsewhere herein. In an embodiment, the silk fibroin fragments comprise one or more of low molecular weight silk fibroin fragments and medium molecular weight silk fibroin fragments. In an embodiment, the solid formulation is substantially pure silk fibroin fragments.

In an embodiment, the disclosure may include an article prepared by a method described elsewhere herein.

In one aspect, the present disclosure relates to a pouch comprising a plurality of holes, wherein the pouch encloses a substantially solid formulation comprising silk fibroin fragments having an average weight average molecular weight selected from between about 1 kDa and about 5 kDa, from between about 5 kDa and about 10 kDa, from between about 6 kDa and about 17 kDa, from between about 10 kDa and about 15 kDa, from between about 14 kDa and about 30 kDa, from between about 15 kDa and about 20 kDa, from between about 17 kDa and about 39 kDa, from between about 20 kDa and about 25 kDa, from between about 25 kDa and about 30 kDa, from between about 30 kDa and about 35 kDa, from between about 35 kDa and about 40 kDa, from between about 39 kDa and about 54 kDa, from between about 39 kDa and about 80 kDa, from between about 40 kDa and about 45 kDa, from between about 45 kDa and about 50 kDa, from between about 50 kDa and about 55 kDa, from between about 55 kDa and about 60 kDa, from between about 60 kDa and about 100 kDa, or from between about 80 kDa and about 144 kDa, and a polydispersity ranging from 1 to about 5. In an embodiment, the silk fibroin fragments have a polydispersity from 1 to about 1.5, from about 1.5 to about 2.0, from about 2.0 to about 2.5, from about 2.5 to about 3.0, from about 3.0 to about 3.5, from about 3.5 to about 4.0, from about 4.0 to about 4.5, or from about 4.5 to about 5.0. In an embodiment, the silk fibroin fragments have a polydispersity from about 1.5 to about 3.0. In an embodiment, the silk fibroin fragments comprise one or more of low molecular weight silk fibroin fragments and medium molecular weight silk fibroin fragments. In an embodiment, the pouch comprises a polymer selected from the group consisting of: nylon, polyglycolide (PGA), polylactic acid (PLA), poly(lactide-co-glycolide) (PLGA), polycaprolactone (PCL), poly(butylene succinate) (PBS), polybutylene succinate adipate, poly(p-dioxanone) (PPDO), poly(butylene adipate-co-terephthalate) (PBAT), a copolyester of terephthalic acid and lactic acid, a copolyester of terephthalic acid and glycolic acid, a copolyester of terephthalic acid and succinic acid, poly(hydroxybutyrate), poly(hydroxyvalerate), polyhydroxyhexanoate, a poly(hydroxyalkanoate) (PHA), polymethylene adipate/terephthalate, and combinations thereof. In an embodiment, the pouch comprises nylon, polylactic acid, or a combination thereof. In an embodiment, the pouch is biodegradable. In an embodiment, the pouch encloses between about 0.1 g and about 500 g, between about

0.1 g and about 100 g, between about 0.1 g and about 50 g, between about 0.1 g and about 20 g, between about 0.1 g and about 15 g, between about 0.1 g and about 10 g, between about 1 g and about 10 g, or between about 1 g and about 5 g of the formulation. In an embodiment, the formulation comprises loose formulation fragments or loose formulation particles. In an embodiment, the loose formulation fragments or loose formulation particles have a fragment or particle size of between about 0.1 mm and about 30 mm, about 0.1 mm and about 25 mm, about 0.1 mm and about 20 mm, about 0.1 mm and about 15 mm, about 0.1 mm and about 10 mm, about 0.1 mm and about 5 mm, about 1 mm and about 5 mm, or about 1 mm and about 3 mm. In an embodiment, the formulation comprises cryo-pelletized silk fibroin fragments. In an embodiment, the cryo-pelletized silk fibroin fragments comprise dry, solid silk fibroin fragments molded using a hand press and a mold to form a disc.

In another aspect, the present disclosure relates to a method of reconstituting a substantially liquid formulation comprising silk fibroin fragments, the method comprising: contacting a pouch enclosing a substantially solid formulation comprising silk fibroin fragments with a solvent, wherein the pouch comprises a plurality of holes. In an embodiment, the contacting comprises placing the pouch in a vessel and adding the solvent to the vessel. In an embodiment, the solvent comprises water. In an embodiment, the solvent dissolves a substantial portion of the silk fibroin fragments and does not dissolve one or more impurities in the solid formulation. In an embodiment, the solid formulation dissolves in the solvent at room temperature in between about 30 minutes and about 12 hours, between about 30 minutes and about 10 hours, between about 30 minutes and about 8 hours, between about 30 minutes and about 6 hours, between about 30 minutes and about 4 hours, between about 1 hour and about 4 hours, or between about 1.5 hours and about 2.5 hours. In an embodiment, the silk fibroin fragments have an average weight average molecular weight selected from between about 1 kDa and about 5 kDa, from between about 5 kDa and about 10 kDa, from between about 6 kDa and about 17 kDa, from between about 10 kDa and about 15 kDa, from between about 14 kDa and about 30 kDa, from between about 15 kDa and about 20 kDa, from between about 17 kDa and about 39 kDa, from between about 20 kDa and about 25 kDa, from between about 25 kDa and about 30 kDa, from between about 30 kDa and about 35 kDa, from between about 35 kDa and about 40 kDa, from between about 39 kDa and about 54 kDa, from between about 39 kDa and about 80 kDa, from between about 40 kDa and about 45 kDa, from

between about 45 kDa and about 50 kDa, from between about 50 kDa and about 55 kDa, from between about 55 kDa and about 60 kDa, from between about 60 kDa and about 100 kDa, or from between about 80 kDa and about 144 kDa, and a polydispersity ranging from 1 to about 5. In an embodiment, the silk fibroin fragments have a polydispersity from 1 to about 1.5, from about 1.5 to about 2.0, from about 2.0 to about 2.5, from about 2.5 to about 3.0, from about 3.0 to about 3.5, from about 3.5 to about 4.0, from about 4.0 to about 4.5, or from about 4.5 to about 5.0. In an embodiment, the silk fibroin fragments have a polydispersity from about 1.5 to about 3.0. In an embodiment, the silk fibroin fragments comprise one or more of low molecular weight silk fibroin fragments and medium molecular weight silk fibroin fragments. In an embodiment, the pouch comprises a polymer selected from the group consisting of: nylon, polyglycolide (PGA), polylactic acid (PLA), poly(lactide-co-glycolide) (PLGA), polycaprolactone (PCL), poly(butylene succinate) (PBS), polybutylene succinate adipate, poly(p-dioxanone) (PPDO), poly(butylene adipate-co-terephthalate) (PBAT), a copolyester of terephthalic acid and lactic acid, a copolyester of terephthalic acid and glycolic acid, a copolyester of terephthalic acid and succinic acid, poly(hydroxybutyrate), poly(hydroxyvalerate), polyhydroxyhexanoate, a poly(hydroxyalkanoate) (PHA), polymethylene adipate/terephthalate, and combinations thereof. In an embodiment, the pouch comprises nylon, polylactic acid, or a combination thereof. In an embodiment, the pouch is biodegradable. In an embodiment, the plurality of holes have a diameter of between about 0.1 μm and about 1,000 μm , between about 10 μm and about 1,000 μm , between about 10 μm and about 900 μm , between about 10 μm and about 800 μm , between about 10 μm and about 700 μm , between about 10 μm and about 600 μm , between about 10 μm and about 500 μm , between about 10 μm and about 400 μm , between about 10 μm and about 300 μm , between about 10 μm and about 200 μm , between about 30 μm and about 180 μm , between about 50 μm and about 160 μm , between about 70 μm and about 140 μm , between about 90 μm and about 120 μm , or between about 90 μm and about 110 μm . In an embodiment, the pouch encloses between about 0.1 g and about 500 g, between about 0.1 g and about 100 g, between about 0.1 g and about 50 g, between about 0.1 g and about 20 g, between about 0.1 g and about 15 g, between about 0.1 g and about 10 g, between about 1 g and about 10 g, or between about 1 g and about 5 g of the formulation. In an embodiment, the solid formulation comprises loose formulation fragments or loose formulation particles. In an embodiment, the

loose formulation fragments or loose formulation particles have a fragment or particle size of between about 0.1 mm and about 30 mm, about 0.1 mm and about 25 mm, about 0.1 mm and about 20 mm, about 0.1 mm and about 15 mm, about 0.1 mm and about 10 mm, about 0.1 mm and about 5 mm, about 1 mm and about 5 mm, or about 1 mm and about 3 mm. In an embodiment, the solid formulation comprises cryo-pelletized silk fibroin fragments. In an embodiment, the cryo-pelletized silk fibroin fragments comprise dry, solid silk fibroin fragments molded using a hand press and a mold to form a disc.

In yet another aspect, the present disclosure relates to a method of making a pouch enclosing a substantially solid formulation comprising silk fibroin fragments, the method comprising: folding a rectangular piece of mesh in half; sealing the edges of two sides of the folded mesh, leaving the top of the folded mesh unsealed; loading the formulation into the folded mesh through the unsealed top; and sealing the top edge of the folded mesh to form the pouch. In an embodiment, one or more edges of the folded mesh are sealed using heat sealing. In an embodiment, the pouch encloses between about 0.1 g and about 500 g, between about 0.1 g and about 100 g, between about 0.1 g and about 50 g, between about 0.1 g and about 20 g, between about 0.1 g and about 15 g, between about 0.1 g and about 10 g, between about 1 g and about 10 g, or between about 1 g and about 5 g of the formulation. In an embodiment, the silk fibroin fragments have an average weight average molecular weight selected from between about 1 kDa and about 5 kDa, from between about 5 kDa and about 10 kDa, from between about 6 kDa and about 17 kDa, from between about 10 kDa and about 15 kDa, from between about 14 kDa and about 30 kDa, from between about 15 kDa and about 20 kDa, from between about 17 kDa and about 39 kDa, from between about 20 kDa and about 25 kDa, from between about 25 kDa and about 30 kDa, from between about 30 kDa and about 35 kDa, from between about 35 kDa and about 40 kDa, from between about 39 kDa and about 54 kDa, from between about 39 kDa and about 80 kDa, from between about 40 kDa and about 45 kDa, from between about 45 kDa and about 50 kDa, from between about 50 kDa and about 55 kDa, from between about 55 kDa and about 60 kDa, from between about 60 kDa and about 100 kDa, or from between about 80 kDa and about 144 kDa, and a polydispersity ranging from 1 to about 5. In an embodiment, the silk fibroin fragments have a polydispersity from 1 to about 1.5, from about 1.5 to about 2.0, from about 2.0 to about 2.5, from about 2.5 to about 3.0, from about 3.0 to about 3.5, from about 3.5 to about 4.0, from about 4.0

to about 4.5, or from about 4.5 to about 5.0. In an embodiment, the silk fibroin fragments have a polydispersity from about 1.5 to about 3.0. In an embodiment, the silk fibroin fragments comprise one or more of low molecular weight silk fibroin fragments and medium molecular weight silk fibroin fragments. In an embodiment, the piece of mesh comprises a polymer selected from the group consisting of: nylon, polyglycolide (PGA), polylactic acid (PLA), poly(lactide-co-glycolide) (PLGA), polycaprolactone (PCL), poly(butylene succinate) (PBS), polybutylene succinate adipate, poly(p-dioxanone) (PPDO), poly(butylene adipate-co-terephthalate) (PBAT), a copolyester of terephthalic acid and lactic acid, a copolyester of terephthalic acid and glycolic acid, a copolyester of terephthalic acid and succinic acid, poly(hydroxybutyrate), poly(hydroxyvalerate), polyhydroxyhexanoate, a poly(hydroxyalkanoate) (PHA), polymethylene adipate/terephthalate, and combinations thereof. In an embodiment, the piece of mesh comprises nylon, polylactic acid, or a combination thereof. In an embodiment, the pouch is biodegradable. In an embodiment, the plurality of holes have a diameter of between about 0.1 μm and about 1,000 μm , between about 10 μm and about 1,000 μm , between about 10 μm and about 900 μm , between about 10 μm and about 800 μm , between about 10 μm and about 700 μm , between about 10 μm and about 600 μm , between about 10 μm and about 500 μm , between about 10 μm and about 400 μm , between about 10 μm and about 300 μm , between about 10 μm and about 200 μm , between about 30 μm and about 180 μm , between about 50 μm and about 160 μm , between about 70 μm and about 140 μm , between about 90 μm and about 120 μm , or between about 90 μm and about 110 μm . In an embodiment, the formulation comprises loose formulation fragments or loose formulation particles. In an embodiment, the loose formulation fragments or loose formulation particles have a fragment or particle size of between about 0.1 mm and about 30 mm, about 0.1 mm and about 25 mm, about 0.1 mm and about 20 mm, about 0.1 mm and about 15 mm, about 0.1 mm and about 10 mm, about 0.1 mm and about 5 mm, about 1 mm and about 5 mm, or about 1 mm and about 3 mm. In an embodiment, the formulation comprises cryo-pelletized silk fibroin fragments. In an embodiment, the cryo-pelletized silk fibroin fragments comprise dry, solid silk fibroin fragments molded using a hand press and a mold to form a disc. In yet another aspect, the present disclosure provides an article prepared by the above method.

The disclosure provides peptide compositions, e.g., silk fibroin derived peptide compositions.

The disclosure provides a lyophilized or sprayable peptide or protein fragment comprising a plurality of amino acids selected from M, R, V, K, T, F, I, L, C, A, Q, Y, N, D, E, G, S, H, P, and W, wherein at least one of the amino acids is modified, substituted, or replaced. In some embodiments, the peptide or protein fragment is a fibroin peptide or protein fragment comprising an amino acid modification, substitution, or replacement of an amino acid from of amino acids selected from M, R, V, K, T, F, I, L, C, A, Q, Y, N, D, E, G, S, H, P, and W. In some embodiments, the fibroin is a fibroin heavy chain, a fibroin light chain, or a fibrohexamerin. In some embodiments, the peptide or protein fragment comprises between about 2 and about 100 amino acids. In some embodiments, the peptide or protein fragment comprises between about 2 and about 25 amino acids. In some embodiments, the peptide or protein fragment comprises between about 25 and about 50 amino acids. In some embodiments, the peptide or protein fragment comprises between about 50 and about 75 amino acids. In some embodiments, the peptide or protein fragment comprises between about 75 and about 100 amino acids. In some embodiments, the peptide or protein fragment comprises between about 100 and about 125 amino acids. In some embodiments, the peptide or protein fragment comprises between about 125 and about 150 amino acids. In some embodiments, the peptide or protein fragment comprises between about 150 and about 200 amino acids. In some embodiments, the peptide or protein fragment comprises between about 200 and about 250, 300, 350, 400, 450, or 500 amino acids. In some embodiments, the peptide or protein fragment comprises between one and five modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein fragment comprises one modification, substitution, and/or replacement. In some embodiments, the peptide or protein fragment comprises two modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein fragment comprises three modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein fragment comprises four modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein fragment comprises five modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein fragment comprises six modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein fragment comprises seven modifications,

substitutions, and/or replacements. In some embodiments, the peptide or protein fragment comprises eight modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein fragment comprises nine modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein

5 fragment comprises ten modifications, substitutions, and/or replacements. In some embodiments, a modification, substitution, and/or replacement is selected from an asparagine to aspartic acid modification, substitution, and/or replacement, a glutamine to glutamic acid modification, substitution, and/or replacement, and a methionine to methionine oxide modification, substitution, and/or replacement. In some

10 embodiments, the fibroin is a fibroin heavy chain, and wherein a modification, substitution, and/or replacement is at a position corresponding to any one position from 1 to 5263 of the fibroin heavy chain. In some embodiments, a modification, substitution, and/or replacement is at Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, and/or N5262 position of fibroin heavy

15 chain. In some embodiments, the fibroin is a fibroin light chain, and wherein a modification, substitution, and/or replacement is at a position corresponding to any one position from 1 to 262 of the fibroin light chain. In some embodiments, a modification, substitution, and/or replacement is at N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, and/or

20 Q255 position of fibroin light chain. In some embodiments, the fibroin is a fibrohexamerin (p25), and wherein a modification, substitution, and/or replacement is at a position corresponding to any one position from 1 to 220 of the fibrohexamerin (p25). In some embodiments, a modification, substitution, and/or replacement is at Q62, N93, M120, N149, N172, N174, and/or N202 position of fibrohexamerin (p25).

25 The disclosure provides a lyophilized or sprayable composition comprising a plurality of peptides or protein fragments, each comprising a plurality of amino acids selected from M, R, V, K, T, F, I, L, C, A, Q, Y, N, D, E, G, S, H, P, and W, wherein at least one of the amino acids is modified, substituted, or replaced. In some

embodiments, the plurality of peptides or protein fragments comprises a fibroin

30 peptide or protein fragment comprising an amino acid modification, substitution, or replacement of an amino acid selected from M, R, V, K, T, F, I, L, C, A, Q, Y, N, D, E, G, S, H, P, and W. In some embodiments, the fibroin is a fibroin heavy chain, a fibroin light chain, or a fibrohexamerin. In some embodiments, the peptide or protein fragment comprises between about 2 and about 100 amino acids. In some

embodiments, the peptide or protein fragment comprises between about 2 and about 25 amino acids. In some embodiments, the peptide or protein fragment comprises between about 25 and about 50 amino acids. In some embodiments, the peptide or protein fragment comprises between about 50 and about 75 amino acids. In some
5 embodiments, the peptide or protein fragment comprises between about 75 and about 100 amino acids. In some embodiments, the peptide or protein fragment comprises between about 100 and about 125 amino acids. In some embodiments, the peptide or protein fragment comprises between about 125 and about 150 amino acids. In some
10 embodiments, the peptide or protein fragment comprises between about 150 and about 200 amino acids. In some embodiments, the peptide or protein fragment comprises between about 200 and about 250, 300, 350, 400, 450, or 500 amino acids. In some
embodiments, the peptide or protein fragment comprises between one and five
modifications, substitutions, and/or replacements. In some embodiments, the peptide
or protein fragment comprises one modification, substitution, and/or replacement. In
15 some embodiments, the peptide or protein fragment comprises two modifications,
substitutions, and/or replacements. In some embodiments, the peptide or protein
fragment comprises three modifications, substitutions, and/or replacements. In some
embodiments, the peptide or protein fragment comprises four modifications,
substitutions, and/or replacements. In some embodiments, the peptide or protein
20 fragment comprises five modifications, substitutions, and/or replacements. In some
embodiments, the peptide or protein fragment comprises six modifications,
substitutions, and/or replacements. In some embodiments, the peptide or protein
fragment comprises seven modifications, substitutions, and/or replacements. In some
embodiments, the peptide or protein fragment comprises eight modifications,
25 substitutions, and/or replacements. In some embodiments, the peptide or protein
fragment comprises nine modifications, substitutions, and/or replacements. In some
embodiments, the peptide or protein fragment comprises ten modifications,
substitutions, and/or replacements. In some embodiments, a modification,
substitution, and/or replacement is selected from an asparagine to aspartic acid
30 modification, substitution, and/or replacement, a glutamine to glutamic acid
modification, substitution, and/or replacement, and a methionine to methionine oxide
modification, substitution, and/or replacement. In some embodiments, the fibroin is a
fibroin heavy chain, and wherein a modification, substitution, and/or replacement is at
a position corresponding to any one position from 1 to 5263 of the fibroin heavy

chain. In some embodiments, a modification, substitution, and/or replacement is at Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, and/or N5262 position of fibroin heavy chain. In some embodiments, the fibroin is a fibroin light chain, and wherein a modification, substitution, and/or

5 replacement is at a position corresponding to any one position from 1 to 262 of the fibroin light chain. In some embodiments, a modification, substitution, and/or replacement is at N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, and/or Q255 position of fibroin light chain. In some embodiments, the fibroin is a fibrohexamerin (p25), and wherein a

10 modification, substitution, and/or replacement is at a position corresponding to any one position from 1 to 220 of the fibrohexamerin (p25). In some embodiments, a modification, substitution, and/or replacement is at Q62, N93, M120, N149, N172, N174, and/or N202 position of fibrohexamerin (p25). In some embodiments, each modification, substitution, and/or replacement is independently ranging in the

15 composition between about 1% to about 99%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 1% to about 10%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 10% to about 20%. In some embodiments, each

20 modification, substitution, and/or replacement is independently ranging in the composition between about 20% to about 30%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 30% to about 40%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the

25 composition between about 40% to about 50%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 50% to about 60%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 60% to about 70%. In some embodiments, each

30 modification, substitution, and/or replacement is independently ranging in the composition between about 70% to about 80%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 80% to about 90%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the

composition between or about 90% to about 99%. As used herein, a % modification, substitution, and/or replacement is defined as (number of peptide or protein fragments comprising a modification, substitution, and/or replacement at a specific position, divided by the total number of peptide or protein fragments which include the specific position, whether comprising a modification, substitution, and/or replacement, or not) x 100.

The disclosure provides a lyophilized or sprayable composition comprising a plurality of peptides or protein fragments of fibroin heavy chain, fibroin light chain, and/or fibrohexamerin (p25), the composition comprising one or more fractions, wherein the plurality of peptides or protein fragments comprises a fibroin peptide or protein fragment comprising an amino acid modification, substitution, or replacement. In some embodiments, the plurality of peptides or protein fragments having a weight average molecular weight (M_w) selected from between about 1 kDa and about 20 kDa, from between about 20 kDa and about 40 kDa, from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, from between about 120 kDa and about 140 kDa, or from between about 140 kDa and about 160 kDa, from between about 160 kDa and about 180 kDa, from between about 180 kDa and about 200 kDa, or from between about 200 kDa and about 250 kDa, and a polydispersity between 1 and about 1.7, between 1 and less than 3, or between 1 and less than 5. In some embodiments, the plurality of peptides or protein fragments in a fraction having a weight average molecular weight (M_w) selected from between about 10 kDa and about 20 kDa, from between about 20 kDa and about 40 kDa, from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, from between about 120 kDa and about 140 kDa, from between about 140 kDa and about 160 kDa, or from between about 160 kDa and about 180 kDa, and a polydispersity between 1 and about 1.1, or 1 and about 1.2, or between 1 and less than 3, or between 1 and less than 5. In some embodiments, the plurality of peptides or protein fragments in a fraction having a weight average molecular weight (M_w) selected from between about 10 kDa and about 20 kDa, from between about 20 kDa and about 40 kDa, from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, or from between about 120 kDa and

about 140 kDa, and a polydispersity between 1 and about 1.1, or 1 and about 1.2, or between 1 and less than 3, or between 1 and less than 5. In some embodiments, the plurality of peptides or protein fragments in a fraction having a weight average molecular weight (M_w) selected from between about 60 kDa and about 80 kDa, from
5 between about 80 kDa and about 100 kDa, or from between about 100 kDa and about 120 kDa, and a polydispersity between 1 and about 1.1, or between 1 and less than 3, or between 1 and less than 5. In some embodiments, the plurality of peptides or protein fragments in a fraction having a weight average molecular weight (M_w) selected from between about 10 kDa and about 20 kDa, from between about 20 kDa
10 and about 40 kDa, from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, or from between about 100 kDa and about 110 kDa, and a polydispersity between 1 and about 1.1, or 1 and about 1.2, or between 1 and less than 3, or between 1 and less than 5. In some embodiments, the plurality of peptides or protein fragments in a fraction having
15 a weight average molecular weight (M_w) selected from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, or from between about 120 kDa and about 140 kDa, and a polydispersity between 1 and about 1.1, or between 1 and less than 3, or between 1 and less than 5. In some embodiments, the plurality of peptides or protein fragments
20 in a fraction having a weight average molecular weight (M_w) selected from between about 20 kDa and about 40 kDa, or from between about 40 kDa and about 60 kDa, and a polydispersity between 1 and about 1.1, or 1 and about 1.2, or between 1 and less than 3, or between 1 and less than 5. In some embodiments, the one or more fractions are selected from AS77, AS78, AS79, AS80, and AS81. In some
25 embodiments, the one or more fractions are selected from AS82, AS83, AS84, AS85, AS86, AS87, AS88, and AS89. In some embodiments, the one or more fractions are selected from AS90, AS91, AS92, AS93, and AS94. In some embodiments, the one or more fractions are selected from AS95, AS96, AS97, AS98, AS99, and AS100. In some embodiments, the plurality of peptides or protein fragments having a weight
30 average molecular weight (M_w) selected from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, from between about 120 kDa and about 140 kDa, from between about 140 kDa and about 160 kDa, from between about 160 kDa and about 180 kDa, from between about 180 kDa and about

200 kDa, or from between about 200 kDa and about 220 kDa, and a polydispersity between 1 and about 1.7, or between 1 and less than 3, or between 1 and less than 5. In some embodiments, the plurality of peptides or protein fragments in a fraction having a weight average molecular weight (M_w) selected from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, from between about 120 kDa and about 140 kDa, from between about 140 kDa and about 160 kDa, from between about 160 kDa and about 180 kDa, from between about 180 kDa and about 200 kDa, or from between about 200 kDa and about 210 kDa, and a polydispersity between 1 and about 1.2, or 1 and about 1.3, or between 1 and less than 3, or between 1 and less than 5. In some embodiments, the plurality of peptides or protein fragments in a fraction having a weight average molecular weight (M_w) selected from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, or from between about 100 kDa and about 110 kDa, and a polydispersity between 1 and about 1.1, or 1 and about 1.2, or between 1 and less than 3, or between 1 and less than 5. In some embodiments, the plurality of peptides or protein fragments in a fraction having a weight average molecular weight (M_w) selected from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, from between about 120 kDa and about 140 kDa, from between about 140 kDa and about 160 kDa, from between about 160 kDa and about 180 kDa, from between about 180 kDa and about 200 kDa, or from between about 200 kDa and about 210 kDa, and a polydispersity between 1 and about 1.2, or 1 and about 1.3, or between 1 and less than 3, or between 1 and less than 5. In some embodiments, the one or more fractions are selected from AS101, AS102, AS103, AS104, and AS105. In some embodiments, the one or more fractions are selected from AS106, AS107, AS108, AS109, AS110, and AS111. In some embodiments, an amino acid is selected from M, R, V, K, T, F, I, L, C, A, Q, Y, N, D, E, G, S, H, P, and W. In some embodiments, a peptide or protein fragment comprises between about 2 and about 100 amino acids. In some embodiments, the peptide or protein fragment comprises between about 2 and about 25 amino acids. In some embodiments, the peptide or protein fragment comprises between about 25 and about 50 amino acids. In some embodiments, the peptide or protein fragment comprises between about 50 and about 75 amino acids. In some embodiments, the peptide or protein fragment

comprises between about 75 and about 100 amino acids. In some embodiments, the peptide or protein fragment comprises between about 100 and about 125 amino acids. In some embodiments, the peptide or protein fragment comprises between about 125 and about 150 amino acids. In some embodiments, the peptide or protein fragment

5 comprises between about 150 and about 200 amino acids. In some embodiments, the peptide or protein fragment comprises between about 200 and about 250, 300, 350, 400, 450, or 500 amino acids. In some embodiments, a peptide or protein fragment comprises between one and five modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein fragment comprises one modification,

10 substitution, and/or replacement. In some embodiments, the peptide or protein fragment comprises two modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein fragment comprises three modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein fragment comprises four modifications, substitutions, and/or replacements. In some

15 embodiments, the peptide or protein fragment comprises five modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein fragment comprises six modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein fragment comprises seven modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein

20 fragment comprises eight modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein fragment comprises nine modifications, substitutions, and/or replacements. In some embodiments, the peptide or protein fragment comprises ten modifications, substitutions, and/or replacements. In some embodiments, the fibroin is a fibroin heavy chain, and wherein a modification,

25 substitution, and/or replacement is at a position corresponding to any one position from 1 to 5263 of the fibroin heavy chain. In some embodiments, the fibroin is a fibroin light chain, and wherein a modification, substitution, and/or replacement is at a position corresponding to any one position from 1 to 262 of the fibroin light chain. In some embodiments, the fibroin is a fibrohexamerin (p25) chain, and wherein a

30 modification, substitution, and/or replacement is at a position corresponding to any one position from 1 to 220 of the fibrohexamerin (p25) chain. In some embodiments, a modification, substitution, and/or replacement is selected from an asparagine to aspartic acid modification, substitution, and/or replacement, a glutamine to glutamic acid modification, substitution, and/or replacement, and a methionine to methionine

oxide modification, substitution, and/or replacement. In some embodiments, a modification, substitution, and/or replacement is at fibroin heavy chain position selected from Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, and/or N5262. In some embodiments, a modification, substitution, and/or replacement is at fibroin light chain position selected from N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, and/or Q255. In some embodiments, a modification, substitution, and/or replacement is at fibrohexamerin (p25) position selected from Q62, N93, M120, N149, N172, N174, and/or N202. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 1% to about 99%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 1% to about 10%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 10% to about 20%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 20% to about 30%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 30% to about 40%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 40% to about 50%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 50% to about 60%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 60% to about 70%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 70% to about 80%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between about 80% to about 90%. In some embodiments, each modification, substitution, and/or replacement is independently ranging in the composition between or about 90% to about 99%. As used herein, a % modification, substitution, and/or replacement is defined as (number of peptide or protein fragments comprising a modification, substitution, and/or replacement at a specific position, divided by the total number of peptide or protein fragments which include the specific

position, whether comprising a modification, substitution, and/or replacement, or not) x 100. In some embodiments, a molecular weight is determined by MALS.

In some embodiments, the disclosure provides a lyophilized or sprayable composition disclosed herein, a plurality of substantially solid silk fibroin particles disclosed herein, a silk fibroin nanoclay composite or film disclosed herein, a stabilized silk fibroin solution disclosed herein, a liquid in air suspension disclosed herein, or a plurality of drops or droplets disclosed herein, each independently comprising a plurality of peptides or protein fragments, e.g., a plurality of fibroin heavy chain peptides or fibroin heavy chain fragments, and comprising a plurality of amino acid modifications, substitutions, and/or replacements, and further comprising one or more ratios of modifications, substitutions, and/or replacements at specific positions selected from:

- a ratio of Q58 to M64 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of Q58 to N68 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of Q58 to N70 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of Q58 to N77 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of Q58 to M80 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about

6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

5 - a ratio of Q58 to N93 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

10 - a ratio of Q58 to M103 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

15 - a ratio of Q58 to Q125 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

20 - a ratio of Q58 to N132 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

25 - a ratio of Q58 to Q139 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

30 - a ratio of Q58 to Q275 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of Q58 to N4191 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 5 about 1:20, or about 1:25;
- a ratio of Q58 to Q5216 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 10 about 1:20, or about 1:25;
- a ratio of Q58 to N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 15 about 1:20, or about 1:25;
- a ratio of M64 to N68 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 20 about 1:20, or about 1:25;
- a ratio of M64 to N70 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 25 about 1:20, or about 1:25;
- a ratio of M64 to N77 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 30 about 1:20, or about 1:25;
- a ratio of M64 to M80 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about

1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M64 to N93 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
5 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M64 to M103 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
10 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M64 to Q125 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
15 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M64 to N132 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
20 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M64 to Q139 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
25 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M64 to Q275 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
30 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M64 to N4191 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about

6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

5 - a ratio of M64 to Q5216 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

10 - a ratio of M64 to N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

15 - a ratio of N68 to N70 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

20 - a ratio of N68 to N77 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

25 - a ratio of N68 to M80 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

30 - a ratio of N68 to N93 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of N68 to M103 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 5 about 1:20, or about 1:25;
- a ratio of N68 to Q125 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 10 about 1:20, or about 1:25;
- a ratio of N68 to N132 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 15 about 1:20, or about 1:25;
- a ratio of N68 to Q139 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 20 about 1:20, or about 1:25;
- a ratio of N68 to Q275 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 25 about 1:20, or about 1:25;
- a ratio of N68 to N4191 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 30 about 1:20, or about 1:25;
- a ratio of N68 to Q5216 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about

1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N68 to N5262 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
5 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N70 to N77 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
10 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N70 to M80 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
15 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N70 to N93 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
20 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N70 to M103 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
25 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N70 to Q125 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
30 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N70 to N132 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about

6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

5 - a ratio of N70 to Q139 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

10 - a ratio of N70 to Q275 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

15 - a ratio of N70 to N4191 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

20 - a ratio of N70 to Q5216 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

25 - a ratio of N70 to N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

30 - a ratio of N77 to M80 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of N77 to N93 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 5 about 1:20, or about 1:25;
- a ratio of N77 to M103 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 10 about 1:20, or about 1:25;
- a ratio of N77 to Q125 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 15 about 1:20, or about 1:25;
- a ratio of N77 to N132 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 20 about 1:20, or about 1:25;
- a ratio of N77 to Q139 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 25 about 1:20, or about 1:25;
- a ratio of N77 to Q275 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 30 about 1:20, or about 1:25;
- a ratio of N77 to N4191 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about

1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N77 to Q5216 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
5 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;
- a ratio of N77 to N5262 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
10 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;
- a ratio of M80 to N93 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
15 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;
- a ratio of M80 to M103 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
20 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;
- a ratio of M80 to Q125 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
25 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;
- a ratio of M80 to N132 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
30 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;
- a ratio of M80 to Q139 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about

6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

5 - a ratio of M80 to Q275 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

10 - a ratio of M80 to N4191 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

15 - a ratio of M80 to Q5216 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

20 - a ratio of M80 to N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

25 - a ratio of N93 to M103 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

30 - a ratio of N93 to Q125 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of N93 to N132 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 5 about 1:20, or about 1:25;
- a ratio of N93 to Q139 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 10 about 1:20, or about 1:25;
- a ratio of N93 to Q275 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 15 about 1:20, or about 1:25;
- a ratio of N93 to N4191 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 20 about 1:20, or about 1:25;
- a ratio of N93 to Q5216 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 25 about 1:20, or about 1:25;
- a ratio of N93 to N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 30 about 1:20, or about 1:25;
- a ratio of M103 to Q125 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about

1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M103 to N132 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
5 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M103 to Q139 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
10 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M103 to Q275 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
15 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M103 to N4191 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
20 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M103 to Q5216 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
25 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M103 to N5262 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
30 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q125 to N132 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about

6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

5 - a ratio of Q125 to Q139 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

10 - a ratio of Q125 to Q275 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

15 - a ratio of Q125 to N4191 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

20 - a ratio of Q125 to Q5216 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

25 - a ratio of Q125 to N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

30 - a ratio of N132 to Q139 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of N132 to Q275 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 5 about 1:20, or about 1:25;
- a ratio of N132 to N4191 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 10 about 1:20, or about 1:25;
- a ratio of N132 to Q5216 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 15 about 1:20, or about 1:25;
- a ratio of N132 to N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 20 about 1:20, or about 1:25;
- a ratio of Q139 to Q275 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 25 about 1:20, or about 1:25;
- a ratio of Q139 to N4191 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 30 about 1:20, or about 1:25;
- a ratio of Q139 to Q5216 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about

1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q139 to N5262 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
5 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q275 to N4191 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
10 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q275 to Q5216 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
15 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q275 to N5262 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
20 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N4191 to Q5216 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
25 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N4191 to N5262 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
30 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q5216 to N5262 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about

6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- In some embodiments, the disclosure provides a lyophilized or sprayable composition disclosed herein, a plurality of substantially solid silk fibroin particles disclosed herein, a silk fibroin nanoclay composite or film disclosed herein, a stabilized silk fibroin solution disclosed herein, a liquid in air suspension disclosed herein, or a plurality of drops or droplets disclosed herein, each independently comprising a plurality of peptides or protein fragments, e.g., a plurality of fibroin heavy chain and light chain peptides or fibroin heavy chain and light chain fragments, and comprising a plurality of amino acid modifications, substitutions, and/or replacements, and further comprising one or more ratios of modifications, substitutions, and/or replacements at specific positions selected from:
- a ratio of heavy chain Q58 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
 - a ratio of heavy chain M64 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
 - a ratio of heavy chain N68 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
 - a ratio of heavy chain N70 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about

- 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1,
about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6,
about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain N77 to light chain N23, Q24, N28, M69, N105, N108,
5 N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255
modifications, substitutions, and/or replacements of about 25:1, about 20:1, about
15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1,
about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6,
about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
10 - a ratio of heavy chain M80 to light chain N23, Q24, N28, M69, N105, N108,
N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255
modifications, substitutions, and/or replacements of about 25:1, about 20:1, about
15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1,
about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6,
15 about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain N93 to light chain N23, Q24, N28, M69, N105, N108,
N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255
modifications, substitutions, and/or replacements of about 25:1, about 20:1, about
15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1,
20 about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6,
about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain M103 to light chain N23, Q24, N28, M69, N105, N108,
N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255
modifications, substitutions, and/or replacements of about 25:1, about 20:1, about
25 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1,
about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6,
about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain Q125 to light chain N23, Q24, N28, M69, N105, N108,
N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255
30 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about
15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1,
about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6,
about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of heavy chain N132 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain Q139 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain Q275 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain N4191 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain Q5216 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain N5262 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1,

about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25.

In some embodiments, the disclosure provides a lyophilized or sprayable composition disclosed herein, a plurality of substantially solid silk fibroin particles disclosed herein, a silk fibroin nanoclay composite or film disclosed herein, a stabilized silk fibroin solution disclosed herein, a liquid in air suspension disclosed herein, or a plurality of drops or droplets disclosed herein, each independently comprising a plurality of peptides or protein fragments, e.g., a plurality of fibroin heavy chain and p25 peptides or fibroin heavy chain and p25 fragments, and comprising a plurality of amino acid modifications, substitutions, and/or replacements, comprising one or more ratios of modifications, substitutions, and/or replacements at specific positions selected from:

- a ratio of heavy chain Q58 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain M64 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain N68 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain N70 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain N77 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1,

- about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain M80 to p25 Q62, N93, M120, N149, N172, N174, or
- 5 N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain N93 to p25 Q62, N93, M120, N149, N172, N174, or
- 10 N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain M103 to p25 Q62, N93, M120, N149, N172, N174, or
- 15 N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain Q125 to p25 Q62, N93, M120, N149, N172, N174, or
- 20 N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain N132 to p25 Q62, N93, M120, N149, N172, N174, or
- 25 N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain Q139 to p25 Q62, N93, M120, N149, N172, N174, or
- 30 N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of heavy chain Q275 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain N4191 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain Q5216 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of heavy chain N5262 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25.

- In some embodiments, the disclosure provides a lyophilized or sprayable composition disclosed herein, a plurality of substantially solid silk fibroin particles disclosed herein, a silk fibroin nanoclay composite or film disclosed herein, a stabilized silk fibroin solution disclosed herein, a liquid in air suspension disclosed herein, or a plurality of drops or droplets disclosed herein, each independently comprising a plurality of peptides or protein fragments, e.g., a plurality of light chain fibroin peptides or fibroin fragments, and comprising a plurality of amino acid modifications, substitutions, and/or replacements, comprising one or more ratios of modifications, substitutions, and/or replacements at specific positions selected from:
- a ratio of N23 to Q24 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of Q24 to N28 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 5 about 1:20, or about 1:25;
- a ratio of Q24 to M69 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 10 about 1:20, or about 1:25;
- a ratio of Q24 to N105 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 15 about 1:20, or about 1:25;
- a ratio of Q24 to N108 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 20 about 1:20, or about 1:25;
- a ratio of Q24 to N118 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 25 about 1:20, or about 1:25;
- a ratio of Q24 to N136 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 30 about 1:20, or about 1:25;
- a ratio of Q24 to N138 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about

1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q24 to Q149 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
5 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q24 to N186 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
10 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q24 to N200 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
15 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q24 to Q202 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
20 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q24 to N204 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
25 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q24 to N240 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
30 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q24 to N248 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about

6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

5 - a ratio of Q24 to Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

10 - a ratio of N28 to M69 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

15 - a ratio of N28 to N105 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

20 - a ratio of N28 to N108 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

25 - a ratio of N28 to N118 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

30 - a ratio of N28 to N136 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of N28 to N138 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 5 about 1:20, or about 1:25;
- a ratio of N28 to Q149 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 10 about 1:20, or about 1:25;
- a ratio of N28 to N186 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 15 about 1:20, or about 1:25;
- a ratio of N28 to N200 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 20 about 1:20, or about 1:25;
- a ratio of N28 to Q202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 25 about 1:20, or about 1:25;
- a ratio of N28 to N204 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 30 about 1:20, or about 1:25;
- a ratio of N28 to N240 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about

1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N28 to N248 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
5 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N28 to Q255 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
10 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M69 to N105 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
15 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M69 to N108 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
20 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M69 to N118 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
25 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M69 to N136 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
30 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M69 to N138 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about

6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

5 - a ratio of M69 to Q149 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

10 - a ratio of M69 to N186 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

15 - a ratio of M69 to N200 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

20 - a ratio of M69 to Q202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

25 - a ratio of M69 to N204 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

30 - a ratio of M69 to N240 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of M69 to N248 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 5 about 1:20, or about 1:25;
- a ratio of M69 to Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 10 about 1:20, or about 1:25;
- a ratio of N105 to N108 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 15 about 1:20, or about 1:25;
- a ratio of N105 to N118 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 20 about 1:20, or about 1:25;
- a ratio of N105 to N136 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 25 about 1:20, or about 1:25;
- a ratio of N105 to N138 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 30 about 1:20, or about 1:25;
- a ratio of N105 to Q149 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about

1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N105 to N186 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
5 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N105 to N200 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
10 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N105 to Q202 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
15 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N105 to N204 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
20 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N105 to N240 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
25 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N105 to N248 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
30 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N105 to Q255 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about

6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

5 - a ratio of N108 to N118 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

10 - a ratio of N108 to N136 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

15 - a ratio of N108 to N138 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

20 - a ratio of N108 to Q149 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

25 - a ratio of N108 to N186 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

30 - a ratio of N108 to N200 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of N108 to Q202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
5 about 1:20, or about 1:25;
- a ratio of N108 to N204 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
10 about 1:20, or about 1:25;
- a ratio of N108 to N240 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
15 about 1:20, or about 1:25;
- a ratio of N108 to N248 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
20 about 1:20, or about 1:25;
- a ratio of N108 to Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
25 about 1:20, or about 1:25;
- a ratio of N118 to N136 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
30 about 1:20, or about 1:25;
- a ratio of N118 to N138 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about

1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N118 to Q149 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
5 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N118 to N186 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
10 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N118 to N200 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
15 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N118 to Q202 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
20 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N118 to N204 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
25 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N118 to N240 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
30 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N118 to N248 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about

6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

5 - a ratio of N118 to Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

10 - a ratio of N136 to N138 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

15 - a ratio of N136 to Q149 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

20 - a ratio of N136 to N186 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

25 - a ratio of N136 to N200 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

30 - a ratio of N136 to Q202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of N136 to N204 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 5 about 1:20, or about 1:25;
- a ratio of N136 to N240 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 10 about 1:20, or about 1:25;
- a ratio of N136 to N248 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 15 about 1:20, or about 1:25;
- a ratio of N136 to Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 20 about 1:20, or about 1:25;
- a ratio of N138 to Q149 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 25 about 1:20, or about 1:25;
- a ratio of N138 to N186 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 30 about 1:20, or about 1:25;
- a ratio of N138 to N200 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about

1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N138 to Q202 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
5 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N138 to N204 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
10 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N138 to N240 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
15 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N138 to N248 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
20 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N138 to Q255 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
25 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q149 to N186 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
30 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q149 to N200 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about

6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

5 - a ratio of Q149 to Q202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

10 - a ratio of Q149 to N204 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

15 - a ratio of Q149 to N240 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

20 - a ratio of Q149 to N248 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

25 - a ratio of Q149 to Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

30 - a ratio of N186 to N200 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of N186 to Q202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
5 about 1:20, or about 1:25;
- a ratio of N186 to N204 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
10 about 1:20, or about 1:25;
- a ratio of N186 to N240 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
15 about 1:20, or about 1:25;
- a ratio of N186 to N248 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
20 about 1:20, or about 1:25;
- a ratio of N186 to Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
25 about 1:20, or about 1:25;
- a ratio of N200 to Q202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
30 about 1:20, or about 1:25;
- a ratio of N200 to N204 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about

1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N200 to N240 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
5 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N200 to N248 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
10 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N200 to Q255 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
15 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q202 to N204 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
20 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q202 to N240 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
25 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q202 to N248 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
30 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of Q202 to Q255 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about

6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

5 - a ratio of N204 to N240 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

10 - a ratio of N204 to N248 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

15 - a ratio of N204 to Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

20 - a ratio of N240 to N248 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

25 - a ratio of N240 to Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

30 - a ratio of N248 to Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

In some embodiments, the disclosure provides a lyophilized or sprayable composition disclosed herein, a plurality of substantially solid silk fibroin particles disclosed herein, a silk fibroin nanoclay composite or film disclosed herein, a stabilized silk fibroin solution disclosed herein, a liquid in air suspension disclosed

5 herein, or a plurality of drops or droplets disclosed herein, each independently comprising a plurality of peptides or protein fragments, e.g., a plurality of fibroin light chain and heavy chain peptides or fibroin light chain and heavy chain fragments, and comprising a plurality of amino acid modifications, substitutions, and/or replacements, and further comprising one or more ratios of modifications,

10 substitutions, and/or replacements at specific positions selected from:

- a ratio of light chain N23 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1,

15 about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain Q24 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1,

20 about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N28 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications,

25 substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain M69 to heavy chain Q58, M64, N68, N70, N77, M80,

30 N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of light chain N105 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N108 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N118 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N136 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N138 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain Q149 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1,

about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of light chain N186 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N200 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain Q202 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N204 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N240 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N248 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications,

substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- 5 - a ratio of light chain Q255 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25.

In some embodiments, the disclosure provides a lyophilized or sprayable composition disclosed herein, a plurality of substantially solid silk fibroin particles disclosed herein, a silk fibroin nanoclay composite or film disclosed herein, a stabilized silk fibroin solution disclosed herein, a liquid in air suspension disclosed herein, or a plurality of drops or droplets disclosed herein, each independently comprising a plurality of peptides or protein fragments, e.g., a plurality of fibroin light chain and p25 peptides or fibroin light chain and p25 fragments, and comprising a plurality of amino acid modifications, substitutions, and/or replacements, comprising one or more ratios of modifications, substitutions, and/or replacements at specific positions selected from:

- 20 - a ratio of light chain N23 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- 25 - a ratio of light chain Q24 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- 30 - a ratio of light chain N28 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about

- 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain M69 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1,
- 5 about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N105 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1,
- 10 about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N108 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1,
- 15 about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N118 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1,
- 20 about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N136 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1,
- 25 about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N138 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1,
- 30 about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain Q149 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1,

- about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N186 to p25 Q62, N93, M120, N149, N172, N174, or
- 5 N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N200 to p25 Q62, N93, M120, N149, N172, N174, or
- 10 N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain Q202 to p25 Q62, N93, M120, N149, N172, N174, or
- 15 N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N204 to p25 Q62, N93, M120, N149, N172, N174, or
- 20 N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N240 to p25 Q62, N93, M120, N149, N172, N174, or
- 25 N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of light chain N248 to p25 Q62, N93, M120, N149, N172, N174, or
- 30 N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of light chain Q255 to p25 Q62, N93, M120, N149, N172, N174, or N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25.

In some embodiments, the disclosure provides a lyophilized or sprayable composition disclosed herein, a plurality of substantially solid silk fibroin particles disclosed herein, a silk fibroin nanoclay composite or film disclosed herein, a stabilized silk fibroin solution disclosed herein, a liquid in air suspension disclosed herein, or a plurality of drops or droplets disclosed herein, each independently comprising a plurality of peptides or protein fragments, e.g., a plurality of p25 fibroin peptides or fibroin fragments, and comprising a plurality of amino acid modifications, substitutions, and/or replacements, comprising one or more ratios of modifications, substitutions, and/or replacements at specific positions selected from:

- a ratio of Q62 to N93 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of Q62 to M120 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of Q62 to N149 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of Q62 to N172 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of Q62 to N174 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 5 about 1:20, or about 1:25;
- a ratio of Q62 to N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 10 about 1:20, or about 1:25;
- a ratio of N93 to M120 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 15 about 1:20, or about 1:25;
- a ratio of N93 to N149 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 20 about 1:20, or about 1:25;
- a ratio of N93 to N172 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 25 about 1:20, or about 1:25;
- a ratio of N93 to N174 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, 30 about 1:20, or about 1:25;
- a ratio of N93 to N202 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about

1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M120 to N149 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
5 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M120 to N172 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
10 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M120 to N174 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
15 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of M120 to N202 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
20 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N149 to N172 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
25 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N149 to N174 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about
30 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about
1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15,
about 1:20, or about 1:25;

- a ratio of N149 to N202 modifications, substitutions, and/or replacements of
about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about

6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of N172 to N174 modifications, substitutions, and/or replacements of
5 about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of N172 to N202 modifications, substitutions, and/or replacements of
10 about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of N174 to N202 modifications, substitutions, and/or replacements of
15 about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25.

In some embodiments, the disclosure provides a lyophilized or sprayable
20 composition disclosed herein, a plurality of substantially solid silk fibroin particles disclosed herein, a silk fibroin nanoclay composite or film disclosed herein, a stabilized silk fibroin solution disclosed herein, a liquid in air suspension disclosed herein, or a plurality of drops or droplets disclosed herein, each independently comprising a plurality of peptides or protein fragments, e.g., a plurality of p25 and
25 heavy chain peptides or p25 and heavy chain fragments, and comprising a plurality of amino acid modifications, substitutions, and/or replacements, comprising one or more ratios of modifications, substitutions, and/or replacements at specific positions selected from:

- a ratio of p25 Q62 to heavy chain Q58, M64, N68, N70, N77, M80, N93,
30 M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

- a ratio of p25 N93 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of p25 M120 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of p25 N149 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of p25 N172 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of p25 N174 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
- a ratio of p25 N202 to heavy chain Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, or N5262 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1,

about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25.

- In some embodiments, the disclosure provides a lyophilized or sprayable composition disclosed herein, a plurality of substantially solid silk fibroin particles disclosed herein, a silk fibroin nanoclay composite or film disclosed herein, a stabilized silk fibroin solution disclosed herein, a liquid in air suspension disclosed herein, or a plurality of drops or droplets disclosed herein, each independently comprising a plurality of peptides or protein fragments, e.g., a plurality of p25 and light chain peptides or fibroin light chain and light chain fragments, and comprising a plurality of amino acid modifications, substitutions, and/or replacements, and further comprising one or more ratios of modifications, substitutions, and/or replacements at specific positions selected from:
- a ratio of p25 Q62 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
 - a ratio of p25 N93 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
 - a ratio of p25 M120 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;
 - a ratio of p25 N149 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1,

about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

5 - a ratio of p25 N172 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

10 - a ratio of p25 N174 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25;

15 a ratio of p25 N202 to light chain N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, or Q255 modifications, substitutions, and/or replacements of about 25:1, about 20:1, about 15:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1, about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:15, about 1:20, or about 1:25.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the described embodiments, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperature, *etc.*) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1: Scaleup Methods

In some embodiments, a scaleup method comprises a lower gradient of temperature (Δ°) between any two points, two planes, or two voxels in a scaleup setup, e.g., and without limitation, a scaleup reaction vessel, compared to any
5 similarly situated two points, two planes, or two voxels in a bench setup, e.g., and without limitation, a bench reaction vessel. In some embodiments, the scaleup gradient is lower than the bench gradient by about 1 °C, about 2 °C, about 3 °C, about 4 °C, about 5 °C, about 6 °C, about 7 °C, about 8 °C, about 9 °C, about 10 °C, about 11 °C, about 12 °C, about 13 °C, about 14 °C, about 15 °C, about 16 °C, about
10 17 °C, about 18 °C, about 19 °C, or about 20 °C.

15

Extraction:				
	Bench	Scaleup		
Cocoon mass	95.3 g	3750 g		
Ext water vol	8 L (RODI)	88.2 L (city)		
Sodium carbonate mass	21.2 g	834.2 g		
Ext water temp	100 C	100 C		
Extraction time	60 min	30 min		
Rinse water vol	8 L (RODI)	88.2 L (city)		
Rinse water temp	60 C	60 C		
Rinse time	20 min	15 min		
Number of rinses	3	3		
Notes	Intermittent agitation done by hand. Silk is wrung by hand to remove extraction and rinse water between steps	Intermittent agitation done by washer drum rotation. Water removal done by spin cycle after final rinse (no spin cycles between washes/rinses)		
Dissolution:				
	Bench (mid)	Bench (low)	Scaleup (mid)	Scaleup (low)
Fibroin mass	100 g	100 g	10375 g	10375 g
9.3 M LiBr vol	400 mL	400 mL	41.5 L	41.5 L
Dissolution temp	100 C	134 C	103 C	125 C

Combination time	1-3 minutes	1-3 minutes	30-60 min	
Reaction time	60 min	360 min	60 min	360 min
Notes	Reaction done in 5 L beaker fully contained in an oven. Beaker is mostly unagitated after initial combination	Reaction done in 5 L beaker fully contained in an oven. Beaker is mostly unagitated after initial combination	Reaction done in 50 L jacketed glass reactor. Combination carried out by repetitively submerging and agitating fibroin until all fibroin is added. Mixture is agitated at 25 RPM for duration of reaction time.	Reaction done in 50 L jacketed glass reactor. Combination carried out by repetitively submerging and agitating fibroin until all fibroin is added. Mixture is agitated at 25 RPM for duration of reaction time.

Example 2: Derivatized or Modified Peptides

A novel method is disclosed to generate compositions of polypeptides that are derived from *B. mori* silkworm cocoons and comprised of natural and modified polypeptides. These two novel compositions are called Low Skid and Mid Skid
5 silk/modified polypeptide compositions.

The novel production method involves removing sericin through several washing steps with an organic sodium carbonate salt with tightly controlled multi-stage temperature cycles and agitation as the first step in forming natural/modified polypeptide composition. Next the silk is dried to remove remaining water at
10 controlled temperature to maintain polypeptide composition. The silk is then dissolved in high concentration of Lithium salt at two different temperatures and times to achieve the different compositions for Mid and Low silk. The liquid solution is then filtered and purified to remove the Lithium salt leaving only the natural/modified silk compositions in solution with pure water.

15 Low Skid and Mid Skid silk/modified polypeptide compositions comprise of populations of silk/modified polypeptides with distinctive properties. Low Skid silk/modified polypeptide composition does not self-assemble at 5 mg/mL. Low Skid silk/modified polypeptide composition comprises of two main populations of silk/modified polypeptides; one population (AS22) that does not self-assemble under
20 conditions that promote self-assembly at 5 mg/mL and is rich in negatively charged amino acids; a second population of polypeptides (AS12) that self-assemble fast at 5 mg/mL and are depleted on negatively charged amino acids.

When AS12 and AS22 are combined at a ratio of 50% each the average molecular weight of the mixture becomes the same as Low Skid silk/modified
25 polypeptide composition. Thus Low Skid silk/modified polypeptide composition consists of 50% AS12 and 50% AS22 silk/modified polypeptide compositions.

Mid Skid silk/modified polypeptide composition comprises of two main populations of silk/modified polypeptides; one population (AS11) that self-assemble slower than Mid Skid silk/modified polypeptides, under conditions that promote self-
30 assembly at 5 mg/mL and is rich in negatively charged amino acids; a second population of polypeptides (AS1) that self-assemble faster than Mid Skid silk/modified polypeptides, under conditions that promote self-assembly at 5 mg/mL and are depleted on negatively charged amino acids.

When AS11 and AS11 are combined at a ratio of 50% each the average molecular weight of the mixture becomes the same as Mid Skid silk/modified polypeptide composition. Thus, Low Skid silk/modified polypeptide composition consists of 50% AS1 and 50% AS11 silk/modified polypeptide compositions.

5 Both Low Skid and Mid Skid silk/modified polypeptide compositions contain modified peptides that were determined after analysis with Mass Spectrometry.

Low and Mid Skid silk/modified polypeptide compositions described in this disclosure are never produced before compositions of silk-derived and modified polypeptides that when isolated display a wide range of behaviors, from extreme self-assembly to solubility and stability over time in various buffers and various average molecular weights and polydispersities. These novel silk-derived polypeptide compositions contain unique modified amino acid sequences that result from a unique silk processing method and scaleup. The tight controls around temperature, silk concentration, salt concentrations, physical agitation and purification allow us to tune at each step of the process the unique peptide compositions in the natural/modified silk species to design for specific performance criteria. Some of constituent polypeptide compositions display biological activity and could be used as therapeutic candidates.

Silk is a versatile material that can be used in many applications from development of implantable medical devices to the development of soluble polypeptide formulations of medicinal value. A major challenge with silk polypeptides in solution is their tendency to self-assemble and aggregate, making the control of their solubility very difficult. Also, the kinetics of gel/film formation cannot be controlled in a predictable way. This novel silk/modified peptide compositions contain populations of peptides that allows to control their properties and develop products with predictable and desired properties.

The disclosed compositions contain a collection of many polypeptides with different properties. Silk has been characterized mostly based on its molecular weight and polydispersity, and no mixture of silk/modified polypeptides has been characterized or has been generated. As disclosed herein, a unique large scale process is used to generate compositions of silk/modified polypeptides.

Low/Mid skid silks begin their process to remove sericin using sodium carbonate at specific silk mass, sodium carbonate, and water ratios. Multiple different temperature washing cycles 100 °C and 60 °C and agitation is also key in producing

the specific natural/modified compositions. The silk is then dried to remove water at a specific temperature that maintains the silk composition. Next the silk is dissolved in a high concentration of Lithium Bromide at 103 °C and 125 °C for 1 and 6 hours respectively. The time and temperature allow for fine tuning the degree of post translational modifications that give the unique polypeptide compositions. The silk is then purified to remove Lithium Bromide and optionally concentrate the silk. For the downstream characterization of isolation of the various silk/modified polypeptide compositions chromatographic techniques were employed, biochemical/biophysical techniques, and cell biology methods.

10 To characterize/separate novel Low and Mid Skid silk/modified polypeptide compositions a combinations of Ion Exchange Chromatography fractionation, analytical methods, and biochemical dissection were used to characterize its properties.

Generation of Low and Mid Skid silk/modified polypeptide compositions.

15 Silk is washed to remove sericin at 100 °C and 60 °C with sodium carbonate and then dried at 60 °C. The silk is then dissolved in 9.3 M Lithium Bromide at 103 °C for 1 hour for Mid silk and 9.3 M Lithium Bromide at 125 °C for 6 hours for Low silk. This dissolution step controls not only molecular weight but also the polypeptide modifications creating the natural/modified silk compositions. The silk is then filtered
20 to remove undissolved debris and purified using 10 KDa cutoff PES hollow fiber membranes, and concentrated using the same process leaving only natural/modified silk composite in solution with pure water. Every unit ops is tightly controlled for temperature, time, concentrations, agitation, and shear.

Isolation of Low and Mid Skid/modified polypeptide compositions

25 ***Isolation of the AS22 silk/modified polypeptide composition component of Low Skid silk/modified polypeptide composition.***

To isolate AS22 it was fractionated Low Skid silk/modified polypeptide compositions using HiTrap Q Sepharose Anion Exchanger (Figures 1,2). Tris was added in the silk preparations to a final concentration of 50 mM Tris-HCl pH=8.0.
30 Silk was centrifuged before loading the HiTrap Q Sepharose column to remove any preformed aggregates. Low Skid silk preparation solutions have a characteristic yellow hue. The flow through from the HiTrap Q Sepharose column was transparent. AS22 eluted with 1 M NaCl and it has an intense yellow hue.

When AS22 formulation is analyzed with an analytical SEC column (see materials and methods) with HPLC it has an average molecular weight (MW) of about 35 kDa and Polydispersity (PDI) of 2.3 (Figures 3, 4). This molecular weight and polydispersity are distinctive and different from Low Skid silk/modified polypeptide composition (MW about 19.5 kDa and PDI 2.2) and Mid Skid silk/modified polypeptide composition (MW about 38 kDa and PDI 2.4) (Figures 3, 4).

When AS22 and Low Skid silk were analyzed in Isoelectric Focusing Polyacrylamide gels, it was found that 22 M silk peptides had pIs 3-6 and some species with pIs of about 9.6, whereas Low Skid silk also contains peptides that span the whole range of pIs, from 3 to 9.6 (Figure 5).

Isolation of the AS12 silk/modified polypeptide composition component of Low Skid silk/modified polypeptide composition.

To develop AS12 a combination of Ion Exchange Chromatography (IEX) fractionation was used to purify the formulation, analytical methods and biochemical dissection to characterize its properties.

To isolate AS12 Low Skid silk preparations was fractionated using HiTrap Q Sepharose Anion Exchanger (Figure 1,2). Tris was added in the silk preparations to a final concentration of 50 mM Tris-HCl pH=8.0. Silk was centrifuged before loading the HiTrap Q Sepharose column to remove any preformed aggregates. The flow through from the HiTrap Q Sepharose column was collected and was designated AS12. AS12 is colorless.

AS12 silk formulation is composed of short silk/modified polypeptides depleted in negative charges.

When AS12 was analyzed with analytical Size Exclusion Chromatography, it has an average molecular weight (MW) of about 12 kDa and Polydispersity (PDI) of 1.7 (Figure 3,4). This molecular weight and polydispersity are distinctive and different from Low Skid silk/modified peptide composition (MW about 19.5 kDa and PDI 2.2) and Mid Skid silk/modified peptide composition (MW about 38 kDa and PDI 2.4) (Figure 3,4). When AS12 and Low Skid silk/modified peptide composition were analyzed in Isoelectric Focusing Polyacrylamide gels, it was found that AS12 silk peptides had a pI of about 9-10, whereas Low Skid silk also contains peptides that span the whole range of pIs, from 3 to 9.6 (Figure 5) with most of them concentrated around pI 3-5.5.

Isolation of the AS1 silk/modified polypeptide composition component of Mid Skid silk/modified polypeptide composition.

To isolate AS1 Low Skid silk preparations were fractionated using HiTrap Q Sepharose Anion Exchanger (Figure 1, 2). Tris was added in the silk preparations to a final concentration of 50 mM Tris-HCl pH=8.0. Silk was centrifuged before loading the HiTrap Q Sepharose column to remove any preformed aggregates. The flow through from the HiTrap Q Sepharose column was collected and was designated AS1. AS1 has a MW of about 28 kDa and PDI of 1.7-2.1 (Figure 3,4). Mid Skid silk/modified peptide composition has MW of 37 kDa PDI 2.0 (Figure 3,4).

Isolation of the AS11 silk/modified polypeptide composition component of Mid Skid silk/modified polypeptide composition.

To develop AS11 a combination of Ion Exchange Chromatography (IEX) fractionation to purify the formulation, analytical methods and biochemical dissection to characterize its properties was used. To isolate AS11 Mid Skid silk/modified peptide composition preparations was fractionated using HiTrap Q Sepharose Anion Exchanger (Figure 1, 2). Tris was added in the silk preparations to a final concentration of 50 mM Tris-HCl pH=8.0. Silk was centrifuged before loading the HiTrap Q Sepharose column to remove any preformed aggregates. The elution from the HiTrap Q Sepharose column was collected and was designated AS11. AS11 formulation has a molecular weight (MW) of about 53 kDa and Polydispersity (PDI) of 2.8 (Figure 3, 4). This molecular weight and polydispersity are distinctive and different from Mid Skid silk/modified peptide composition (MW, about 37 kDa and PDI 2.4) (Figure 3, 4).

Self-Assembly of Low and Mid Skid/modified polypeptide compositions

Self assembly assay and data derived from it.

To study the stability of AS1 in solution self-assembly assays was performed. AS1 silk at 5 mg/mL self assembles very fast. The absorbance at 550 nm curves of the self-assembly assays are sigmoid and they can be described as logistic curves. The typical logistic function is:

$$f_x = A_{max} / (1 + e^{-k(t-t_{0.5})})$$

A_{max} is the maximum density of the gel formed

k is the Self Assembly Rate Factor

$t_{0.5}$ is the time point at which 50% of the gel has formed

e is the exponential equation for the specific curve

(see figure 8 A the red dotted lines for a better demonstration of how these factors were calculated from the self-assembly experiments)

Another parameter introduced to characterize the propensity of silk to form gels is the Self Assembly Factor which is:

$$f_{SAF} = 1/t_{0.5} \times A_{max} \times 1000$$

Using the experimental data from the self-assemble assays performed with the various novel isolated silk polypeptides these parameters calculated and use to dissect their properties (Figure 6, 7, 8). Four parameters were in focus, collectively referred to as Self-assembly kinetic factors; the Self-Assembly Rate Factor (SARF), A_{max} , $t_{0.5}$, and the Self Assembly Factor (SAF) (Figure 6, 7, 8). The SARF shows how fast silk self-assembles to form gel after the reaction begins or the gelation nuclei have formed; A_{max} shows how dense is the gel that is formed after self-assembly is complete, $t_{0.5}$ shows how long it takes for the self-assembly reaction to reach the point where gel density is $A_{max}/2$ and SAF shows the propensity of silk to self-assemble (Figure 6, 7, 8).

AS1 silk/modified polypeptide composition has the fastest self-assembly kinetics.

Self-assembly assays as described before, revealed that AS1 self-assembles very fast, much faster than Mid Skid silk/modified peptide compositions (Figure 6, 7, 8). Mid Skid silk/modified peptide compositions was used as a positive control and shows fast self-assembly kinetics (Figure 6, 7, 8).

AS11 silk/modified polypeptide composition has the fastest self-assembly kinetics.

Self-assembly assays as described before, revealed that AS11 self-assembles fast but not as fast as Mid Skid silk/modified peptide compositions (Figure 6, 7, 8). Mid Skid silk/modified peptide compositions was used as a positive control and shows fast self-assembly kinetics (Figure 6, 7, 8).

AS12 silk/modified polypeptide composition is the component of Low Skid silk/modified polypeptide composition that promotes self-assembly.

Self-assembly assays as described before, revealed that AS12 self-assembles fast but not as fast as Mid Skid silk/modified peptide compositions (Figure 6, 7, 8). Mid Skid silk/modified peptide compositions was used as a positive control and shows fast self-assembly kinetics (Figure 6, 7, 8).

AS22 silk/modified polypeptide composition is very stable in aqueous solution and doesn't self-assemble.

Self-assembly assays as described before, revealed that AS22 displays a remarkable stability and doesn't self-assemble even in conditions that promote silk self-assembly. Mid Skid silk was used as a positive control and shows fast self-assembly kinetics (Figure 6, 7, 8).

When AS1, AS11, AS12 and AS22 silk/modified polypeptide compositions are combined at different ratios they result in compositions with unique properties.

To better understand the properties of the silk/modified polypeptide compositions (Figure 6, 7, 8) a series of mixtures were created (see table 1). The resulting compositions display a unique combination of properties in self-assembly assays (Figure 6, 7, 8). Mixtures of AS1 and AS11 (AS2-10) and AS12 with AS22 (AS13-AS21) all display a unique combination of self-assembly kinetics (Figure 6, 7, 8). This information can be used to create silk/modified polypeptide compositions with specific desired properties.

Materials and Methods used for the generation and characterization of AS1-AS28.

Anion Exchange Fractionation of Silk.

Low Skid silk was provided at a concentration of 60 mg/mL. Low Skid silk was centrifuged at 20,000 x g, at 4 °C for 15 min to remove any aggregated material. For the silk fractionation Q-Sepharose prepacked columns connected to an AKTA pure 25 L (Cytiva, Serial Number 2747908) or columns packed with Q-Big Beads resin (Cytiva) were used. All buffers used were filtered through a 0.45µm PES filter and degassed with sonication. Centrifuged Low or Mid Skid silk was loaded on 5 x 5mL HiTrap Q HP columns (Cytiva 17115401, Lot:10305274) washed with 10 column volumes of 50mM Tris pH=8.0, 10 column volumes of 50mM Tris pH=8.0 (Tris Base, Goldbio CAT# T-400-5, CAS# 77-86-1, Lot # 250501T400), 1M NaCl (NaCl, VWR High Purity Grade, X190-5kg, Lot # 20G2756431) and finally 10 column volumes of 50mM Tris pH=8.0. 100mL of centrifuged Low Skid silk were loaded on the column with a flow rate of 5mL/min. The flow-through was collected. The column was washed with 50 mM Tris pH=8.0 until the absorbance at 280nm [A₂₈₀] got to 100 AU. Bound protein was eluted in one step with 50mM Tris pH=8.0, 1M NaCl and all fractions with absorbance [A₂₈₀] >500AU were pooled together. 20mL of the Low Skid silk (LS) that was used for the fractionation, the flow through

(QFT) and the pooled elution fractions (QE) were placed in dialysis bags (Sigma D9652-100FT, Dialysis tubing cellulose membrane avg flat width 33mm, 1.3in., PCode1003241246, Lot#3110) and were immersed in 50 x volumes of 50mM Tris pH=8.0. Samples were flash frozen in liquid nitrogen and stored at -20°C until they
5 were used. Before use samples were thawed slowly at 4 °C or on ice.

Analytical/Protein Characterization methods.

Protein concentration determination.

Protein concentration was determined by absorbance at 220nm or 280nm. Solubilized silk preparations were diluted until A_{280} was between 0.1-1. In this range
10 the absorbance correlates linearly with the concentration of silk in the solution and the correlation is 1AU=1mg/mL soluble silk proteins. Final concentrations in the initial silk solution were calculated after adjustment for the dilution used for the absorbance measurement.

Analytical Size Exclusion Chromatography.

15 Analysis was performed in a PolySep GFC P-4000 LC Column, 300 mm x 7.8 mm (Phenomenex, Part No. CH0-9229) connected to a Agilent 1260 Infinity II HPLC system with an Agilent G7162A RID Refractive Index Detector. The mobile phase used for the analysis was a solution of 0.1M NaCl, 12.5mM Na₂HPO₄, pH 7 (the pH was adjusted with phosphoric acid and filtered through a 0.2µm PES filter into a clean
20 glass media bottle). 25µL of sample were loaded on the column and the analysis was performed at 25 °C with a flow rate of 1mL/min for 20min. Calculation of the molecular weight of each sample was done using Agilent Technologies Open LAB CDS ChemStation Edition for LC & LC/MS Systems software Cirrus SEC data collection and molecular weight analysis software. (For more details see Report
25 XXX).

Analytical Anion Exchange Chromatography.

For the analysis an HPLC Dionex Ultimate 3000 and a TSKgel (07163) DEAE-3SW column 7.5mm ID x 7.5cm, 10µm was used. Chromatography was performed at 25 °C. 10% Trifluoroethanol, 45% Acetonitrile in water was used as a
30 blank solution. The column was equilibrated with 20mM Na₂HPO₄/KH₂PO₄ (Na₂HPO₄: Fisher Chemical, Lot# 188298, PN# S374-500, KH₂PO₄: Fisher Chemical, Lot# 187270, PN# P285-500), pH 6 at 1 mL/min for 10 min. 30µL of sample were loaded on the column. Bound silk polypeptides were eluted with a linear gradient of 500mM NaCl (NaCl: Fisher Chemical, Lot# 206719, PN# BP358-1). All solutions

were made in LCMS water: Fisher Chemical, Lot# 216650, PN# W5-4. Collected data were analyzed with XCalibur™ Software.

Isoelectric Point determination of silk.

To determine the isoelectric point of the new silk compositions Isoelectric

5 Focusing gels were used. These separate proteins based on their net charge and not their molecular weight. For the analysis, a BIO-RAD Criterion Precast Gels was used, IEF standards pI 4.45-9.6 (BIO-RAD, Cat#1610310, Batch 64417452, L0040178). Silk protein samples from new silk compositions were mixed with IEF Sample Buffer (BIO-RAD Cat # 16110763, Batch 64345676, L004138B) (make sure that you

10 have at least 5% v/v glycerol in the final mix). The mixtures were loaded on a Criterion Precast Gel. For the electrophoresis a 1x IEF Anode Buffer (BIO-RAD Cat # 1610761, Batch 64376614) and 1x IEF Cathode Buffer (BIO-RAD, Cat # 1610762, Batch 64364827) were used. The running conditions for the electrophoresis were: 100V constant for 60min, 250V constant for 60min and 500V constant for 30min.

15 After the electrophoresis was complete proteins were fixed on the gel with a solution of 40% v/v Methanol (Sigma Aldrich, Methanol ACS reagent >99.8%, 179337-4L-Pb, Source SHBN0806, Pcode 1003210445), 10% v/v Acetic acid (VWR Acetic Acid, Glacial, ACS Grade, BDH3092-500MLP, Lot: 2018071399), for 30min to overnight at room temperature with rocking.

LC/MS analysis of polypeptides.

Samples were stored at 4 °C until used for analysis. For each sample, an aliquot was taken and mixed with an equal volume of 6 M guanidine hydrochloride (GuHCl) in a new tube. From that mix, an aliquot was taken again to create 120-fold and 240-fold further dilutions for determining protein concentrations using the BCA

25 assay. Using the concentrations determined above, samples were diluted to 20µg/µL with 6 M GuHCl. An aliquot of 1,000µg total protein was transferred to a new tube. 50 mM dithiothreitol (DTT) was added to a final concentration of 5mM and the samples were incubated at 60 °C for 30 minutes. After a brief equilibration period to room temperature, 100mM iodoacetamide (IAM) was added to a final concentration

30 of 10mM and the samples were incubated at room temperature in the dark for 30 minutes. The IAM reaction was quenched by the addition of 50mM DTT to a final concentration of 5mM DTT, followed by a further incubation at room temperature for 30minutes. Three aliquots corresponding to 100µg of total protein were taken in separate tubes and diluted in PBS to get a final concentration of 0.2M GuHCl.

Samples were then treated with enzyme at a protease to protein ratio of 1:50 (2 μ g of each protease) overnight at either room temperature (chymotrypsin) or 37 °C (trypsin/Lys-C and Glu-C). The protease reactions were quenched by the addition of TFA to a final concentration of 1% (v/v). Samples were centrifuged for 10 minutes at 14,000rpm and supernatant was transferred to HPLC autosampler vials for LC-MS analysis. LC

Column: C18 column (100 μ m x 150 mm, 3 μ m)

Mobile Phase A: Water 0.1% formic acid

Mobile Phase B: Acetonitrile 0.1% formic acid

10 **Flow Rate:** 600 nL/min (micro pump) and 15 μ L/min (loading pump)

Chromatographic time: 60 minutes

Elution Gradient: 7% B (0 to 3.3 min); 7% to 35% B (3.3 to 35 min); 35% to 95% B (35-37 min); 95% to 80% B (37-39 min); 80% B (39-41 min); 80% to 7% B (41 to 44 min); 7% B (44 to 60 min)

15 **Injection volume:** 4 μ L

Acquisition for full MS ranges from 350 to 1600m/z. The MS method is based on data-dependent acquisition (DDA) for the top 10 ions with an isolation window of 3.0m/z and a normalized collision energy of 26.

20 Data was acquired using Thermo XcaliburTM Software. Data analysis was performed using Thermo Proteome DiscovererTM Software. To unequivocally assign a specific protein from the identified peptides, a minimum of 2 unique peptides per protein are required upon searching against SwissProt database.

Gel Staining methods.

Silver Staining

25 SDS and IEF polyacrylamide gels were stained using ProteoSilver Silver Stain Kit (Sigma, PROTSIL-1-IKT, Lot # SLCH2293, Pcode: 1003135372) following the manufacturer's instructions. Briefly, gels were immersed in fixing solution (50% v/v Ethanol, 10% Glacial Acetic Acid), washed with water, sensitizer solution, silver solution and developer solution. Gel band development was terminated with
30 ProteoSilver Stop Solution.

Self Assembly Assay.

The silk Self Assembly Assay (SAF) was performed in 35% v/v 2-propanol (Sigma-Aldrich 2-Propanol ACS reagent >99.5%. 190764-4L, Lot # SHBK7164, PCode 1002789344) and 50mM CH₃COONa pH=5 (Sodium acetate anhydrous,

VWR life sciences Product number 0602-1Kg, Lot # 0677C055). Each reaction was done in a final volume of 200 μ L. Total silk protein concentration was 5 mg/mL. First the buffer of 50 mM CH₃COONa pH=5, 35% v/v 2-propanol was prepared. Then DI/RO water was added so that after the addition of the volume of silk protein required to reach a final concentration of 5 mg/mL the total volume would be 200 μ L. The protein was added last and mixed with very gentle pipetting to reduce shearing force. The protein mixtures were placed in wells of flat-bottom 96-well plates and a layer of 100 μ L of Mineral Oil (Sigma, Mineral Oil BioReagent, for molecular biology, light oil, M5904-500mL, Lot # MKCC7596, PCode 1002883254) carefully so as to not create any bubbles. Absorbance was recorded at 550 nm for 16-24 h (depending on the sample). Recorded values were exported in Excel files for storage and further analysis.

Data analysis.

Data were analyzed using GraphPad Prism 9 for macOS Version 9.2.0 (283), July 15, 2021. Figures were prepared with Adobe illustrator 25.4.1.

Table 1. Detailed composition of all AS products generated herein. The composition of each product is given in % per mass (mg/mL).

(*use the AS number to locate more information about the preparations in the AS Library inventory in the ELN)

<i>AS number</i>	Low Skid Q-Elution (%)	Low Skid Q Flow Through (%)	Mid Skid Q-Elution (%)	Mid Skid Q Flow Through (%)
1	0	0	0	100
2	0	0	90	10
3	0	0	80	20
4	0	0	70	30
5	0	0	60	40
6	0	0	50	50
7	0	0	40	60
8	0	0	30	70
9	0	0	20	80
10	0	0	10	90
11	0	0	100	0
12	0	100	0	0
13	5	95	0	0

14	2	90	0	0
15	5	90	0	0
16	6	90	0	0
17	8	90	0	0
18	10	90	0	0
19	15	85	0	0
20	20	80	0	0
21	30	70	0	0
22	100	0	0	0
23	40	60	0	0
24	50	50	0	0
25	60	40	0	0
26	70	30	0	0
27	80	20	0	0
28	90	10	0	0

Example 3. Skid Manufacturing Process Examples for specific new Low, Mid & High silk fragment populations each having a single weight average Molecular Weight Average (MW) with polydispersity less than 5 and/or less than 3

5 *Fibroin isolation*

Fibroin isolation requires separation of sericin from raw *B. mori* silk fibers and cocoons. This separation is typically carried out in a single stage and facilitated in a solid substrate extraction operation. The unit consists of a vessel vented to atmosphere and encloses a perforated drum which rotates on a horizontal axis. Any vessel or drum type, vented to atmosphere or operated under pressure, may be used so long as the controls and gradients described are achieved with or without agitation in the reactor or mixing of the reaction components on any axis of rotation.

The raw silk fibers are introduced into the rotating drum via a sealable access port. Raw silk may be added to the vessel or enclosed in a secondary container, such as silk cocoons packed loosely into permeable mesh bags. This secondary containment step minimizes product loss, protects the equipment by preventing silk strands from entangling with rotating components in the vessel, and protects the drain lines from plugging with solids that would otherwise escape from the drum during processing. The reaction vessel fills with extraction solvent to partially submerge the perforated drum. The reaction extraction solvent is composed of 0.7% - 0.95% by weight sodium

carbonate in water to partially submerge the perforated drum. Example concentrations of extraction solvent are 0.94% by weight for fragment populations below 40 kDa and 0.70% by weight for fragments below 96 kDa as measured by HPLC with size exclusion chromatography with a refractive index detector (SEC-RI). Concentrations
5 may be varied to generate different specific weight average molecule weight fragment size populations independently of or in combination with other process parameters at any single weight average MW between 1 kDa and 250 kDa. This holds true for every parameter detailed here (**please see Example 29 Process Parameters**).

This solvent composition has been shown effective at dissolving and stabilizing
10 sericin in solution such that it can be removed with the solvent. The cocoon/solvent ratio is 0.040 kg/kg – 0.070 kg/kg (typically 0.042 kg/kg). An electric heater located at the base of the vessel is used to maintain temperature of the extraction solvent in the range of 30 °C to 110 °C. For examples below, a measured temperature range between 94.5 °C – 97 °C, or 2.5 °C total temperature gradient was used. The elevated
15 extraction solvent temperature, its maintenance, consistency and gradient within the reactor vessel drives sericin removal and final product creation with targetable specificity.

The rotating drum turns periodically throughout an isothermal phase of the extraction. It could rotate continuously, or intermittently, so long as the temperature gradient is
20 maintained. In the examples below (figures 9 and 10), a 30 minute isothermal phase was utilized. In another example, a 15 minute isothermal phase was utilized to generate a higher specific weight average molecule weight fragment size population of 96 kDa. This action serves to expose all fiber surfaces to the extraction solvent and to increase the precision of the product's final polydispersity, degree of modification,
25 charge density, molecular weight and/or other critical features and benefits of the final product.

Rinsing with copious hot water follows, between 0.5X to 20X initial reaction vessel reaction solvent volume. The vessel is filled with non-potable water to partially submerge the perforated drum. Rinse water temperature is maintained in the range of
30 55 °C – 65 °C with an ideal gradient of less than 10 degrees for 20 minutes with intermittent drum rotation, then the rinse water is drained to waste. This is repeated two additional times. Additional ranges are presented in Example 29 to detail expanded specific weight average molecule weight fragment size populations.

The gradient or consistency or boundary of a specific temperature range is critical at every stage of the overall process or specific reaction step where increased or decreased temperature from ambient is employed, as these specific parameters and their associated levels or variable levels serve to produce differentiated final product from both processes and products described in the art.

Once rinsed, the drum rotates at high speed to remove water retained in the cocoons by centrifugal action. Fibroin with moisture content from 15% - 65%wt (average 47%) is then manually removed from the washer and distributed evenly onto perforated trays. The residual moisture is driven off the fibroin by storage in a dryer with internal temperature maintained at 55 °C - 60 °C until moisture content of the material is less than 1% of the total mass. It is also possible to continue to forward process the material immediately forward into the solvation process without drying. Extraction efficacy is verified by measuring the change in mass of the dry material before and after processing in the extraction unit. Typically, the amount of sericin removed from the cocoons is 30-36%wt of the total mass of the raw cocoons. The composition of the raw cocoons has been characterized by liquid chromatography-mass spec (LCMS). Using LCMS, sericin concentration in raw cocoons was determined to be ~35.3%wt. Sericin was undetectable in fibroin after processing in the extraction unit. This result suggests that the sericin extraction method is effective, and the fraction of sericin detected in the raw cocoons corresponds well with the observed mass loss of the cocoons in the field.

Fibroin solvation and modification

Control over the solubilization and modification of fibroin is achieved by dispersing the solid protein into a solvent and thermally treating the mixture at variable time and temperature. In one example, a 9.3 M lithium bromide solution in water is used as a solvent. The solvent is prepared in a vessel with or without baffles. The solution is blended to uniformity in the vessel using a center-mounted agitator with stacked 45° pitched blade turbines. Heat transfer oil circulates through the vessel jacket to stabilize bulk fluid temperature at the required reaction temperature while the solvent mixes. Typically, the reaction temperature is stabilized in the ranges of 100 °C – 103 °C (103 °C target) or 122 °C - 125 °C (125 °C target).

Fibroin is loaded into the vessel through an access port in the vessel head once the solvent reaches the required reaction temperature. The mass ratio of fibroin to solvent is typically 0.16 kg/kg. Downward force is applied to the floating protein mat to fully

submerge the material and clear the headspace for additional material to be added.

Once the headspace is cleared, agitation is briefly employed to disperse the wetted silk mat before addition is continued. Loading the vessel with the full mass of fibroin occurs over the course of 40-60 minutes, during which time reaction temperature is maintained in the vessel. The fibroin may also be placed in secondary containment and loaded at a single time point without the extended loading.

The reaction time begins after the fibroin addition is completed. Agitation is carried out over the full course of the reaction period. Reaction time varies depending on the desired properties of the resulting solution.

10 A first reaction process shows reactions carried out at multiple times between 0 – 63 minutes with working fluid temperature held at 100 °C – 103 °C. Figure 1 displays the typical predictable evolution of the average molecular weight average (average MW) of the solubilized fibroin as a function of reaction time.

A second reaction process shows reactions carried out at multiple times between 40 – 15 420 min with temperature held at 122 °C – 125 °C. Figure 2 displays the typical evolution of the average molecular weight average (average MW) of the solubilized fibroin as a function of reaction time.

The contents of the vessel are subsequently cooled. Cooling is accomplished by either of two methods. In one method, cooling is carried out by immediate removal of the solution from the vessel, dividing the solution into small volumes, and storing the containers in a refrigerator held at 4 °C. In another method, cooling is carried out in place by recirculating chilled heat transfer oil through the vessel jacket. If cooling using a jacketed vessel, the temperature may be reduced to below 60 °C rapidly and within 70 minutes of the reaction period elapsing. Cooling to room temperature from 25 60 °C may be carried out rapidly or more slowly by environmental radiation or by forced cooling. When using forced cooling, the solution can be brought to room temperature within 3 hours, or less or more, to control the reaction outputs depending on desired product outcomes.

Purification

30 The cooled reaction mixture is a viscous liquid composed of water, solvent or stabilizing salt (typically LiBr), fibroin, and miscellaneous undissolved organic solids. Fibroin is isolated from this mixture. Purification occurs through three filtration stages.

- First, the reaction mixture undergoes dead-end filtration through a needle felt polypropylene filter media with nominal particle size rejection in the range of 1 mm – 200 mm to remove relatively large undissolved contaminants. The filtered reaction mixture is transferred through the filtration media to a holding vessel with or without baffles, which is pre-charged with some volume of reverse osmosis/de-ionized (RODI) water. The volume of water charged to the holding vessel is determined by multiplication of the reaction mixture volume against a water-to-reaction mixture volumetric ratio. This ratio ranges from 1 – 7 L/L depending on the desired product and required downstream processing conditions.
- 10 The reaction mixture is blended to uniformity with the dilution water using a center-mounted agitator with stacked 45° pitched blade turbines or a propeller. Chilled propylene glycol circulates through the vessel jacket to cool the diluted mixture if the diluted material is to be stored for greater than 24 hours. Agitation for blending is limited to the bare minimum to achieve homogeneity, as excessive or prolonged shear on the fluid increases risk of product loss due to precipitation or foaming.
- 15 The diluted reaction mixture undergoes additional dead-end filtration through either a melt-blown and spun-bonded pleated poly propylene media with nominal 0.2 mm rejection or a resin bonded cellulose/diatomaceous earth lenticular media with absolute 2.5 mm rejection to reduce solution turbidity below a desired threshold.
- 20 The diluted reaction mixture is transferred through the filtration media to a tangential flow filtration (TFF) unit outfitted with a jacketed retentate vessel, a rotary lobe pump, 10 kDa molecular weight cutoff hollow fiber ultrafiltration membranes, or any molecular weight cut off between 1 kDa and 80 kDa, and an automatically controlled backpressure valve used to stabilize transmembrane pressure (TMP) during processing. TMP is defined as the average internal pressure of the TFF unit minus the permeate line pressure. The diluted reaction mixture recirculates between the retentate vessel and the membrane bank via the lobe pump and backpressure valve. The pump operates to maintain a constant recirculation flowrate, typically in the range of 200 – 500 L/min depending on application. The backpressure valve actuates to maintain
- 25 TMP in the range of 7 – 35 psig depending on application. Chilled or heated propylene glycol or water is circulated through the retentate vessel to maintain working fluid temperature between 20 °C and 35 °C depending on application and with a filtration reaction temperature standard deviation of less than 10°C ideally. For
- 30

final product concentrations (protein by weight, in water) greater than 6% by weight, temperature greater than room temperature are typically used.

Operating under these conditions drives permeation of permeate such as water and LiBr through the membrane selective layers to waste. The TFF operation begins
5 diafiltration, where volume is maintained in the retentate vessel by backfilling with RODI water during as volume is lost to membrane permeate. Diafiltration conditions are maintained until the conductivity of the permeate dips below a desired threshold, typically at any value between 10 - 50 mS/cm. LiBr concentration can be at any useful value, or typically below 1,000 ppm, or ideally be below 150 ppm. Diafiltration
10 ceases once this condition is satisfied, at which point RODI water flow to the system stops and the working fluid is allowed to concentrate as permeation continues under maintained TMP and flow conditions. Protein concentration is monitored over the course of the concentration phase of operation. Concentration conditions are maintained until the protein concentration is within the range of 5 – 17% wt
15 depending on application but can be any value between 1% to 40% by weight. Total residence time in the TFF unit ideally ranges from 12 – 35 hours depending on application.

The purified soluble silk fibroin fragment solution is drained from the TFF unit. The solution may be stored in either HDPE carboys or stainless-steel totes as work in
20 progress or final product, or used in any further downstream process or processes, for example to further separate out ideal peptides, sub-populations of fragments or to purify the fragment population further, or complex the fragments with additional molecules or entities, or alter the form of the silk fibroin fragment solution from solubilized in water into dry powder by means of solvent extraction, lyophilization
25 and freeze drying.

Process Yield, Reproducibility, Consistency and Optimization

Process development has resulted in product yield improvements. Relative to the existing process described in *Silk protein fragment compositions and articles manufactured therefrom* (taken as the baseline case in this context), silk fibroin
30 fragment yield was increased by at least 2X (200% yield) and up to at least 100x (10,000% yield improvement) for multiple fragment populations with specific average molecular weight averages between 1 and 250 kDa.

Additionally, process development has resulted in significant quality improvements exemplified by reduced variation in critical quality parameters such, specifically in

production reproducibility between manufacturing production runs of weight average molecular weight and polydispersity characteristics of the protein fragment population in the final product. In reference to the standard bench top processes known in the art without the specific extraction reactor vessel and dissolution and filtration process parameters mentioned above, the scaleup of production of final product volumes of greater than 1 liter of soluble silk fragments in water resulted in up to 75% reduction in the standard deviation of molecular weight measurements and a 70% reduction in the standard deviation of dispersity measurements for a single silk fragment population with a single average MW between 1 and 250 kDa between 2 or more production runs.

Example 4. Modified single silk fragment weight average Molecular Weight Average (MW) populations “Low” and “Mid” with degree of amino acid modifications produced using new “Skid” silk production processes.

Modified Low and Mid Skid silk polypeptide compositions which are each a single silk fragment weight average Molecular Weight Average (MW) or average molecular weight average silk fragment population of silk-derived peptides, with each population having a polydispersity less than 5 and/or less than 3 as desired, described in this invention are never produced before compositions of silk-derived and modified polypeptides that when isolated display a wide range of behaviors, from extreme self-assembly to superb solubility and stability over time in various buffers and various weight average molecular weights and polydispersities. These novel silk-derived polypeptide compositions contain unique modified amino acids that result from the unique silk “skid” processing method and scale. The tight controls around temperature, silk concentration, salt concentrations, physical agitation and purification allow us to tune at each step of the process the unique peptide compositions in the silk species to design for specific performance criteria. Some of constituent polypeptide compositions display biological activity and could be used as therapeutic candidates.

Silk is a versatile material that can be used in many applications from development of implantable medical devices to the development of soluble polypeptide formulations of medicinal value. A major challenge with silk polypeptides in solution is their tendency to self-assemble and aggregate, making the control of their solubility very difficult. Also, the kinetics of gel/film formation cannot be controlled in a predictable way. The novel silk

peptide compositions contain populations of peptides that allow us to control their properties and develop products with predictable and desired properties.

Development of Low and Mid Skid silk/modified polypeptide compositions.

Activated silk contains a collection of many polypeptides with different properties. Silk
5 fragment populations has been characterized mostly based on its average molecular weight average and polydispersity, referred to here as a new silk fragment population or “silk polypeptide”. So far no mixture of silk polypeptides (i.e., multiple silk fragment populations) have been characterized or has been generated. Here, a unique large-scale process known as the Skid Silk Process or “skid: silk” was used to generate
10 compositions of silk polypeptides.

Low/Mid skid silks were produced using process parameters and conditions described. Briefly, in summary, they begin their process to remove sericin using sodium carbonate at specific silk mass, sodium carbonate, and water ratios. Multiple different temperature washing cycles between 100 °C and 60 °C and agitation is also key in producing the
15 specific natural/modified compositions. The silk is then dried to remove water at a specific temperature that maintains the silk composition. Next the silk is dissolved in a high concentration of Lithium Bromide at 103 °C and 125 °C for 1 and 6 hours respectively. The time and temperature allow for fine tuning the degree of post translational modifications that give the unique polypeptide compositions. The silk is
20 then purified to remove Lithium Bromide and concentrate the silk.

Generation of Low and Mid Skid silk/modified polypeptide compositions.

Silk was washed to remove sericin at 100 °C and 60 °C with sodium carbonate and then dried at 60 °C. The silk was then dissolved in 9.3 M Lithium Bromide at 103 °C for 1 hour for Mid silk and 9.3 M Lithium Bromide at 125 °C for 6 hours for Low silk. This
25 dissolution step controls not only molecular weight but also the polypeptide modifications creating the natural/modified silk compositions. The silk was then filtered to remove undissolved debris and purified using 10 kDa cutoff PES hollow fiber membranes and concentrated using the same process leaving only natural/modified silk composite in solution with pure water. Every unit ops was tightly controlled for
30 temperature, time, concentrations, agitation, and shear.

The silk preparations have unique modifications depending on the production method.

The dissolution of degummed silk cocoons was performed in high concentration of chaotropic salts (9M LiBr) and at very high temperatures that exceed 100°C (see previous sections). The unique thermal treatment that occurs during the production method described herein, promotes the deamidation of Asparagine and Glutamine residues and the oxidation of Methionines. The deamidation of Asparagine and Glutamine residues and the oxidation of Methionines is referred to as “modifications” from now on. To determine the degree of amino acid modification during the various silk preparation methods LC/MS approaches were used (see LC/MS analysis of polypeptides for more details). The Skid process is run in facilities known as Walpole and Medford. A former process not employing the new process conditions is referred to as “benchtop” silk, or the “bench silk process” known in the art.

When Low Skid Silk was compared with Mid Skid silk produced with differing process conditions in the Walpole facility, it was found that Low Skid silk was more modified than Mid Skid silk (Figs. 12A- 12C, Table 2). When silk produced in Walpole was lyophilized it retained the same modification trend; lyophilized Low Skid silk was more modified than lyophilize Mid Skid silk (Figs. 13A-13B, Table 3). Low Skid silk produced in the Walpole facility is unique and less modified than the Low Skid silk produced in Medford (Figs. 14A- 14B, Table 4). When silk was produced using a benchtop setup, the resulting silk preparation was different than its Skid counterpart. On average Low Benchtop is less deamidated than Low Skid silk. (Figs. 15A-15C, Table 5) Mid Benchtop on average is also less modified than Mid Skid silk (Figure 15D, Table 5 comparing Mid Skid silk with Mid Benchtop silk).

LC/MS analysis of polypeptides.

Materials

Reagents

- Guanidine hydrochloride (GuHCl) (Sigma cat# G3272-1KG)
- Dithiothreitol (DTT) (ThermoFisher cat# 20290)
- Iodoacetamide (IAM) (Sigma cat# I1149-5G)
- HPLC-grade water (FisherChemical cat# W5-4)
- Acetonitrile (ACN) (FisherChemical cat# A955-4)
- Formic acid (FA) (FisherChemical cat# A117-10X1AMP)
- Trifluoroacetic acid (TFA) (FisherChemical cat# A116-10X1AMP)

- Sodium acetate (Sigma cat# S5636-250G)
- Proteases
- Trypsin/Lys-C mix (Promega cat# V5073)
- Chymotrypsin (Promega cat# V1061)
- 5 ● Glu-C (Promega cat# V1651)
- Solutions
- 6 M GuHCl
- 50 mM DTT (10X)
- 100 mM IAM (10X)
- 10 ● 50 mM sodium acetate

Methods

Denaturation, Reduction and Alkylation

Samples were stored at 4 °C until used for analysis.

- 15 For each sample, an aliquot was taken and mixed with an equal volume of 6 M guanidine hydrochloride (GuHCl) in a new tube. 50 mM dithiothreitol (DTT) was added to a final concentration of 5 mM and the samples were incubated at 60 °C for 30 minutes. After a brief equilibration period to room temperature, 100 mM iodoacetamide (IAM) was added to a final concentration of 10 mM and the samples were incubated at
- 20 room temperature in the dark for 30 minutes. The IAM reaction was quenched by the addition of 50 mM DTT to a final concentration of 5 mM DTT. The samples were diluted in 50 mM sodium acetate to get a final concentration of 0.18 M GuHCl.

Protease digestion

- Using the sample concentrations provided, 3 aliquots corresponding to 30 µg of total
- 25 protein were taken in separate tubes. Samples were then treated with enzymes at a protease to protein ratio of 1:30 (1 µg of each protease) overnight at either room temperature (chymotrypsin) or 37 °C (trypsin/Lys-C and Glu-C). The aliquots treated with trypsin/Lys-C and Glu-C were boosted with the same amount of enzyme and incubated at 37 °C for 3 hours the next day. The protease reactions were quenched by
- 30 the addition of TFA to a final concentration of 1% (v/v). Samples were centrifuged for 10 minutes at 14,000 rpm and supernatant was transferred to HPLC autosampler vials for LC-MS analysis.

LC Conditions

Column: C18 column (100 µm x 200 mm, 3 µm)

Mobile Phase A: Water 0.1% formic acid

Mobile Phase B: Acetonitrile 0.1% formic acid

Flow Rate: 300 nL/min (micro pump) and 15 µL/min (loading pump)

Chromatographic time: 60 minutes

- 5 **Elution Gradient:** 14% B (0 to 3.3 min); 14% to 30% B (3.3 to 35 min); 30% to 95% B (35-37 min); 95% to 80% B (37-39 min); 80% B (39-41 min); 80% to 14% B (41 to 44 min); 14% B (44 to 60 min)

Injection amount: 2 µg

MS Conditions

- 10 Acquisition for full MS ranges from 350 to 1600 m/z. The MS method is based on data-dependent acquisition(DDA) for the top 10 ions with an isolation window of 3.0 m/z and a normalized collision energy of 27.

Data Acquisition and Analysis

- Data was acquired using Thermo Xcalibur™ Software. Data analysis was performed using Thermo Proteome Discoverer™ Software. In order to unequivocally assign a specific protein from the identified peptides, a minimum of 2 unique peptides per protein are required upon searching against *Bombyx mori* database.

- For each modification site, all the peptides containing that amino acid were categorized into modified vs unmodified. The modification percentage is then calculated using the formula below:
- 20

$$\text{modification \%} = \frac{\text{sum of modified peptides}}{\text{sum of modified and unmodified peptides}}$$

Tables

- 25 **Table 2.** Percentage of amino acid modifications in Low Skid Silk and Mid Skid silk produced in the facility in Walpole. N are Asparagines that become aspartic acid and Q are Glutamines that become deamidated. M corresponds to Methionines that become oxidized.

Fibroin Heavy Chain

	N68	N70	N77	N93	N13	N526	Q5	Q13	Q27	Q521	M64
LS	95.3	92.9	85.3	33.7	69.0		3.5	15.9			86.5
Walpole	7	9	3	3	8	21	6	7	4.01	6.98	4

MS	37.2	42.1		15.8	45.4		2.9				
Walpole	5	8	0	1	3	10.14	5	6.9	0.91	2.49	70.7

Fibroin Light Chain

	N23	N28	N108	N118	N136	N186	N200	N204	N240	N248	Q24	Q149	Q202
LS													
Walpole	90.69	28.26	34.4	45.7	48.88	29.79	100	53.44	70.34	63.91	0.74	3.58	35.16
MS													
Walpole	75.49	1.58	12.92	28.46	22.06	3.98	87.36	45.88	25.12	20.86	0.04	2.65	2.58

Fibrohexamerin (p25)

	N93	N149	N172	N174	N202
LS Walpole	9.76	59.34	74.42	15.42	20.19
MS Walpole	4.38	34.42	50.03	2.58	9.45

5

Table 3. Percentage of amino acid modifications in Low Skid Silk and Mid Skid silk produced in the facility in Walpole and lyophilized. N are Asparagines that become aspartic acid and Q are Glutamines that become deamidated. M corresponds to Methionies that become oxidized.

10 ***Fibroin Heavy Chain***

	N68	N70	N93	N132	N5262
Low Skid Lyo	100	100	11.87	86.34	39.13
Mid Skid Lyo	86.1	26.57	5.95	63.12	4.37

Fibroin Light Chain

	N28	N105	N108	N136	N200	N204	N240	N248	Q255
Low Skid Lyo	32.21	69.79	22.84	34.57	87.31	87.1	90.45	56.16	7.02
Mid Skid Lyo	4.06	56.15	17.14	15.53	52.87	54.44	52.37	17.79	2.4

Table 4. Percentage of amino acid modifications in Low Skid silk produced in the facility in Walpole and Medford under differing process parameters and variable levels. N are Asparagines that become aspartic acid and Q are Glutamines that become deamidated. M corresponds to Methionies that become oxidized.

15 ***Fibroin Heavy Chain***

	N70	N132	N4191	Q125	Q275	Q5216
Low Skid Walp	25.02	47.21	18.78	2.82	2.92	4.39
Low Skid Med	83.82	82.88	96.01	5.82	6.7	13.67

Fibroin Light Chain

	N28	N108	N200
Low Skid Walp	27.94	26.16	54.96
Low Skid Med	35.38	42.92	80.99

Table 5. Percentage of amino acid modifications in Mid silk produced in between the different Skid and Benchtop processes. N are Asparagines that become aspartic acid and Q are Glutamines that become deamidated. M corresponds to Methionies that become oxidized.

Fibroin Heavy Chain (Low Skid and Low Benchtop)

	N70	N132	N526 2	Q12 5	Q13 9	M64	M80	M10 3
Low Skid Walpole	85.1 2	66.9 1	48.31	2.66	0.62	27.9	17.2 9	16.1
Low Benchtop	90.5 6	74.6 4	21.29	1.2	2.59	47.5 4	43.1 4	33.3

Fibroin Light Chain (Low Skid and Low Benchtop)

	N28	N118	N138	N200	N204	N240	N248	M69
Low Skid Walpole	21.09	43.51	5.37	87.48	74.02	88.49	31.93	28.61
Low Benchtop	4.72	38.96	0.83	68.27	66.14	75.25	12.87	69.6

Fibrohexamerin (p25)(Low Skid and Low Benchtop)

	N174	Q62	M120
Low Skid Walpole	77.6	87.34	0
Low Benchtop	0	2.74	59.28

Fibroin Heavy Chain (Mid Skid and Mid Benchtop)

	N68	N4191	Q113	Q125
Mid Skid Walpole	94.29	100	7.07	17.95
Mid Benchtop	77.81	81.3	0	0.57

*No significant differences in the other proteins between Mid Skid and Mid Benchtop.

Calculation of the percentage of amino acid modification per location along the amino acid sequence of each protein chain.

To determine the percentage of modified amino acids within the sequences of individual protein chains in the silk preparations, LC/MS analysis was employed. Through this analytical method, it was not only discerned the sequence of all peptides present in the solution but also quantified their respective concentrations. LC/MS methodology possesses the capability to pinpoint amino acid modifications at specific positions within these sequences, a phenomenon induced by the production techniques. Upon successfully identifying and quantifying all the peptides within the mixture, they were aligned with the protein sequences present in the silk cocoons. This alignment process allowed for the ascertainment of the origin of each peptide along the polypeptide chain of fibroin heavy and light chains, as well as fibrohexamerin (p25). Consequently, the exact positions of amino acids were pinpointed on these polypeptide chains.

To calculate the percentage of modified amino acids at specific locations, all peptides containing these amino acids were quantified, both modified and unmodified. The calculation involved dividing the quantity of peptides containing modified amino acids by the total quantity of peptides containing those particular amino acids (modified and unmodified) and then multiplying the result by 100. This yielded the percentage of modified amino acids at the designated location. Refer to Fig. 16.

Example 5. Low Skid Silk/Modified Polypeptide Compositions Isolated by Charge and Size Properties

Described herein is a novel method to generate compositions of polypeptide that are derived from *B. mori* silkworm cocoons and comprise of natural and modified polypeptides. This novel composition is called Low Skid silk/modified polypeptide compositions.

The novel production method involves removing sericin through several washing steps with an organic sodium carbonate salt with tightly controlled multi-stage temperature cycles and agitation as the first step in forming natural/modified polypeptide composition. Next the silk is dried to remove remaining water at controlled temperature to maintain polypeptide composition. The silk is then dissolved in high concentration of Lithium salt at 125 °C for 6 hours to achieve the compositions of Low Skid silk. The liquid solution is then filtered and purified to remove the Lithium salt leaving only the natural/modified silk compositions in solution with pure water.

Low Skid silk/modified polypeptide compositions comprise of populations of silk/modified polypeptides with distinctive properties.

Low Skid silk/modified polypeptide composition does not self-assemble at 5 mg/mL.

Low Skid silk/modified polypeptide composition comprises of a variety of populations
5 of silk/modified polypeptides; distinct populations were isolated based on charge and size, by fractionating Low Skid silk/modified polypeptides by anion exchange chromatography and size exclusion chromatography. A high-resolution separation of five negatively-charged silk compositions was achieved— AS77, AS78, AS79, AS80, and AS81. These silk compositions differ from one another by their average size, when
10 AS77 is the largest, and AS81 is the smallest. These silk compositions do not self-assemble under conditions that promote self-assembly at 5 mg/mL.

The Low Skid silk/modified polypeptide compositions described in this invention are novel compositions of silk and modified polypeptides composed of a variety of silk polypeptide populations, generated by the exclusive treatment method of natural silk
15 produced by *B. mori*. These silk compositions contain modified amino acid sequences that result from silk processing method and scale. The tight controls over temperature, silk concentration, buffers and salt concentrations, physical agitation, and purification allow for the precise development of silk compositions with a variety of performance criteria. Isolation of these populations by charge and size reveals new characteristics,
20 like high solubility and stability in solution over time in these populations. The purification method allows for the isolation of silk/modified polypeptide compositions that display biological activities and could be used for therapeutic purposes.

Silk is a complex natural biomaterial that has the potential to be utilized in various applications such as the development of implantable medical devices, and the
25 development of soluble polypeptide compositions of medical value. Additionally, it was demonstrated that silk peptides have anti-genotoxic effects. However, silk, in its natural form, is not soluble, and silk polypeptide compositions, without the proper processing, display poor solubility in solution and tend to self-assemble and aggregate over time. The kinetics of this self-assembly is unpredictable, and highly depends on the composition of
30 the silk polypeptides/modified composition. Novel silk/modified polypeptide compositions were produced and the silk/modified polypeptide compositions isolated specific populations within these compositions. The isolation process allows for the control of the properties of the silk compositions and development of products with predictable and desired characteristics.

Generation of Low Skid silk/modified polypeptide compositions.

Silk is washed to remove sericin at 100 °C and 60 °C with sodium carbonate and then dried at 60 °C. The silk is then dissolved in 9.3 M Lithium Bromide at 125 °C for 6 hours. This dissolution step controls not only molecular weight but also the polypeptide modifications creating the natural/modified silk compositions. The silk is then filtered to remove undissolved debris and purified using 10 kDa cutoff PES hollow fiber membranes and concentrated using the same process leaving only natural/modified silk composite in solution with pure water. Every unit ops is tightly controlled for temperature, time, concentrations, agitation, and shear.

Isolation of Low Skid/modified polypeptide compositions***Isolation of the AS77-AS81 silk/modified polypeptide composition component of Low Skid silk/modified polypeptide composition.***

To isolate AS77-AS81 Low Skid silk/modified polypeptide compositions were fractionated using anion exchange chromatography (Q-Sepharose chromatography), following HiLoad 26/600 Superdex 200 pg size exclusion chromatography of the Q-eluate (figure 1, 2). Prior to chromatography, Tris was added to the silk preparations to a final concentration of 50 mM Tris-HCl, pH=8.0. The silk was centrifuged and filtered before loading to the Q-Sepharose column, to remove any preformed aggregates. The silk compositions were loaded onto the Q-Sepharose column, and the flowthrough fraction was collected. The negatively charged silk compositions were eluted using high salt buffer (50 mM Tris, 500 mM CaCl₂). The eluted fractions were pulled together and are referred to as the Q-elution fraction. The Q-elution was further fractionated by the HiLoad 26/600 Superdex 200 pg, where the largest polypeptide compositions were eluted first, and each following fraction had a population of lower molecular weight of silk compositions (Figs. 17, 18A, 18B). Low Skid silk preparation solutions have a characteristic yellow hue. The Q-elution fraction has a strong yellow hue, while the flowthrough fraction is transparent, and tends to self-assemble very quickly. The Q-elution silk compositions that are fractionated by size exclusion also had a yellow hue. When silk formulations AS77-AS81 are analyzed with an analytical SEC column (see materials and methods) with HPLC, each of the silk formulations demonstrates a different average Mw, and a different Polydispersity (PDI) value (Figs. 19A-19B, Table 7). In general, AS77 has the highest Mw (55714 Da), while AS81 has the lowest Mw (28750 Da). The PDI values display a differential change as well. The PDI value of AS77 is relatively low (1.1836), and AS81 is higher (1.3648) (Figs. 19A-19B, Table

7). Unfractionated Low Skid silk has an average Mw of ~19500, indicating that most of the peptide population tends to have lower molecular weight than fractions AS77-AS81. The polydispersity of unfractionated Low Skid silk is ~2.2 – significantly higher than the values of fractions AS77-AS81. This indicated that the unfractionated Low Skid silk is composed of a much diverse peptide population compared to fractions AS77-AS81.

AS77-AS81 silk compositions demonstrate relative uniformity by dynamic light scattering and show gradual particle size distribution.

In dynamic light scattering analysis (Zetasizer Pro, Figures 22A-22B, Table 6), AS77-AS81 demonstrated relatively uniform, though broad, peaks where AS77 has the largest Z-average (17.16 nm), then AS78 (15.14 nm), and so on (Table 6), demonstrating the efficiency of the fractionation by size of the Q-elution fraction.

Self-Assembly of Low and Mid Skid/modified polypeptide compositions

Self-assembly assay and data derived from it.

To study the stability of silk/modified peptide compositions in solution, Self-Assembly assays at a concentration of 5 mg/mL were performed.

The absorbance at 550nm curves of the self-assembly assays are sigmoid and they can be described as logistic curves. The typical logistic function is:

$$f(x) = \frac{A_{max}}{1 + e^{-k(t-t_{0.5})}}$$

A_{max} is the maximum density of the gel formed

k is the Self-Assembly Rate Factor (SARF)

$t_{0.5}$ is the time point at which 50% of the gel has formed

e is the exponential equation for the specific curve

(see Fig. 21A the red dotted lines for a better demonstration of how these factors from the Self-Assembly experiments were calculated)

Another parameter introduced to characterize the propensity of silk to form gels is the Self-Assembly Factor (F_{SAF}) which is:

$$F_{SAF} = \frac{1}{t_{0.5}(min)} * A_{max}(Abs) * 1000$$

Using the experimental data from the Self-Assembly assays that were performed with the various novel isolated silk polypeptides, these parameters were calculated and used

to dissect their properties (Figs. 21A- 21B). Four parameters were focused on, collectively referred to as Self-Assembly kinetic factors; the Self-Assembly Rate Factor (SARF), A_{max} , $t_{0.5}$, and the Self-Assembly Factor (SAF) (Figs. 21A- 21B). The SARF shows how fast silk self-assembles to form gel after the reaction begins or the gelation nuclei have formed; A_{max} shows how dense is the gel that is formed after self-assembly is complete, $t_{0.5}$ shows how long it takes for the self-assembly reaction to reach the point where gel density is $\frac{A_{max}}{2}$ and SAF shows the propensity of silk to self-assemble (Figs. 21A- 21B).

AS77-AS81 silk compositions do not self-assemble.

Self-assembly assays revealed that Low Skid silk/modified peptide compositions do not self-assemble under the experimental system conditions (Figure 22A). No self-assembly occurred after 24 hours, and no self-assembly was detected even 12 days post-assay (Figure 22B). Mid Skid/modified peptide compositions was used as a positive control and shows fast self-assembly kinetics (Figs. 21A- 21B).

Materials and Methods used for the generation and characterization of AS77-AS81.

Anion exchange chromatography of silk.

Low Skid silk was provided at a concentration of 60 mg/mL. 50 mM Tris, pH=8.0 buffer was added to the Low Skid silk, and the silk was centrifuged at 16000 rpm (rotor JA-18, Beckman coulter, average of 28100 xg), at 4°C, for 30 min to separate formed aggregates from soluble silk. The supernatant was collected and filtered through a 0.22 μ m PES filter. For silk fractionation, Q-Sepharose prepacked columns connected to an AKTA pure 25L or HiPrep Q FF 16/10 20 mL Column, or HiTrap™ Capto™ Q 1 mL column was used. All buffers used were filtered through a 0.22 μ m PES filter and degassed with sonication. Centrifuged and filtered Low Skid silk was loaded on 5 x 5mL HiTrap Q HP columns washed with 10 column volumes of 50 mM Tris pH=8.0, 10 column volumes of 50 mM Tris pH=8.0, 500 mM CaCl₂ and finally 10 column volumes of 50 mM Tris pH=8.0. 170 mL of centrifuged Low Skid silk were loaded on the column with a flow rate of 5 mL/min. The flow-through was collected. The column was washed with 50 mM Tris pH=8.0 until the absorbance at 280 nm [A_{280}] got to 100 AU. Bound protein was eluted in one step with 50 mM Tris pH=8.0, 500 mM CaCl₂ and all fractions with absorbance [A_{280}] >500AU were pooled together. The Q-elution

fraction (the eluate) was then used for further fractionation by size exclusion chromatography.

Size Exclusion Chromatography of Silk.

The Low Skid silk eluate fraction of the Q-Sepharose anion exchange chromatography (Q-elution) was the starting material for size exclusion chromatography. The eluate was loaded onto a HiLoad 26/600 Superdex 200 pg gel filtration column for fractionation, using the AKTA Pure 25L system. All buffers used during fractionation were filtered through 0.22 µm PES filter as well and were degassed. The Low Skid silk was loaded on the Superdex 200 gel filtration column, and was run with 50 mM Tris, 200 mM CaCl₂, pH=8.0, to fractionate the Q-elution Low Skid silk. The eluted silk compositions were collected in 10 ml fractions. Fractions 6-10 (AS77, AS78, AS79, AS80, AS81) were collected, and have relatively narrow range of molecular weight. The fractions were placed in 3.5 kDa cutoff dialysis bags, and were concentrated by covering the dialysis bags with polyethylene glycol 35000 Da. Then, fractions in the dialysis bags were immersed in 160X volumes of 50 mM Tris pH=8.0 overnight, and then were immersed in a new batch of 160X volume of 50 mM Tris pH=8.0. Samples were kept at 4°C until they were used.

Analytical/Protein Characterization methods.

Protein concentration determination.

Protein concentration was determined by absorbance at 220nm or 280nm. Solubilized silk preparations were diluted until A₂₈₀ was between 0.1-1. In this range the absorbance correlates linearly with the concentration of silk in the solution and the correlation is 1AU=1mg/mL soluble silk proteins. Final concentrations in the initial silk solution were calculated after adjustment for the dilution used for the absorbance measurement.

Analytical Size Exclusion Chromatography.

Analytical Size Exclusion Chromatography is performed as described in detail in below Example 23.. Analysis was performed in a PolySep GFC P-4000 LC Column, 300 mm x 7.8 mm connected to an Agilent 1260 Infinity II HPLC system with an Agilent G7162A RID Refractive Index Detector. The mobile phase used for the analysis was a solution of 0.1M NaCl, 12.5mM Na₂HPO₄, pH 7 (the pH was adjusted with phosphoric

acid and filtered through a 0.2µm PES filter into a clean glass media bottle). 25µL of sample were loaded on the column and the analysis was performed at 25°C with a flow rate of 1 mL/min for 20 min. Calculation of the molecular weight of each sample was done using Agilent Technologies Open LAB CDS ChemStation Edition for LC & LC/MS Systems software Cirrus SEC data collection and molecular weight analysis software.

SDS polyacrylamide gel.

Low Skid silk fractions were uploaded onto a Mini-Protean TGX precast gel, 4-20%, with a protein marker Trident Prestained Protein Ladder for molecular weight reference. The SDS polyacrylamide gel was stained using ReadyBlue™ Protein stain gel. Gels were immersed in ReadyBlue™ solution for 1 h, then destained with DI/RO water.

Self-Assembly Assay.

The silk Self Assembly Assay (SAF) was performed in 35% v/v 2-propanol and 50mM CH₃COONa pH=5. Each reaction was done in a final volume of 200 µL. Total silk protein concentration was 5 mg/mL. First the buffer of 50 mM CH₃COONa pH=5.0, 35% v/v 2-propanol was prepared. Then DI/RO water was added so that after the addition of the volume of silk protein required to reach a final concentration of 5 mg/mL the total volume would be 200 µL. The protein was added last and mixed with very gentle pipetting to reduce shearing force. The protein mixtures were placed in wells of flat-bottom 96-well plates and a layer of 100 µL of Mineral Oil carefully, so as to not create any bubbles. Absorbance was recorded at 550 nm for 24h. Recorded values were exported in Excel files for storage and further analysis.

Dynamic Light Scattering analysis of silk compositions.

Low Skid silk compositions were diluted to a concentration of 1 mg/mL and filtered with a 0.22 µm PES syringe filter. All measurements were performed with a Malvern Zetasizer Pro Red Label, detection angle of 173°. The Red Label system operates with a 10 mW He-Ne laser (633 nm). The software used is ZS XPLOER version 3.2.1.11. All measurements were done with 4.2 ml polystyrol/polystyrene transparent cuvettes. samples were measured at 25°C, with 120 sec of equilibration time. The intensity size distributions, autocorrelation, and Z-average were measured.

Tables Low Skid silk/modified polypeptide composition	Z-average (d. nm)
AS77	17.16
AS78	15.14
AS79	14.27
AS80	13.07
AS81	12.19

Table 6: Z-average of AS77-AS81 calculated by Dynamic Light Scattering. The Z-average value of each silk/modified polypeptide composition was calculated by the Zetasizer Pro. Shown here are the Z-average values of each silk composition. The abbreviation d. nm refers to the diameter in nanometers.

Low Skid silk/modified polypeptide composition	Mw	PDI
AS77	55714	1.1836
AS78	45350	1.1725
AS79	38633	1.2549
AS80	34476	1.2366
AS81	28750	1.3648
Low Skid silk	19500	2.2

Table 7: Molecular weight (Mw) and Polydispersity (PDI) values of silk compositions AS77-AS81. Silk/modified polypeptide compositions AS77, AS78, AS79, AS80, and AS81 were analyzed by size exclusion chromatography (SEC) column with HPLC, and values of molecular weights (Mw) and Polydispersity (PDI) are indicated.

Example 6. Low Skid Silk/Modified Polypeptide Compositions Isolated by Size Properties

Described herein is a novel method to generate compositions of polypeptide that are derived from *B. mori* silkworm cocoons and comprise of natural and modified polypeptides. This novel composition is called Low Skid silk/modified polypeptide compositions.

The novel production method involves removing sericin through several washing steps with an organic sodium carbonate salt with tightly controlled multi-stage temperature cycles and agitation as the first step in forming natural/modified polypeptide composition. Next the silk is dried to remove remaining water at controlled temperature to maintain polypeptide composition. The silk is then dissolved in high concentration

of Lithium salt at 125°C for 6 hours to achieve the compositions of Low Skid silk. The liquid solution is then filtered and purified to remove the Lithium salt leaving only the natural/modified silk compositions in solution with pure water.

Low Skid silk/modified polypeptide compositions comprise of populations of silk/modified polypeptides with distinctive properties.

Low Skid silk/modified polypeptide composition does not self-assemble at 5 mg/mL.

Low Skid silk/modified polypeptide composition comprises of a variety of populations of silk/modified polypeptides; here distinct populations were isolated based on size, by fractionating Low Skid silk/modified polypeptides by size exclusion chromatography.

A high-resolution separation of five silk compositions – AS82, AS83, AS84, AS85, and AS86 was achieved. These silk compositions differ from one another by their average size, when AS82 is the largest, and AS86 is the smallest. These silk compositions do not self-assemble under conditions that promote self-assembly at 5 mg/mL.

In addition to the well-separated silk compositions, lower-molecular-weight silk compositions (AS87, AS88, AS89) were generated that are less well-resolved, but composed of significantly smaller polypeptide populations. These silk compositions self-assemble into a gel within a few days under conditions that promote self-assembly at 5 mg/mL, but not as quickly as Mid Skid silk (starting to self-assemble within 3 h).

Low Skid silk/modified polypeptide compositions isolated by size properties.

The Low Skid silk/modified polypeptide compositions described in this invention are novel compositions of silk and modified polypeptides composed of a variety of silk polypeptide populations, generated by the exclusive treatment method of natural silk produced by *B. mori*. These silk compositions contain modified amino acid sequences that result from the silk processing method and scale. The tight controls over temperature, silk concentration, buffers and salt concentrations, physical agitation, and purification allow us to precisely develop silk compositions with a variety of performance criteria. Isolation of these populations by size reveals different characteristics, like high solubility and stability in solution over time in some populations, and the tendency to self-assemble in others. the purification method allows us to isolate silk/modified polypeptide compositions that display biological activities and could be used for therapeutic purposes.

Silk is a complex natural biomaterial that has the potential to be utilized in various applications such as the development of implantable medical devices, and the development of soluble polypeptide compositions of medical value. Additionally, it was

demonstrated that silk peptides have anti-genotoxic effects. However, silk, in its natural form, is not soluble, and silk polypeptide compositions, without the proper processing, display poor solubility in solution and tend to self-assemble and aggregate over time. The kinetics of this self-assembly is unpredictable, and highly depends on the composition of the silk polypeptides/modified composition. Novel silk/modified polypeptide compositions were produced and specific populations were isolated within these compositions. The isolation process allows us to control the properties of the silk compositions and develop products with predictable and desired characteristics.

Generation of Low Skid silk/modified polypeptide compositions.

Silk is washed to remove sericin at 100 °C and 60 °C with sodium carbonate and then dried at 60 °C. The silk is then dissolved in 9.3 M Lithium Bromide at 125 °C for 6 hours. This dissolution step controls not only molecular weight but also the polypeptide modifications creating the natural/modified silk compositions. The silk is then filtered to remove undissolved debris and purified using 10 kDa cutoff PES hollow fiber membranes and concentrated using the same process leaving only natural/modified silk composite in solution with pure water. Every unit ops is tightly controlled for temperature, time, concentrations, agitation, and shear.

Isolation of Low Skid/modified polypeptide compositions

Isolation of the AS82-AS89 silk/modified polypeptide composition component of Low Skid silk/modified polypeptide composition.

To isolate AS82-AS89 Low Skid silk/modified polypeptide compositions were fractionated using HiLoad 26/600 Superdex 200 pg size exclusion chromatography column (Figs. 23 & 24). Tris was added to the silk preparations to a final concentration of 50 mM Tris-HCl, pH=8.0. The silk was centrifuged and filtered before loading to the HiLoad 26/600 Superdex 200 pg column, to remove any preformed aggregates. The silk compositions were fractionated by the HiLoad 26/600 Superdex 200 pg, where the largest polypeptide compositions were eluted first, and each following fraction had a population of lower molecular weight of silk compositions (Fig. 24). Low Skid silk preparation solutions have a characteristic yellow hue, and the fractionated silk compositions had a yellow hue. When silk formulations AS82-AS89 are analyzed with an analytical SEC column (see materials and methods) with HPLC, each of the silk formulations demonstrates a different average Mw, and a different Polydispersity (PDI) value (Fig. 24, Table 9). In general, AS82 has the highest Mw (51936 Da), while AS89 has the lowest Mw (6826 Da). The PDI values display a differential change as well.

The PDI value of AS82 is relatively low (1.1738), and AS89 is higher (1.3544) (Figs. 25A-25B, Table 9).

AS82-AS86 silk compositions demonstrate relative uniformity by dynamic light scattering, while AS87-AS89 silk compositions contain multiple peptide populations sizes.

In dynamic light scattering analysis (Zetasizer Pro, Figs. 28A-28C, Table 8), AS82-AS86 demonstrated relatively uniform, though broad, peaks where AS82 has the largest Z-average (18.174 nm), then AS83 (15.659 nm), and so on (Table 9). AS87, AS88, and AS89 are of lower molecular weight, and are eluted later during the chromatography, where the resolution of the Superdex 200 is not optimal and cannot resolve the peptide populations very well (the column resolution is reduced for proteins smaller than average size of ~44 kDa), as can be observed by SDS gel electrophoresis in Fig. 26 (fraction 11 and on). Dynamic light scattering shows two peaks for these fractions, indicating the presence of several populations (Fig. 28A).

15 **Self-Assembly of Low and Mid Skid/modified polypeptide compositions**

Self-Assembly assay and data derived from it.

To study the stability of silk/modified peptide compositions in solution Self-Assembly assays were performed at a concentration of 5 mg/mL.

The absorbance at 550nm curves of the self-assembly assays are sigmoid and they can be described as logistic curves. The typical logistic function is:

$$f(x) = \frac{A_{max}}{1 + e^{-k(t-t_{0.5})}}$$

A_{max} is the maximum density of the gel formed

25 k is the Self-Assembly Rate Factor (SARF)

$t_{0.5}$ is the time point at which 50% of the gel has formed

e is the exponential equation for the specific curve

(see Fig. 27A the red dotted lines for a better demonstration of how these factors from the Self-Assembly experiments were calculated)

30

Another parameter that was introduced to characterize the propensity of silk to form gels is the Self-Assembly Factor (F_{SAF}) which is:

$$F_{SAF} = \frac{1}{t_{0.5}(\text{min})} * A_{max}(\text{Abs}) * 1000$$

Using the experimental data from the Self-Assembly assays that were performed with the various novel isolated silk polypeptides, these parameters were calculated and used to dissect their properties (Figs. 27A- 27B). Four parameters were focused on, collectively referred to as Self-Assembly kinetic factors; the Self-Assembly Rate Factor (SARF), A_{max} , $t_{0.5}$, and the Self-Assembly Factor (SAF) (Figs. 27A- 27B). The SARF shows how fast silk self-assembles to form gel after the reaction begins or the gelation nuclei have formed; A_{max} shows how dense is the gel that is formed after self-assembly is complete, $t_{0.5}$ shows how long it takes for the self-assembly reaction to reach the point where gel density is $\frac{A_{max}}{2}$ and SAF shows the propensity of silk to self-assemble (Figs. 27A- 27B).

AS82-AS86 silk compositions do not self-assemble.

Self-assembly assays revealed that Low Skid silk/modified peptide compositions do not self-assemble under the experimental system conditions (Fig. 27A). No self-assembly occurred after 24 hours, and no self-assembly was detected even 18 days post-assay (Fig. 27B). Mid Skid/modified peptide compositions was used as a positive control and shows fast self-assembly kinetics (Figs. 27A- 27B).

AS87-AS89 silk compositions self-assemble within few days.

Self-assembly assays as described before, revealed that AS87-AS89 do not self-assemble within 24 hours (Fig. 27A). However, if left for an extra 4-5 days, self-assembly occurs in these silk/modified polypeptide compositions (Fig. 27B). Mid Skid/modified peptide compositions was used as a positive control and shows fast self-assembly kinetics (Figs. 27A- 27B).

Materials and Methods used for the generation and characterization of AS82-AS89.

Size Exclusion Chromatography of Silk.

The starting material, Low Skid silk at a concentration of 60 mg/mL, was provided by the manufacturing team. The Low Skid silk was transferred to 50 mM Tris, pH=8.0 buffer, and centrifuged at 16000 rpm, at 4°C, for 30 min to separate formed aggregates from soluble silk. The supernatant was collected and filtered through a 0.22 µm PES filter. Then, the silk was loaded onto a HiLoad 26/600 Superdex 200 pg gel filtration

column for fractionation, using the AKTA Pure 25L system. All buffers used during fractionation were filtered through 0.22 μ m PES filter as well and were degassed. The Low Skid silk was loaded on the Superdex 200 gel filtration column, and was run with 50 mM Tris, 200 mM CaCl₂, pH8, to fractionate the Low Skid silk. The eluted silk compositions were collected in 10 ml fractions. Fractions 6-10 (AS82, AS83, AS84, AS85, AS86) were collected, and have relatively narrow range of molecular weight, while fractions 18-20 (AS87, AS88, AS89) have a broader molecular weight range, since these molecular sizes are outside of the HiLoad 26/600 Superdex 200 pg separation range. The fractions were placed in 3.5 kDa cutoff dialysis bags, and were concentrated by covering the dialysis bags with polyethylene glycol 35000 Da. Then, fractions in the dialysis bags were immersed in 160X volumes of 50 mM Tris pH=8.0 overnight, and then were immersed in a new batch of 160X volume of 50 mM Tris pH=8.0. Samples were kept at 4°C until they were used.

Analytical/Protein Characterization methods.

Protein concentration determination.

Protein concentration was determined by absorbance at 220nm or 280nm. Solubilized silk preparations were diluted until A₂₈₀ was between 0.1-1. In this range the absorbance correlates linearly with the concentration of silk in the solution and the correlation is 1AU=1mg/mL soluble silk proteins. Final concentrations in the initial silk solution were calculated after adjustment for the dilution used for the absorbance measurement.

Analytical Size Exclusion Chromatography.

Analytical Size Exclusion Chromatography is performed as described in detail in Example 23. Analysis was performed in a PolySep GFC P-4000 LC Column, 300 mm x 7.8 mm connected to an Agilent 1260 Infinity II HPLC system with an Agilent G7162A RID Refractive Index Detector. The mobile phase used for the analysis was a solution of 0.1M NaCl, 12.5mM Na₂HPO₄, pH 7 (the pH was adjusted with phosphoric acid and filtered through a 0.2 μ m PES filter into a clean glass media bottle). 25 μ L of sample were loaded on the column and the analysis was performed at 25°C with a flow rate of 1 mL/min for 20 min. Calculation of the molecular weight of each sample was done using Agilent Technologies Open LAB CDS ChemStation Edition for LC &

LC/MS Systems software Cirrus SEC data collection and molecular weight analysis software.

SDS polyacrylamide gel.

Low Skid silk fractions were uploaded onto a Mini-Protean TGX precast gel, 4-20%,
5 with a protein marker Trident Prestained Protein Ladder for molecular weight reference. The SDS polyacrylamide gel was stained using ReadyBlue™ Protein stain gel. Gels were immersed in ReadyBlue™ solution for 1 h, then destained with DI/RO water.

Self-Assembly Assay.

10 The silk Self Assembly Assay (SAF) was performed in 35% v/v 2-propanol and 50mM CH₃COONa pH=5. Each reaction was done in a final volume of 200μL. Total silk protein concentration was 5mg/mL. First the buffer of 50mM CH₃COONa pH=5, 35% v/v 2-propanol was prepared. Then DI/RO water was added so that after the addition of the volume of silk protein required to reach a final concentration of 5mg/mL the total
15 volume would be 200μL. The protein was added last and mixed with very gentle pipetting to reduce shearing force. The protein mixtures were placed in wells of flat-bottom 96-well plates and a layer of 100μL of Mineral Oil carefully so as to not create any bubbles. Absorbance was recorded at 550nm for 24h.

Dynamic Light Scattering analysis of silk compositions.

20 Low Skid silk compositions were diluted to a concentration of 1 mg/mL and filtered with a 0.22 μm PES syringe filter. All measurements were performed with a Malvern Zetasizer Pro Red Label, detection angle of 173°. The Red Label system operates with a 10 mW He-Ne laser (633 nm). The software used is ZS XPLOER version 3.2.1.11. All measurements were done with 4.2 mL polystyrol/polystyrene transparent cuvettes.
25 samples were measured at 25°C, with 120 sec of equilibration time. The intensity size distributions, autocorrelation, and Z-average were measured.

Tables

Low Skid silk/modified polypeptide composition	Z-average (d. nm)
AS82	18.147

AS83	15.659
AS84	14.836
AS85	13.473
AS86	13.467
AS87	8.577
AS88	7.843
AS89	7.681

Table 8: Z-average of AS82-AS89 calculated by Dynamic Light Scattering. The Z-average value of each silk/modified polypeptide composition was calculated by the Zetasizer Pro. Shown here are the Z-average values of each silk composition.

Low Skid silk/modified polypeptide composition	Mw	PDI
AS82	51936	1.1738
AS83	42028	1.1543
AS84	36098	1.1755
AS85	29089	1.2223
AS86	23574	1.235
AS87	8668	1.425
AS88	7972	1.3537
AS89	6826	1.3544
Low Skid silk	19500	2.2

Table 9: Molecular weight (Mw) and Polydispersity (PDI) values of silk compositions AS82-AS89. Silk/modified polypeptide compositions AS82, AS83, AS84, AS85, AS86, AS87, AS88, and AS89 were analyzed by size exclusion chromatography (SEC) column with HPLC, and values of molecular weights (Mw) and Polydispersity (PDI) are indicated.

Example 7. Low Skid silk/modified polypeptide compositions isolated by charge, hydrophobicity levels, and size properties

Described herein is a novel method to generate compositions of polypeptide that are derived from *B. mori* silkworm cocoons and comprise of natural and modified polypeptides. This novel composition is called Low Skid silk/modified polypeptide compositions.

The novel production method involves removing sericin through several washing steps with an organic sodium carbonate salt with tightly controlled multi-stage temperature cycles and agitation as the first step in forming natural/modified polypeptide

composition. Next the silk is dried to remove remaining water at controlled temperature to maintain polypeptide composition. The silk is then dissolved in high concentration of Lithium salt at 125°C for 6 hours to achieve the compositions of Low Skid silk. The liquid solution is then filtered and purified to remove the Lithium salt leaving only the natural/modified silk compositions in solution with pure water.

Low Skid silk/modified polypeptide compositions comprise of populations of silk/modified polypeptides with distinctive properties

Low Skid silk/modified polypeptide composition does not self-assemble at 5 mg/mL.

Low Skid silk/modified polypeptide composition comprises of a variety of populations of silk/modified polypeptides; here it was sought to isolate distinct populations based on charge, hydrophobicity, and size, by fractionating Low Skid silk/modified polypeptides by anion exchange chromatography, followed by hydrophobic interaction chromatography and size exclusion chromatography. A high-resolution separation of ten distinct Low Skid silk/modified polypeptides compositions was achieved: five are negatively-charged silk compositions that display hydrophobicity characteristics as well – AS90, AS91, AS92, AS93, and AS94. These silk compositions differ from one another by their average size, when AS90 is the largest, and AS94 is the smallest. These silk compositions do not self-assemble under conditions that promote self-assembly at 5 mg/mL.

Additional five compositions are negatively charged silk compositions as well, that are less hydrophobic compared to AS90-AS94, and have relatively lower molecular weights (AS95, AS96, AS97, AS98, AS99, AS100). These silk compositions differ from one another by their average size, when AS95 is the largest, and AS100 is the smallest. These silk compositions may have the tendency to aggregate in solution, as can be demonstrated by dynamic light scattering and loss of material after filtration with 0.2 µm PES filter.

The Low Skid silk/modified polypeptide compositions described in this invention are novel compositions of silk and modified polypeptides composed of a variety of silk polypeptide populations, generated by the exclusive treatment method of natural silk produced by *B. mori*. These silk compositions contain modified amino acid sequences that result from the silk processing method and scale. The tight controls over temperature, silk concentration, buffers and salt concentrations, physical agitation, and purification allow us to precisely develop silk compositions with a variety of performance criteria. Isolation of these populations by charge, hydrophobicity, and size

reveals new characteristics, like high solubility and stability in solution over time in some populations, and the tendency to aggregate in others. the purification method allows us to isolate silk/modified polypeptide compositions that display biological activities and could be used for therapeutic purposes.

- 5 Silk is a complex natural biomaterial that has the potential to be utilized in various applications such as the development of implantable medical devices, and the development of soluble polypeptide compositions of medical value. Additionally, it was demonstrated that silk peptides have anti-genotoxic effects. However, silk, in its natural form, is not soluble, and silk polypeptide compositions, without the proper processing, display poor solubility in solution and tend to self-assemble and aggregate over time. The kinetics of this self-assembly is unpredictable, and highly depends on the composition of the silk polypeptides/modified composition. Novel silk/modified polypeptide compositions were produced and specific populations were isolated within these compositions. The isolation process allows us to control the properties of the silk compositions and develop products with predictable and desired characteristics.

Generation of Low Skid silk/modified polypeptide compositions.

- Silk is washed to remove sericin at 100 °C and 60 °C with sodium carbonate and then dried at 60 °C. The silk is then dissolved in 9.3 M Lithium Bromide at 125 °C for 6 hours. This dissolution step controls not only molecular weight but also the polypeptide modifications creating the natural/modified silk compositions. The silk is then filtered to remove undissolved debris and purified using 10 kDa cutoff PES hollow fiber membranes and concentrated using the same process leaving only natural/modified silk composite in solution with pure water. Every unit ops is tightly controlled for temperature, time, concentrations, agitation, and shear.

25 **Isolation of Low Skid/modified polypeptide compositions**

Isolation of the AS90-AS100 silk/modified polypeptide composition component of Low Skid silk/modified polypeptide composition.

- To isolate AS90-AS100 Low Skid silk/modified polypeptide compositions were fractionated using anion exchange chromatography (Q-Sepharose chromatography), following hydrophobic interactions (HIC) chromatography (Butyl ImpRes column), followed by HiLoad 26/600 Superdex 200 pg size exclusion chromatography of the Q-HIC-eluate (AS90-94) and of the Q-HIC-flowthrough (AS95-100) (figure 1, 2).

Prior to chromatography, Tris was added to the silk preparations to a final concentration of 50 mM Tris-HCl, pH=8.0. The silk was centrifuged and filtered before loading to

the Q-Sepharose column, to remove any preformed aggregates. The silk compositions were loaded onto the Q-Sepharose column, and the flowthrough fraction was collected. The negatively charged silk compositions were eluted using high salt buffer (50 mM Tris, 500 mM CaCl₂) (Figure 2 A). The eluted fractions were pulled together and are referred to as the Q-elution fraction. The Q-flowthrough fraction is colorless and tends to aggregate.

The Q-elution was further fractionated by using a Butyl ImpRes column (Figure 2 B), which separates polypeptides based on hydrophobicity. The chromatography was performed in the presence of 300 mM ammonium sulfate [(NH₄)₂SO₄], to expose hydrophobic regions within the silk polypeptides. The highly charged flowthrough fraction (Q-HIC-flowthrough) was collected for further fractionation by size exclusion chromatography. The more hydrophobic, bound silk peptides were eluted using 50 mM Tris, pH=8.0 in the absence of ammonium sulfate, to reverse the exposure of the hydrophobic regions in silk polypeptides, which results in their release from the Butyl ImpRes column.

The Q-HIC-elution was further fractionated by the HiLoad 26/600 Superdex 200 pg, where the largest polypeptide compositions were eluted first, and each following fraction had a population of lower molecular weight of silk compositions (Fig. 29, Figs. 30C and 30D). The fractions that were isolated are AS90-AS94. Then, the Q-HIC-flowthrough fraction was fractionated as well by the HiLoad 26/600 Superdex 200 pg, resulting in the generation of AS95-AS100. Comparing the size exclusion chromatograms of Q-HIC(elution) and Q-HIC(flowthrough) (Fig. 30E), it is evident that the Q-HIC(elution) fraction is composed of higher-molecular-weight peptide composition, while the silk peptides that compose the Q-HIC(flowthrough) fraction are eluted later, indicating smaller molecular weights (Also see Figs. 32A and 32B for SDS-PAGE of fractions AS90-AS94 (32A) and AS95-AS100 (32B)).

Low Skid silk preparation solutions have a characteristic yellow hue. The Q-elution and the Q-HIC-elution fractions had a strong yellow hue, while the Q-flowthrough fraction is transparent, and tends to self-assemble very quickly. The Q-HIC-elution silk compositions that are fractionated by size exclusion (AS90-94) also had a yellow hue. The Q-HIC-flowthrough fractions that were fractionated by size exclusion chromatography (AS95-AS100) were colorless.

When silk formulations AS90-AS94 and AS95-AS100 are analyzed with an analytical SEC column (see materials and methods) with HPLC, each of the silk formulations

demonstrates a different average Mw, and a different Polydispersity (PDI) value (Figs. 31A-31B, Table 11). Among the Q-HIC-elution fractions, AS90 has the highest Mw (54620 Da), while AS94 has the lowest Mw (21057 Da). The PDI values display a differential change as well. The PDI value of AS90 is the lowest (1.1487), and AS94 is higher (1.3615) (Figs. 31A-31B, Table 11). The Q-HIC-flowthrough fraction is composed of a smaller population of silk peptides, where the first eluted fraction, AS95, has the higher molecular weight among these fractions (45262 Da), 10 kDa smaller than the first eluted fraction of the Q-HIC-elution fraction (AS90). AS100 has the lowest molecular weight among the Q-HIC-flowthrough fractions (22799 Da). In general, AS95-AS100 demonstrate a trend of higher polydispersity compared to AS90-AS94. The PDI value of AS95 is the lowest (1.1988), and AS100 is higher (1.5438) (Figs. 31A-31B, Table 11).

Unfractionated Low Skid silk has an average Mw of ~19500, indicating that most of the peptide population tends to have lower molecular weight than fractions AS90-AS100. The polydispersity of unfractionated Low Skid silk is ~2.2 – significantly higher than the values of fractions AS90-AS100. This suggests that the unfractionated Low Skid silk is composed of a much diverse peptide population compared to fractions AS90-AS100.

AS90-AS94 silk compositions demonstrate relative uniformity by dynamic light scattering and show gradual particle size distribution.

In dynamic light scattering analysis (Zetasizer Pro, Figs. 34A-34F, Table 1), AS90-AS94 demonstrated relatively uniform, though broad, peaks where AS90 has the largest Z-average (17.617 d. nm), then AS91 (16.803 d. nm), and so on (Table 10), demonstrating the efficiency of the fractionation by size of the Q-HIC-elution fraction (Figs. 34C and 34D). Low Skid silk and Mid Skid silk show broad peaks (Figs. 34A and 34B), while the AS90 fraction shows more uniformity and narrower peak compared to the two and to the Q-HIC elution fraction, from which AS90 is derived.

AS95-AS100 silk compositions display non-uniform silk compositions by dynamic light scattering. AS95-AS100 demonstrate by dynamic light scattering the presence of two major broad peaks for each fraction, indicating a wide range of size of peptide populations (Figs. 34E and 34F). The Z-average values of AS95-AS100 are not in descending order like the Z-average of AS90-AS94 and are more variable (Table 10).

AS95-AS100 fractions and Q-HIC (flowthrough) may have the tendency to aggregate. During the generation AS95-AS100 there has been an extensive loss of

protein material. By following the UV280 detection of protein amount that was flowed through the Butyl ImpRes column and did not bind the column, it was expected to find a large amount of silk peptides in solution. However, during size-exclusion chromatography, the amount of silk peptides in the resulting fractions was low (Fig. 32B). It is hypothesized that a large portion of the silk peptides has aggregated or did not go through the filtration step of the Q-HIC(flowthrough), and was lost before loading onto the gel filtration column. SDS-PAGE of AS95-AS100 that was run immediately after size exclusion chromatography (Fig. 32B) showed distinct populations of silk peptides, in a descending order of molecular weight. However, dynamic light scattering analysis that was performed later showed diverse size populations. This can indicate an aggregation over time of silk peptides in fractions AS95-AS100.

Self-Assembly of Low and Mid Skid/modified polypeptide compositions

Self assembly assay and data derived from it.

To study the stability of silk/modified peptide compositions in solution Self-Assembly assays were performed at a concentration of 5 mg/mL.

The absorbance at 550nm curves of the self-assembly assays are sigmoid and they can be described as logistic curves. The typical logistic function is:

$$f(x) = \frac{A_{max}}{1 + e^{-k(t-t_{0.5})}}$$

A_{max} is the maximum density of the gel formed

k is the Self-Assembly Rate Factor (SARF)

$t_{0.5}$ is the time point at which 50% of the gel has formed

e is the exponential equation for the specific curve

(see Fig. 33 the red dotted lines for a better demonstration of how these factors from

the Self-Assembly experiments are calculated)

Another parameter that was introduced to characterize the propensity of silk to form gels is the Self-Assembly Factor (F_{SAF}) which is:

$$F_{SAF} = \frac{1}{t_{0.5}(min)} * A_{max}(Abs) * 1000$$

Using the experimental data from the Self-Assembly assays performed with the various novel isolated silk polypeptides, these parameters were calculated and used to dissect their properties (Fig. 33). Four parameters were focused on, collectively referred to as Self-Assembly kinetic factors; the Self-Assembly Rate Factor (SARF), A_{max} , $t_{0.5}$, and

the Self-Assembly Factor (SAF) (Fig. 33). The SARF shows how fast silk self-assembles to form gel after the reaction begins or the gelation nuclei have formed; A_{max} shows how dense is the gel that is formed after self-assembly is complete, $t_{0.5}$ shows how long it takes for the self-assembly reaction to reach the point where gel density is $\frac{A_{max}}{2}$ and SAF shows the propensity of silk to self-assemble (Fig. 33).

AS90-AS94 silk compositions do not self-assemble.

Self-assembly assays revealed that Low Skid silk/modified peptide compositions do not self-assemble under the experimental system conditions (Fig. 33). Mid Skid/modified peptide compositions was used as a positive control and shows fast self-assembly kinetics (Fig. 33). Fractions AS95-AS100 were not tested for self-assembly since the purification process did not result in sufficient amount of silk peptides to perform the assay.

Materials and Methods used for the generation and characterization of AS90-AS100. Anion exchange chromatography of silk.

Low Skid silk was provided by the manufacturing team at a concentration of 60 mg/mL. 50 mM Tris, pH=8.0 buffer was added to the Low Skid silk, and the silk was centrifuged at 16000 rpm (rotor JA-18, Beckman coulter, average of 28100 xg), at 4°C, for 30 min to separate formed aggregates from soluble silk. The supernatant was collected and filtered through a 0.22 µm PES filter. For silk fractionation Q-Sepharose prepacked columns connected to an AKTA pure 25L or HiPrep Q FF 16/10 20 mL Column, or HiTrap™ Capto™ Q 1 mL column were used. All buffers used were filtered through a 0.22 µm PES filter and degassed with sonication. Centrifuged and filtered Low Skid silk was loaded on 5 x 5mL HiTrap Q HP columns washed with 10 column volumes of 50 mM Tris pH=8.0, 10 column volumes of 50 mM Tris pH=8.0, 500 mM CaCl₂ and finally 10 column volumes of 50 mM Tris pH=8.0. 150 mL of centrifuged Low Skid silk were loaded on the column with a flow rate of 5 mL/min. The flow-through was collected. The column was washed with 50 mM Tris pH=8.0 until the absorbance at 280nm [A_{280}] got to 100 AU. Bound protein was eluted in one step with 50 mM Tris pH=8.0, 500 mM CaCl₂ and all fractions with absorbance [A_{280}] >500AU were pooled together. This process was performed twice (total 300 ml of starting material of Low Skid silk was fractionated). The Q-elution fractions (the eluate) were then used for further fractionation by hydrophobic interactions chromatography.

Hydrophobic Interactions Chromatography of Silk.

The buffer of Q-eluate was exchanged with water with dialysis in x 100 volumes of water. After the buffer exchange was complete, 50mM Tris pH=8.0, 300mM (NH₄)₂SO₄ were added to the Q-eluate. A column of Butyl ImpRes (Cytiva) resin was used for the creation of AS90-AS100. The Butyl ImpRes column was washed with x10 column volumes of degassed and filtered (0.22 µm) 50mM Tris pH=8.0, 300mM (NH₄)₂SO₄, 10 x column volumes of degassed and filtered (0.22 µm) 50mM Tris pH=8.0 and 10 x column volumes of degassed and filtered (0.22 µm) 50mM Tris pH=8.0, 300mM (NH₄)₂SO₄. The Q-eluate (200 mL) was used for the fractionation. Q-eluate silk was in 50mM Tris pH=8.0, 300mM (NH₄)₂SO₄ and loaded on the Butyl ImpRes column. All unbound proteins (the flowthrough fraction) were collected and saved for further analysis. After loading was complete, the Butyl ImpRes column was washed 10 x column volumes of 50mM Tris pH=8.0, 300mM (NH₄)₂SO₄ until the OD280=about 100 AU. After the washing step was complete, bound silk molecules were eluted with 1.5 x column volumes of 50mM Tris pH=8.0. The elution was collected, and both Q-HIC(elution) and Q-HIC(flowthrough) fractions were transferred to dialysis bags and dialyzed against 3 mM Tris, pH=8.0. The two fractions were concentrated by covering the dialysis bags with polyethylene glycol 35000 Da and saved for further fractionation by size exclusion chromatography.

Size Exclusion Chromatography of Silk.

Both the elution fraction of the hydrophobic interactions chromatography (Q-HIC-elution) and the flowthrough fraction (Q-HIC-flowthrough) were filtered (0.22 µm PES filter) to discard preformed aggregates, and fractionated separately by size exclusion chromatography. The eluate or the flowthrough fraction was loaded onto a HiLoad 26/600 Superdex 200 pg gel filtration column for fractionation, using the AKTA Pure 25L system. All buffers used during fractionation were filtered through 0.22 µm PES filter and were degassed. The Q-HIC(elution) or Q-HIC(flowthrough) fractions were loaded on the Superdex 200 gel filtration column, and were run with 50 mM Tris, 200 mM CaCl₂, pH=8.0. The eluted silk compositions were collected in 10 ml fractions. Fractions 6-10 of the Q-HIC(elution) (AS90, AS91, AS92, AS93, AS94) were collected, and have relatively narrow range of molecular weight. Fractions 8-13 (AS95, AS96, AS97, AS98, AS99, AS100) were collected during the fractionation of Q-HIC(flowthrough). The fractions were placed in 3.5 kDa cutoff dialysis bags, and were

concentrated by covering the dialysis bags with polyethylene glycol 35000 Da. Then, fractions in the dialysis bags were immersed in 160X volumes of 50 mM Tris pH=8.0 overnight, and then were immersed in a new batch of 160X volume of 50 mM Tris pH=8.0. Samples were kept at 4°C until they were used.

5 **Analytical/Protein Characterization methods.**

Protein concentration determination.

Protein concentration was determined by absorbance at 220nm or 280nm. Solubilized silk preparations were diluted until A_{280} was between 0.1-1. In this range the absorbance correlates linearly with the concentration of silk in the solution and the correlation is
10 1AU=1mg/mL soluble silk proteins. Final concentrations in the initial silk solution were calculated after adjustment for the dilution used for the absorbance measurement.

Analytical Size Exclusion Chromatography.

Analytical Size Exclusion Chromatography is performed as described in detail in Example 23. Analysis was performed in a PolySep GFC P-4000 LC Column, 300 mm
15 x 7.8 mm connected to an Agilent 1260 Infinity II HPLC system with an Agilent G7162A RID Refractive Index Detector. The mobile phase used for the analysis was a solution of 0.1M NaCl, 12.5mM Na_2HPO_4 , pH 7 (the pH was adjusted with phosphoric acid and filtered through a 0.2µm PES filter into a clean glass media bottle). 25µL of sample were loaded on the column and the analysis was performed at 25°C with a flow
20 rate of 1 mL/min for 20 min. Calculation of the molecular weight of each sample was done using Agilent Technologies Open LAB CDS ChemStation Edition for LC & LC/MS Systems software Cirrus SEC data collection and molecular weight analysis software.

SDS polyacrylamide gel.

25 Low Skid silk fractions were loaded onto a Mini-Protean TGX precast gel, 4-20%, with a protein marker Trident Prestained Protein Ladder for molecular weight reference. The SDS polyacrylamide gel was stained using ReadyBlue™ Protein stain gel. Gels were immersed in ReadyBlue™ solution for 1 h, then destained with DI/RO water.

Self-Assembly Assay.

The silk Self Assembly Assay (SAF) was performed in 35% v/v 2-propanol and 50mM CH₃COONa pH=5. Each reaction was done in a final volume of 200 μ L. Total silk protein concentration was 5 mg/mL. First, the buffer of 50 mM CH₃COONa pH=5.0, 35% v/v 2-propanol was prepared. Then DI/RO water was added so that after the addition of the volume of silk protein required to reach a final concentration of 5 mg/mL the total volume would be 200 μ L. The protein was added last and mixed with very gentle pipetting to reduce shearing force. The protein mixtures were placed in wells of flat-bottom 96-well plates and a layer of 100 μ L of Mineral Oil carefully, so as to not create any bubbles. Absorbance was recorded at 550 nm for 24h.

10 **Dynamic Light Scattering analysis of silk compositions.**

Low and Mid Skid silk compositions were diluted to a concentration of 1 mg/mL and filtered with a 0.22 μ m PES syringe filter. All measurements were performed with a Malvern Zetasizer Pro Red Label, detection angle of 173°. The Red Label system operates with a 10 mW He-Ne laser (633 nm). The software used is ZS XPLOER version 3.2.1.11. All measurements were done with 4.2 ml polystyrol/polystyrene transparent cuvettes. Samples were measured at 25 °C, with 120 sec of equilibration time. The intensity size distributions, autocorrelation, and Z-average were measured.

Tables

Low Skid silk/modified polypeptide composition	Z-average (d. nm)
AS90	17.617
AS91	16.803
AS92	14.489
AS93	12.887
AS94	11.778
AS95	22.01
AS96	16.403
AS97	23.183
AS98	17.824
AS99	13.607
AS100	11.836

20

Table 10: Z-average of AS90-AS100 calculated by Dynamic Light Scattering. The Z-average value of each silk/modified polypeptide composition was calculated by the Zetasizer Pro. Shown here are the Z-average values of each silk composition. The abbreviation d. nm refers to the diameter in nanometers.

Low Skid silk/modified polypeptide composition	Mw	PDI
AS90	54620	1.1487
AS91	43429	1.2309
AS92	35973	1.3634
AS93	32689	1.3752
AS94	21057	1.3615
AS95	45262	1.1988
AS96	36777	1.1995
AS97	35375	1.3112
AS98	31978	1.3351
AS99	26471	1.4107
AS100	22799	1.5438
Low Skid silk	19500	2.2

Table 11: Molecular weight (Mw) and Polydispersity (PDI) values of silk compositions AS90-AS100. Silk/modified polypeptide compositions AS90-AS100 were analyzed by size exclusion chromatography (SEC) column with HPLC, and values of molecular weights (Mw) and Polydispersity (PDI) are indicated.

Example 8. Mid Skid Silk/Modified Polypeptide Compositions Isolated by Size Properties.

Described herein is a novel method to generate compositions of polypeptide that are derived from *B. mori* silkworm cocoons and comprise of natural and modified polypeptides. This novel composition is called Mid Skid silk/modified polypeptide compositions.

The novel production method involves removing sericin through several washing steps with an organic sodium carbonate salt with tightly controlled multi-stage temperature cycles and agitation as the first step in forming natural/modified polypeptide composition. Next the silk is dried to remove remaining water at controlled temperature to maintain polypeptide composition. The silk is then dissolved in high concentration of Lithium salt at 103°C for 1 hour to achieve the compositions of Mid Skid silk. The liquid solution is then filtered and purified to remove the Lithium salt leaving only the natural/modified silk compositions in solution with pure water.

Mid Skid silk/modified polypeptide compositions comprise of populations of silk/modified polypeptides with distinctive properties. Mid Skid silk/modified polypeptide composition self-assembles at 5 mg/mL.

5 Mid Skid silk/modified polypeptide composition comprises of a variety of populations of silk/modified polypeptides; here it was sought to isolate distinct populations based on size, by fractionating Mid Skid silk/modified polypeptides by size exclusion chromatography. A high-resolution separation of six silk compositions was achieved – AS106, AS107, AS108, AS109, AS110, and AS111. These silk compositions differ from one another by their average size, when AS106 is the largest, and AS111 is the
10 smallest. These silk compositions self-assemble under conditions that promote self-assembly at 5 mg/mL.

The Mid Skid silk/modified polypeptide compositions described in this invention are novel compositions of silk and modified polypeptides composed of a variety of silk polypeptide populations, generated by the exclusive treatment method of natural silk
15 produced by *B. mori*. These silk compositions contain modified amino acid sequences that result from the silk processing method and scale. The tight controls over temperature, silk concentration, buffers and salt concentrations, physical agitation, and purification allow for the precise development of silk compositions with a variety of performance criteria. Isolation of these populations by charge and size reveals new
20 characteristics, like high solubility and stability in solution over time in these populations. the purification method allows us to isolate silk/modified polypeptide compositions that display biological activities and could be used for therapeutic purposes.

Silk is a complex natural biomaterial that has the potential to be utilized in various
25 applications such as the development of implantable medical devices, and the development of soluble polypeptide compositions of medical value. Additionally, it was demonstrated that silk peptides have anti-genotoxic effects. However, silk, in its natural form, is not soluble, and silk polypeptide compositions, without the proper processing, display poor solubility in solution and tend to self-assemble and aggregate over time. The
30 kinetics of this self-assembly is unpredictable, and highly depends on the composition of the silk polypeptides/modified composition. ovel silk/modified polypeptide compositions were produced and specific populations were isolated within these compositions. The isolation process allows for control of the properties of the silk compositions and development of products with predictable and desired characteristics.

Generation of Mid Skid silk/modified polypeptide compositions.

Silk is washed to remove sericin at 100 °C and 60 °C with sodium carbonate and then dried at 60 °C. The silk is then dissolved in 9.3 M Lithium Bromide at 103 °C for 1 hour. This dissolution step controls not only molecular weight but also the polypeptide modifications creating the natural/modified silk compositions. The silk is then filtered to remove undissolved debris and purified using 10 kDa cutoff PES hollow fiber membranes and concentrated using the same process leaving only natural/modified silk composite in solution with pure water. Every unit ops is tightly controlled for temperature, time, concentrations, agitation, and shear.

Isolation of Mid Skid/modified polypeptide compositions***Isolation of the AS106-AS111 silk/modified polypeptide composition component of Mid Skid silk/modified polypeptide composition.***

To isolate AS106-AS111, Mid Skid silk/modified polypeptide compositions were fractionated using HiLoad 26/600 Superdex 200 pg size exclusion chromatography column (Figs. 35, 36). Tris was added to the silk preparations to a final concentration of 50 mM Tris-HCl, pH=8.0. The silk was centrifuged and filtered before loading to the HiLoad 26/600 Superdex 200 pg column, to remove any preformed aggregates. The silk compositions were fractionated by the HiLoad 26/600 Superdex 200 pg, where the largest polypeptide compositions were eluted first, and each following fraction had a population of lower molecular weight of silk compositions (Figs. 37A-37B & Fig. 38). Mid Skid silk preparation solutions have a characteristic yellow hue, and the fractionated silk compositions had a light-yellow hue. When silk formulations AS106-AS111 are analyzed with an analytical SEC column (see materials and methods) with HPLC, each of the silk formulations demonstrates a different average molecular weight, and a different Polydispersity (PDI) value (Figs. 37A-37B, Table 13). In general, AS106 has the highest molecular weight (89297 Da), while AS111 has the lowest molecular weight (35474 Da). Unfractionated Mid Skid silk had the lowest Mw (29265 Da), indicating that the majority of peptide population in Mid Skid silk are of lower molecular weight. The PDI values display a differential change as well. The PDI value of AS106 is relatively low (1.2866), and AS111 is higher (1.4702) (Figs. 37A-37B, Table 13). Unfractionated Mid Skid silk has the highest PDI – 1.6985, indicating a broad and diverse peptide population sizes.

AS106-AS111 compositions contain multiple peptide populations sizes.

In dynamic light scattering analysis (Zetasizer Pro, Figs. 40A-40B, Table 12), AS106-AS111 demonstrated multiple peptide size population by having two broad peaks for each fraction, similar to unfractionated Mid Skid silk (Fig. 40A). There is a shift in the molecular size of each fraction, where AS106 had the largest Z-average value (53.71 d. nm), and AS111 silk composition had the lowest (25.34 d. nm). Despite fractionation by size exclusion chromatography, each fraction contains a range of peptides in different molecular sizes, as can be observed by SDS gel electrophoresis in Fig. 38. Dynamic light scattering shows two peaks for these fractions, indicating the presence of several populations (Fig. 40A).

10 **Self-Assembly of Low and Mid Skid/modified polypeptide compositions**

Self-Assembly assay and data derived from it.

To study the stability of silk/modified peptide compositions in solution, Self-Assembly assays were performed at a concentration of 5 mg/mL.

The absorbance at 550 nm curves of the self-assembly assays are sigmoid and they can be described as logistic curves. The typical logistic function is:

$$f(x) = \frac{A_{max}}{1 + e^{-k(t-t_{0.5})}}$$

A_{max} is the maximum density of the gel formed

k is the Self-Assembly Rate Factor (SARF)

$t_{0.5}$ is the time point at which 50% of the gel has formed

20 e is the exponential equation for the specific curve

(see Fig. 39 the red dotted lines for a better demonstration of how these factors from the Self-Assembly experiments were calculated)

Another parameter introduced to characterize the propensity of silk to form gels is the Self-Assembly Factor (F_{SAF}) which is:

$$25 \quad F_{SAF} = \frac{1}{t_{0.5}(min)} * A_{max}(Abs) * 1000$$

Using the experimental data from the Self-Assembly assays that were performed with the various novel isolated silk polypeptides, these parameters were calculated and used to dissect their properties (Fig. 39). Four parameters were focused on, collectively referred to as Self-Assembly kinetic factors; the Self-Assembly Rate Factor (SARF), A_{max} , $t_{0.5}$, and the Self-Assembly Factor (SAF) (Fig. 39). The SARF shows how fast silk self-assembles to form gel after the reaction begins or the gelation nuclei have formed; A_{max} shows how dense is the gel that is formed after self-assembly is complete,

t0.5 shows how long it takes for the self-assembly reaction to reach the point where gel density is $\frac{A_{max}}{2}$ and SAF shows the propensity of silk to self-assemble (Fig. 39). For all parameter calculations, please see Table 14.

AS106-AS111 silk compositions demonstrate high self-assembly characteristics.

- 5 Self-assembly assays revealed that Mid Skid silk/modified peptide compositions AS106-AS111 self-assemble highly efficiently under the experimental system conditions (Fig. 39). All Mid Skid fractions tested form denser gel in shorter time compared to unfractionated Mid Skid silk. This finding may indicate that components that promote self-assembly are of higher molecular weight, since the unfractionated
- 10 Mid Skid silk contains a large populations of lower molecular weight peptides, but silk compositions AS106-AS111 are fractionated by size and contain higher molecular weight peptide populations (Figs. 37A-37B & 38). Low Skid silk was used as a negative control, no self-assembly occurred after 17 hours.

15 **Materials and Methods used for the generation and characterization of AS106-AS111.**

Size Exclusion Chromatography of Silk.

- The starting material, Mid Skid silk at a concentration of 60 mg/mL, was provided by the manufacturing team. The Mid Skid silk was transferred to 50 mM Tris, pH=8.0
- 20 buffer, and centrifuged at 16000 rpm (rotor JA-18, Beckman coulter, average of 28100 xg), at 4°C, for 30 min to separate formed aggregates from soluble silk. The supernatant was collected and filtered through a 0.22 µm PES filter. Then, the silk was loaded onto a HiLoad 26/600 Superdex 200 pg gel filtration column for fractionation, using the AKTA Pure 25L system. All buffers used during fractionation were filtered through
- 25 0.22 µm PES filter as well and were degassed. The Mid Skid silk was loaded on the Superdex 200 gel filtration column, and was run with 50 mM Tris, 200 mM CaCl₂, pH8, to fractionate the Mid Skid silk. The eluted silk compositions were collected in 10 ml fractions. Fractions 5-10 (AS106, AS107, AS108, AS109, AS110, AS111) were collected. The fractions were placed in 3.5 kDa cutoff dialysis bags, and were
- 30 concentrated by covering the dialysis bags with polyethylene glycol 35000 Da. Then, fractions in the dialysis bags were immersed in 160X volumes of 50 mM Tris pH=8.0 overnight, and then were immersed in a new batch of 160X volume of 50 mM Tris pH=8.0. Samples were kept at 4°C until they were used.

Analytical/Protein Characterization methods.**Protein concentration determination.**

5 Protein concentration was determined by absorbance at 220nm or 280nm. Solubilized silk preparations were diluted until A_{280} was between 0.1-1. In this range the absorbance correlates linearly with the concentration of silk in the solution and the correlation is 1AU=1mg/mL soluble silk proteins. Final concentrations in the initial silk solution were calculated after adjustment for the dilution used for the absorbance measurement.

Analytical Size Exclusion Chromatography.

10 Analytical Size Exclusion Chromatography is performed as described in detail in Example 23. Analysis was performed in a PolySep GFC P-4000 LC Column, 300 mm x 7.8 mm connected to an Agilent 1260 Infinity II HPLC system with an Agilent G7162A RID Refractive Index Detector. The mobile phase used for the analysis was a solution of 0.1M NaCl, 12.5mM Na_2HPO_4 , pH 7 (the pH was adjusted with phosphoric acid and filtered through a 0.2 μm PES filter into a clean glass media bottle). 25 μL of sample were loaded on the column and the analysis was performed at 25°C with a flow rate of 1 mL/min for 20 min. Calculation of the molecular weight of each sample was done using Agilent Technologies Open LAB CDS ChemStation Edition for LC & LC/MS Systems software Cirrus SEC data collection and molecular weight analysis software.
20

SDS polyacrylamide gel.

Mid Skid silk fractions were uploaded onto a Mini-Protean TGX precast gel, 4-20%, with a protein marker Trident Prestained Protein Ladder for molecular weight reference. The SDS polyacrylamide gel was stained using ReadyBlue™ Protein stain gel. Gels were immersed in ReadyBlue™ solution for 1 h, then destained with DI/RO water.
25

Self-Assembly Assay.

The silk Self Assembly Assay (SAF) was performed in 35% v/v 2-propanol and 50mM CH₃COONa pH=5. Each reaction was done in a final volume of 200. μ L. Total silk protein concentration was 5 mg/mL. First the buffer of 50 mM CH₃COONa pH=5, 35% v/v 2-propanol was prepared. Then DI/RO water was added so that after the addition of the volume of silk protein required to reach a final concentration of 5 mg/mL the total volume would be 200 μ L. The protein was added last and mixed with very gentle pipetting to reduce shearing force. The protein mixtures were placed in wells of flat-bottom 96-well plates and a layer of 100 μ L of Mineral Oil carefully so as to not create any bubbles. Absorbance was recorded at 550 nm for 17h. Recorded values were exported in Excel files for storage and further analysis.

Dynamic Light Scattering analysis of silk compositions.

Mid Skid silk compositions were diluted to a concentration of 1 mg/mL and filtered with a 0.22 μ m PES syringe filter. All measurements were performed with a Malvern Zetasizer Pro Red Label, detection angle of 173°. The Red Label system operates with a 10 mW He-Ne laser (633 nm). The software used is ZS XPLOER version 3.2.1.11. All measurements were done with 4.2 mL polystyrol/polystyrene transparent cuvettes. samples were measured at 25°C, with 120 sec of equilibration time. The intensity size distributions, autocorrelation, and Z-average were measured.

Tables

Mid Skid silk/modified polypeptide composition	Z-average (d. nm)
AS106	53.71
AS107	39.93
AS108	32.63
AS109	34.87
AS110	26.91
AS111	25.34
Mid Skid silk	32.29

Table 12: Z-average of AS106-AS111 calculated by Dynamic Light Scattering.

The Z-average value of each silk/modified polypeptide composition was calculated by the Zetasizer Pro. Shown here are the Z-average values of each silk composition. MS, Mid Skid silk.

Mid Skid silk/modified polypeptide composition	Mw	PDI
AS106	89297	1.2866
AS107	68997	1.2915
AS108	53964	1.2662
AS109	43762	1.334
AS110	38650	1.3732
AS111	35474	1.4702
Mid Skid silk	29265	1.6985

5 **Table 13: Molecular weight (Mw) and Polydispersity (PDI) values of silk compositions AS106-AS111.** Silk/modified polypeptide compositions AS106, AS107, AS108, AS109, AS110, and AS111 were analyzed by size exclusion chromatography (SEC) column with HPLC, and values of molecular weights (Mw) and Polydispersity (PDI) are indicated.

	Amax (Abs)	T _{0.5} (h)	SARF	F _{SAF} (Abs/min)
AS106	0.7226	1.638	7.599	7.35
AS107	0.8258	1.778	8.270	7.74
AS108	0.7803	2.191	9.959	5.94
AS109	0.7678	2.486	10.66	5.15
AS110	0.8159	2.661	10.38	5.11
AS111	0.7844	3.054	10.47	4.28
MS	0.6710	3.843	7.843	2.91

10 **Table 14: Calculated self-assembly parameters of silk compositions AS106-AS111.** For detailed description of how these parameters are calculated, please see Description of Invention section, Self-Assembly. MS, Mid Skid silk.

Example 9: Mid Skid Silk/Modified Polypeptide Compositions Isolated by Charge and Size Properties.

15 Described herein is a novel method to generate compositions of polypeptide that are derived from B. mori silkworm cocoons and comprise of natural and modified polypeptides. This novel composition is called Mid Skid silk/modified polypeptide compositions.

20 The novel production method involves removing sericin through several washing steps with an organic sodium carbonate salt with tightly controlled multi-stage temperature cycles and agitation as the first step in forming natural/modified

polypeptide composition. Next the silk is dried to remove remaining water at controlled temperature to maintain polypeptide composition. The silk is then dissolved in high concentration of Lithium salt at 103 °C for 1 hour to achieve the compositions of Mid Skid silk. The liquid solution is then filtered and purified to
5 remove the Lithium salt leaving only the natural/modified silk compositions in solution with pure water.

Mid Skid silk/modified polypeptide compositions comprise of populations of silk/modified polypeptides with distinctive properties. Mid Skid silk/modified polypeptide composition self-assembles at 5 mg/mL. Mid Skid silk/modified
10 polypeptide composition comprises of a variety of populations of silk/modified polypeptides; here it was sought to isolate distinct populations based on charge and size, by fractionating Mid Skid silk/modified polypeptides by anion exchange chromatography and size exclusion chromatography. A high-resolution separation of five negatively-charged silk compositions was achieved – AS101, AS102, AS103,
15 AS104, and AS105. These silk compositions differ from one another by their average size, when AS101 is the largest, and AS105 is the smallest. These silk compositions self-assemble under conditions that promote self-assembly at 5 mg/mL.

The Mid Skid silk/modified polypeptide compositions described in this invention are novel compositions of silk and modified polypeptides composed of a variety of silk
20 polypeptide populations, generated by the exclusive treatment method of natural silk produced by *B. mori*. These silk compositions contain modified amino acid sequences that result from the silk processing method and scale. The tight controls over temperature, silk concentration, buffers and salt concentrations, physical agitation, and purification allow us to precisely develop silk compositions with a variety of
25 performance criteria. Isolation of these populations by charge and size reveals new characteristics, like high solubility and stability in solution over time in these populations. the purification method allows us to isolate silk/modified polypeptide compositions that display biological activities and could be used for therapeutic purposes.

30 Silk is a complex natural biomaterial that has the potential to be utilized in various applications such as the development of implantable medical devices, and the development of soluble polypeptide compositions of medical value. Additionally, it was demonstrated that silk peptides have anti-genotoxic effects. However, silk, in its natural form, is not soluble, and silk polypeptide compositions, without the proper

processing, display poor solubility in solution and tend to self-assemble and aggregate over time. The kinetics of this self-assembly is unpredictable, and highly depends on the composition of the silk polypeptides/modified composition. Novel silk/modified polypeptide compositions were produced and specific populations were isolated within these compositions. The isolation process allows for control of the properties of the silk compositions and development of products with predictable and desired characteristics.

Generation of Mid Skid silk/modified polypeptide compositions.

Silk is washed to remove sericin at 100 °C and 60 °C with sodium carbonate and then dried at 60 °C. The silk is then dissolved in 9.3 M Lithium Bromide at 103 °C for 1 hour. This dissolution step controls not only molecular weight but also the polypeptide modifications creating the natural/modified silk compositions. The silk is then filtered to remove undissolved debris and purified using 10 kDa cutoff PES hollow fiber membranes and concentrated using the same process leaving only natural/modified silk composite in solution with pure water. Every unit ops is tightly controlled for temperature, time, concentrations, agitation, and shear.

Isolation of Mid Skid/modified polypeptide compositions

Isolation of the AS101-AS105 silk/modified polypeptide composition component of Mid Skid silk/modified polypeptide composition.

To isolate AS101-AS105, Mid Skid silk/modified polypeptide compositions were fractionated using anion exchange chromatography (Q-Sepharose chromatography), following HiLoad 26/600 Superdex 200 pg size exclusion chromatography of the Q-eluate (Figs. 41, 42A, and 42B). Prior to chromatography, Tris was added to the silk preparations to a final concentration of 50 mM Tris-HCl, pH=8.0. The silk was centrifuged and filtered before loading to the Q-Sepharose column, to remove any preformed aggregates. The silk compositions were loaded onto the Q-Sepharose column, and the flowthrough fraction was collected. The negatively charged silk compositions were eluted using high salt buffer (50 mM Tris, 500 mM CaCl₂). The eluted fractions were pulled together and are referred to as the Q-elution fraction. The Q-elution was further fractionated by the HiLoad 26/600 Superdex 200 pg, where the largest polypeptide compositions were eluted first, and each following fraction had a population of lower molecular weight of silk compositions (Figs. 43A-43B & AJ). Mid Skid silk preparation solutions have a characteristic yellow hue. The Q-elution fraction has a strong yellow hue, while the flowthrough fraction is transparent, and

tends to self-assemble very quickly. The Q-elution silk compositions that are fractionated by size exclusion also had a yellow hue. When silk formulations AS101-AS105 are analyzed with an analytical SEC column (see materials and methods) with HPLC, each of the silk formulations demonstrates a different average Mw, and a different Polydispersity (PDI) value (Figs. 43A-43B, Table 16). In general, AS101 has the highest Mw (60949 Da), while AS105 has the lowest Mw (32804 Da). The PDI values display a differential change as well. The PDI value of AS101 is relatively low (1.1347), and AS105 is higher (1.3937) (Figs. 43A-43B, Table 16).

Unfractionated Mid Skid silk has an average Mw of 29265, indicating that most of the peptide population tends to have lower molecular weight than fractions AS101-AS105. The polydispersity of unfractionated Mid Skid silk is 1.6985 – significantly higher than the values of fractions AS101-AS105. This indicated that the unfractionated Mid Skid silk is composed of a much diverse peptide population compared to fractions AS101-AS105, where the majority of the peptide populations have lower molecular weight.

AS101-AS105 silk compositions demonstrate that majority of the peptide populations are relatively uniform by dynamic light scattering and show gradual particle size distribution.

In dynamic light scattering analysis (Zetasizer Pro, Figs. 45A-45C, Table 15), AS101-AS105 showed two peaks for each fraction, where the intensity is higher for the smaller size distribution compared to the larger size distribution peak. Comparing to unfractionated Mid Skid (Fig. 45B), which has two populations that are very close in intensity, it is evident that the Q-SEC fractionation enriched the populations that are of smaller hydrodynamic radius, and the separation between different fractions is efficient, as can be seen by the gradual decrease in size from fraction to fraction (Figs. 45A-45B, Table 15). AS101 has the largest Z-average (21.905 d. nm), then AS102 (18.735 d. nm), and so on (Table 15), demonstrating the efficiency of the fractionation by size of the Q-elution fraction.

Self-Assembly of Low and Mid Skid/modified polypeptide compositions

Self-assembly assay and data derived from it.

To study the stability of silk/modified peptide compositions in solution, Self-Assembly assays were performed at a concentration of 5 mg/mL.

The absorbance at 550nm curves of the self-assembly assays are sigmoid and they can be described as logistic curves. The typical logistic function is:

$$f(x) = \frac{A_{max}}{1 + e^{-k(t-t_{0.5})}}$$

A_{max} is the maximum density of the gel formed

k is the Self-Assembly Rate Factor (SARF)

$t_{0.5}$ is the time point at which 50% of the gel has formed

5 e is the exponential equation for the specific curve

(see Fig. 44A the red dotted lines for a better demonstration of how these factors from the Self-Assembly experiments were calculated)

Another parameter introduced to characterize the propensity of silk to form gels is the Self-Assembly Factor (FSAF) which is:

$$10 \quad F_{SAF} = \frac{1}{t_{0.5}(min)} * A_{max}(Abs) * 1000$$

Using the experimental data from the Self-Assembly assays that were performed with the various novel isolated silk polypeptides, these parameters were calculated and used to dissect their properties (Fig. 44A). Four parameters were focused on, collectively referred to as Self-Assembly kinetic factors; the Self-Assembly Rate

15 Factor (SARF), A_{max} , $t_{0.5}$, and the Self-Assembly Factor (SAF) (Fig. 44A). The SARF shows how fast silk self-assembles to form gel after the reaction begins or the gelation nuclei have formed; A_{max} shows how dense is the gel that is formed after self-assembly is complete, $t_{0.5}$ shows how long it takes for the self-assembly reaction to reach the point where gel density is $A_{max}/2$ and SAF shows the propensity of

20 silk to self-assemble (Fig. 44A).

AS101-AS105 self-assemble in different kinetics to form a gel, while the Q-elution fraction self-assembles poorly.

Silk compositions AS101-AS105 were tested for their ability to self-assemble and form a gel. The starting material for generating silk compositions AS101-AS105 was

25 the Q-elution fraction (Fig. 41), which did not demonstrate significant self-assembly (Fig. 44A). However, silk compositions AS101-AS105, derived from the Q-elution fraction, self-assembled to form a gel in different kinetics (Fig. 44A, Table 17). This indicates that separating the higher-molecular-weight peptide populations from the lower-molecular-weight peptide population in Q-elution fraction allows them to self-

30 assemble to form a gel. AS101 reached the lowest A_{max} , meaning the formed gel was the least dense. AS105 silk composition had the highest $t_{0.5}$ value: the formation of the gel took the longest, and the gelation nuclei took longer to form.

The collected Q-flowthrough fraction self-assembles exceptionally fast and forms the densest gel of all fractions.

While fractionating silk by anion exchange, the flowthrough fraction (Q-FT) was collected (Fig. 42A) and demonstrated spontaneous slow self-assembly in 4°C. To
5 compare the self-assembly capabilities of Q-FT to Mid Skid silk and the further fractionated silk compositions, the Q-FT in the self-assembly assay was tested. The Q-FT fraction showed extraordinary capability of self-assembly under the assay's conditions and started to self-assemble in less than 1 h (Fig. 44A, Table 17). The density of the formed gel was the highest of all silk compositions tested. These results
10 show that the uncharged/positively charged silk compositions/modified peptides that were separated from the negatively charged population by anion exchange have a much higher tendency to self-assemble.

Materials and Methods used for the generation and characterization of AS101-AS105.

15 Anion exchange chromatography of silk.

Mid Skid silk was provided by the manufacturing team at a concentration of 60 mg/mL. 50 mM Tris, pH=8.0 buffer was added to the Mid Skid silk, and the silk was centrifuged at 16000 rpm (rotor JA-18, Beckman coulter, average of 28100 xg), at 4°C, for 30 min to separate formed aggregates from soluble silk. The supernatant was
20 collected and filtered through a 0.22 µm PES filter. For silk fractionation Q-Sepharose prepacked columns connected to an AKTA pure 25L or HiPrep Q FF 16/10 20 mL Column were used. All buffers used were filtered through a 0.22 µm PES filter and degassed with sonication. Centrifuged and filtered Mid Skid silk was loaded on HiPrep Q FF 16/10 20 mL column, washed with 10 column volumes of 50 mM Tris
25 pH=8.0, 10 column volumes of 50 mM Tris pH=8.0, 500 mM CaCl₂ and finally 10 column volumes of 50 mM Tris pH=8.0. 150 mL of centrifuged Mid Skid silk were loaded on the column with a flow rate of 5 mL/min. The flow-through was collected. The column was washed with 50 mM Tris pH=8.0 until the absorbance at 280nm [A₂₈₀] got to 100 AU. Bound protein was eluted in one step with 50 mM Tris
30 pH=8.0, 500 mM CaCl₂ and all fractions with absorbance [A₂₈₀] >500AU were pooled together. The Q-elution fraction (the eluate) was then used for further fractionation by size exclusion chromatography.

Size Exclusion Chromatography of Silk.

The Mid Skid silk eluate fraction of the Q-Sepharose anion exchange chromatography (Q-elution) was the starting material for size exclusion chromatography. The eluate was loaded onto a HiLoad 26/600 Superdex 200 pg gel filtration column for fractionation, using the AKTA Pure 25L system. All buffers used during fractionation were filtered through 0.22 µm PES filter as well and were degassed. The Mid Skid silk was loaded on the Superdex 200 gel filtration column, and was run with 50 mM Tris, 200 mM CaCl₂, pH=8.0, to fractionate the Q-elution Mid Skid silk. The eluted silk compositions were collected in 10 ml fractions. Fractions 6-10 (AS101, AS102, AS103, AS104, AS105) were collected, and have relatively narrow range of molecular weight. The fractions were placed in 3.5 kDa cutoff dialysis bags, and were concentrated by covering the dialysis bags with polyethylene glycol 35000 Da. Then, fractions in the dialysis bags were immersed in 160X volumes of 50 mM Tris pH=8.0 overnight, and then were immersed in a new batch of 160X volume of 50 mM Tris pH=8.0. Samples were kept at 4°C until they were used.

Analytical/Protein Characterization methods.

Protein Concentration Determination.

Protein concentration was determined by absorbance at 220nm or 280nm. Solubilized silk preparations were diluted until A₂₈₀ was between 0.1-1. In this range the absorbance correlates linearly with the concentration of silk in the solution and the correlation is 1AU=1mg/mL soluble silk proteins. Final concentrations in the initial silk solution were calculated after adjustment for the dilution used for the absorbance measurement.

Analytical Size Exclusion Chromatography.

Analytical Size Exclusion Chromatography is performed as described in detail in Example 23. Analysis was performed in a PolySep GFC P-4000 LC Column, 300 mm x 7.8 mm connected to an Agilent 1260 Infinity II HPLC system with an Agilent G7162A RID Refractive Index Detector. The mobile phase used for the analysis was a solution of 0.1M NaCl, 12.5mM Na₂HPO₄, pH 7 (the pH was adjusted with phosphoric acid and filtered through a 0.2µm PES filter into a clean glass media bottle). 25µL of sample were loaded on the column and the analysis was performed at 25oC with a flow rate of 1 mL/min for 20 min. Calculation of the molecular weight of each sample was done using Agilent Technologies Open LAB CDS ChemStation Edition for LC & LC/MS Systems software Cirrus SEC data collection and molecular weight analysis software.

SDS polyacrylamide gel.

Mid Skid silk fractions were loaded onto a Mini-Protean TGX precast gel, 4-20% (Bio-Rad, CAT# 4561095, Batch# 64518276), with a protein marker Trident Prestained Protein Ladder for molecular weight reference. The SDS polyacrylamide gel was stained using ReadyBlue™ Protein stain gel. Gels were immersed in ReadyBlue™ solution for 1 h, then destained with DI/RO water.

Self-Assembly Assay.

The silk Self Assembly Assay (SAF) was performed in 35% v/v 2-propanol and 50mM CH₃COONa pH=5. Each reaction was done in a final volume of 200 μ L. Total silk protein concentration was 5 mg/mL. First the buffer of 50 mM CH₃COONa pH=5.0, 35% v/v 2-propanol was prepared. Then DI/RO water was added so that after the addition of the volume of silk protein required to reach a final concentration of 5 mg/mL the total volume would be 200 μ L. The protein was added last and mixed with very gentle pipetting to reduce shearing force. The protein mixtures were placed in wells of flat-bottom 96-well plates and a layer of 100 μ L of Mineral Oil carefully, so as to not create any bubbles. Absorbance was recorded at 550 nm for 24h. Recorded values were exported in Excel files for storage and further analysis.

Dynamic Light Scattering analysis of silk compositions.

Mid Skid silk compositions were diluted to a concentration of 1 mg/mL and filtered with a 0.22 μ m PES syringe filter. All measurements were performed with a Malvern Zetasizer Pro Red Label, detection angle of 173°. The Red Label system operates with a 10 mW He-Ne laser (633 nm). The software used is ZS XPLOER version 3.2.1.11. All measurements were done with 4.2 ml polystyrol/polystyrene transparent cuvettes. samples were measured at 25°C, with 120 sec of equilibration time. The intensity size distributions, autocorrelation, and Z-average were measured.

Tables

Mid Skid silk/modified polypeptide composition	Z-average (d. nm)
AS101	21.905
AS102	18.735
AS103	14.27
AS104	13.07
AS105	12.19

Mid Skid silk	30.302
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Table 15: Z-average of AS101-AS105 calculated by Dynamic Light Scattering.

The Z-average value of each silk/modified polypeptide composition was calculated by the Zetasizer Pro. Shown here are the Z-average values of each silk composition. The abbreviation d. nm refers to the diameter in nanometers.

Mid Skid silk/modified polypeptide composition	Mw	PDI
AS101	60949	1.1347
AS102	47772	1.1976
AS103	40743	1.3085
AS104	36269	1.3211
AS105	32804	1.3937
Mid Skid silk	29265	1.6985

Table 16: Molecular weight (Mw) and Polydispersity (PDI) values of silk compositions AS101-AS105. Silk/modified polypeptide compositions AS101,

AS102, AS103, AS104, AS105, and unfractionated Mid Skid silk were analyzed by size exclusion chromatography (SEC) column with HPLC, and values of molecular weights (Mw) and Polydispersity (PDI) are indicated.

	Amax (Abs)	T _{0.5} (h)	SARF	F _{SAF} (Abs/min)
AS101	0.5807	4.120	7.672	2.35
AS102	0.802	4.932	9.398	2.71
AS103	0.8116	5.416	7.734	2.50
AS104	0.8552	6.866	7.450	2.08
AS105	0.9132	10.27	7.132	1.48
Q-FT	1.319	0.9733	7.783	22.59
Mid Skid silk	0.7159	3.533	6.983	3.38

Table 17: Calculated self-assembly parameters of silk compositions/modified peptides. For detailed description of how these factors are calculated, please see Description of Invention section, Self-Assembly. Q-FT, flowthrough fraction collected during anion exchange chromatography using a Q column.

Example 10. Absolute Weight Average Molecular Weight and Polydispersity of Low, Mid, and High Molecular Weight Silk by SEC-MALS.

Summary of Methodologies to Measure Molecular Weight

This example discusses the measurement of molar mass moments, specifically Mw, by SEC-MALS. Molar mass can be used interchangeably with the term “molecular weight”. For the sake of clarity, molar mass will be used in this example.

Measuring Molar Mass Moments by SEC-RI

The methodology previously used for molar mass determination was SEC-RI. The following section is intended to describe the differences of the two methods and how both can exist in future fillings. **As both methods produce a reported value of weight-averaged molecular weight (Mw), it is recommended that the Mw values be redefined as “absolute Mw” for SEC-MALS and “relative Mw” for SEC-RI.**

Summary

SEC is a mode of chromatographic separation and, on its own, cannot provide an absolute molar mass. Instead, SEC can provide a relative molar mass of a protein (or polymer) against a calibration curve of standard; this conventional calibration method provides a relative molar mass. SEC is often paired with a RI detector which is concentration-sensitive and molar-mass-insensitive.

Additional Information: With SEC, molecules are separated by hydrodynamic size (larger molecules having a shorter retention time than smaller molecules) which is correlated to molar mass. Herein, the retention time of the protein of interest is related to the calibration curve comprised of proteins or polymers of known molar mass. The caveat is that the unique structure or shape of a protein will impact the retention time in SEC; this may lead to variance in reported molecular weights from the use of different detectors as a specific protein of interest may differ somewhat or significantly from the calibration curve and molecules used to generate said curve. However, this method is commonly used as an industry-standard in addition to LS detectors as SEC-MALS has not been universally adopted as the singular molecular weight determination method.

Measuring Molar Mass Moments by SEC-MALS

Here molecular weight and polydispersity as measured by Size Exclusion Chromatography-Multi-Angle Light Scattering (SEC-MALS) are reported for fractionated silk. SEC-MALS directly produces a weight averaged molar mass (Mw) measurement as compared to the relative molar mass measurement produced by a conventional calibration method such as SEC-RI.

Two detectors were used in the calculation of molar mass moments by SEC-MALS in this document: a refractive index (RI) detector and light scattering (LS) detector. RI detector directly provides the concentration of the protein. LS detector directly provides the weight-average molar mass (Mw) of the protein; the intensity of scattered light is directly proportionate to the Mw of the molecule.

Calculation of Molar Mass Moments

The calculation of molar mass moments uses the following variables: n_i , M_i , and c_i . M_i and c_i are directly measured by SEC-MALS. Note that the subscript “i” indicates that the value is calculated at each slice of peak of interest.

- n_i is the number of molecules
- M_i is the molecular mass of the molecules (measured by LS detector)
- c_i is the concentration of material as measured by the concentration detector (measured by RI detector)

Number-average molar mass, M_n , is related to an arithmetic mean where the total mass is divided by the number of molecules.

$$\bar{M}_n = \frac{\sum n_i M_i}{\sum n_i} = \frac{\sum c_i}{\sum c_i / M_i}$$

For the weight-average molecular mass, M_w :

$$\bar{M}_w = \frac{\sum n_i M_i^2}{\sum n_i M_i} = \frac{\sum c_i M_i}{\sum c_i}$$

Calculation of Polydispersity Index (PDI)

The ratio of M_w and M_n produces the PDI value as calculated by ASTRA software. A polydisperse macromolecule has a $PDI > 1.05$ and a monodisperse macromolecular has a $PDI < 1.05$ (Figure 46).

Analytical Methods

- 5 Analysis was performed with a PolySep GFC P-4000 LC Column, 300 mm x 7.8 mm with guard column connected to an Agilent 1260 Infinity II HPLC system with Wyatt LS and RI detectors (Table 18).

Table 18. HPLC Instrument Configuration

Instrument Component	Vendor	Part Number
Sample Manager	Agilent	G5668A
Sample Thermostat	Agilent	G7167-60101
Quaternary Pump	Agilent	G5654A
Column Compartment	Agilent	G7116A
UV Detector	Agilent	G1315C
LS Detector	Wyatt	miniDAWN TREOS with online QELS
RI Detector	Wyatt	Optilab TrEX

- The mobile phase used for the analysis was a solution of 12.5 mM Na_2HPO_4 , 100 mM
- 10 NaCl titrated to a final pH of 7.0 ± 0.2 with H_3PO_4 . Before installing mobile phase on the HPLC, the solvent was filtered through a 0.22 μm PES filter into a clean glass bottle. A summary of critical method parameters is described in Table 19.

Table 19. SEC-MALS Critical Method Parameters

Critical Attribute	Value
Target Load Mass ¹ (injection volume)	50 μg (50 μL)
Flow Rate	0.75 mL/min
Column Temperature	25 °C
UV Detection Wavelength	280 nm
Run Time	24 min

Astra Collection Time	23 min
¹ Injection volume is varied to accommodate a 50 µg	

Data Analysis

All silk samples were processed in software ASTRA 7.3.2 following vendor recommended procedures. Reportable values of Mw, Mn, and PDI were derived from software ASTRA 7.3.2. Additional data analysis and production of figures were performed in GraphPad Prism 9 version 9.5.1 and Adobe Illustrator version 27.5.

Tables

Table 20. Summary of Mw and PDI for Low, Mid, and High Molecular Weight Silk Types produced by different processes (BenchTop known in the art and new Skid process, with different conditions)

Production Scale	Target Silk Conc. (%)	Silk Type	Lot Number	Mw (kDa)	PDI
Benchtop	6%	Low	EMED-TFF01-001-0015	38.7	2.051
Benchtop	6%	Mid	TFF-001-0601-M	51.8	1.445
Benchtop (60 min dissolution)	6%	High	TFF-001-0603-IP	67.6	1.566
Benchtop (15 min dissolution)	6%	High	TFF-001-0602-IP	95.1	1.390
Skid	16.7%	Low	Skid-23164-L	36.6	1.462
Skid	16.7%	Low	TFF-002-23212-L	38.7	1.473
Skid	10%	Mid	TFF-002-23177-M	85.5	1.799
Skid	10%	Mid	TFF-002-23226-M	83.3	1.668
Skid	6%	Low	TFF-003-23194-L	35.9	1.416
Skid	6%	Mid	TFF-003-23201-M	71.4	1.527
Lyo Pellet	6%	Low	LYO-004-003L	41.2	1.575
Lyo Pellet	16.7%	Low	0075-23177-001	39.5	1.476

Lyo Pellet	6%	Mid	LYO-004-001M	64.7	1.661
Lyo Pellet	10%	Mid	LYO-004-004M	81.9	1.697
Lyo Pellet	10%	Mid	0092-23193-001	77.8	1.702

Example 11: Weight Average Molecular Weight and Polydispersity of Low Skid silk/modified polypeptide compositions and Mid Skid silk/modified polypeptide compositions by Size Exclusion Chromatography-Multi-Angle Light Scattering (SEC-MALS)

Development of Mid Skid silk/modified polypeptide compositions

The development of modified polypeptide compositions of Low and Mid Skid Silk have been previously discussed as the generation of Low and Mid Skid Silk and the subsequent isolation of their modified polypeptide compositions.

Summary of Methodologies to Measure Molecular Weight

This document discusses the measurement of molar mass moments, specifically Mw, by SEC-MALS. Molar mass can be used interchangeably with the term “molecular weight”. For the sake of clarity, molar mass will be used in this document.

Measuring Molar Mass Moments by SEC-RI

Summary: SEC is a mode of chromatographic separation and, on its own, cannot provide an absolute molar mass. Instead, SEC can provide a relative molar mass of a protein (or polymer) against a calibration curve of standard; this conventional calibration method provides a relative molar mass. SEC is often paired with a refractive index (RI) detector which is concentration-sensitive.

Additional Information: With SEC, molecules are separated by hydrodynamic size (larger molecules having a shorter retention time than smaller molecules) which is correlated to molar mass. Herein, the retention time of the protein of interest is related to the calibration curve comprised of proteins or polymers of known molar mass. The caveat is that the unique structure or shape of a protein will impact the retention time

in SEC; this may lead to variance in reported molecular weights from the use of different detectors as a specific protein of interest may differ somewhat or significantly from the calibration curve and molecules used to generate said curve. However, this method is commonly used as an industry-standard in addition to LS detectors as SEC-

5 MALS has not been universally adopted as the singular molecular weight determination method.

Measuring Molar Mass Moments by SEC-MALS

This document reports molecular weight and polydispersity as measured by SEC-MALS for fractionated silk. SEC-MALS produces a direct weight averaged molar mass

10 (Mw) measurement as compared to the relative molar mass measurement produced by a conventional calibration method used by SEC-RI.

Two detectors were used in the calculation of molar mass moments by SEC-MALS in this document: a refractive index (RI) detector and light scattering (LS) detector. RI detector directly provides the concentration of the protein. LS detector directly provides

15 the weight-average molar mass (Mw) of the protein; the intensity of scattered light is directly proportionate to the Mw of the molecule.

Calculation of Molar Mass Moments

The calculation of molar mass moments uses the following variables: n_i , M_i , and c_i . M_i and c_i are directly measured by SEC-MALS. Note that the subscript “i” indicates that

20 the value is calculated at each slice of peak of interest.

- n_i is the number of molecules
- M_i is the molecular mass of the molecules (measured by LS detector)
- c_i is the concentration of material as measured by the concentration detector (measured by RI detector)

25 Number-average molar mass, M_n , is related to an arithmetic mean where the total mass is divided by the number of molecules.

$$\bar{M}_n = \frac{\sum n_i M_i}{\sum n_i} = \frac{\sum c_i}{\sum c_i / M_i}$$

For the weight-average molecular mass, M_w :

$$\bar{M}_w = \frac{\sum n_i M_i^2}{\sum n_i} = \frac{\sum c_i M_i}{\sum c_i}$$

Calculation of Polydispersity Index (PDI)

The PDI is the ratio of Mw and Mn as calculated by the program ASTRA. A macromolecule is considered to be polydisperse if the PDI > 1.05.

$$PDI = \frac{M_w}{M_n}$$

Results

Low Skid silk/modified polypeptide compositions isolated by charge and size, AS77-AS81

- To isolate AS77-AS81 Low Skid silk/modified polypeptide compositions were fractionated using anion exchange chromatography (Q-Sepharose chromatography), following HiLoad 26/600 Superdex 200 pg size exclusion chromatography of the Q-eluate. Prior to chromatography, Tris was added to the silk preparations to a final concentration of 50 mM Tris-HCl, pH 8.0. The silk was centrifuged and filtered before loading to the Q-Sepharose column, to remove any pre-formed aggregates.
- The silk compositions were loaded onto the Q-Sepharose column, and the flowthrough fraction was collected. The negatively charged silk compositions were eluted using high salt buffer (50 mM Tris, 500 mM CaCl₂). The eluted fractions were pulled together and are referred to as the Q-elution fraction. The Q-elution was further fractionated by the HiLoad 26/600 Superdex 200 pg, where the largest polypeptide compositions were eluted first, and each following fraction had a population of lower molecular weight of silk compositions. Low Skid silk preparation solutions have a characteristic yellow hue. The Q-elution fraction has a strong yellow hue, while the flowthrough fraction is transparent, and tends to self-assemble very quickly. The Q-elution silk compositions that are fractionated by size exclusion also had a yellow hue. When silk formulations AS77-AS81 are analyzed with an analytical SEC-MALS (see materials and methods) with HPLC, the weight average molecular weight range of the 118.2 to 61.1 kDa with AS77 having the highest Mw and AS81 having the lowest Mw (Figure 46 and Table 24). There was not a strong trend relating the PDI and fraction number but all are

polydisperse (PDI > 1.05). The PDI of unfractionated LS skid was significantly higher than the PDI of AS77-81 indicating the unfractionated silk has a more diverse peptide population.

Low Skid silk/modified polypeptide compositions isolated by size: AS82-AS89

- 5 To isolate AS82-AS89 Low Skid silk/modified polypeptide compositions were fractionated using HiLoad 26/600 Superdex 200 pg size exclusion chromatography column. Tris was added to the silk preparations to a final concentration of 50 mM Tris–HCl, pH 8.0. The silk was centrifuged and filtered before loading to the HiLoad 26/600 Superdex 200 pg column, to remove any pre-formed aggregates.
- 10 The silk compositions were fractionated by the HiLoad 26/600 Superdex 200 pg, where the largest polypeptide compositions were eluted first, and each following fraction had a population of lower molecular weight of silk compositions. When silk formulations AS82-AS89 are analyzed with an analytical SEC-MALS (see materials and methods) with HPLC, each of the silk formulations demonstrates a different average Mw and PDI
- 15 values. Distinct populations were isolated by size, by fractionating Low Skid silk/modified polypeptides by preparatory scale size exclusion chromatography by FPLC; these fractions are AS82-AS89. A high-resolution separation of five silk compositions was achieved– AS82, AS83, AS84, AS85, and AS86. These silk compositions differ from one another by their average size; AS82 is the largest (100.9
- 20 kDa) and AS86 is the smallest of this set (51.5 kDa) (Figs. 49A- 49B and Table 25). Three additional fractions, AS87-AS89, were less resolved as the resolution of the Superdex 200 is not optimal for separating proteins smaller than 44 kDa. Fractions AS87, AS88, and AS89 were significantly smaller than AS82-AS86 with Mw of 18.2, 18.5 and 11.1 kDa, respectively. This higher polydispersity of AS87-AS89 is indicative
- 25 of the reduced resolution and in agreement with the size measured by DLS.

Low Skid silk/modified polypeptide compositions isolated by charge, hydrophobicity, and size: AS90-AS94 and AS95-AS100

- To isolate AS90-AS100 Low Skid silk/modified polypeptide compositions were fractionated using anion exchange chromatography (Q-Sepharose chromatography),
- 30 following hydrophobic interactions (HIC) chromatography, followed by HiLoad

26/600 Superdex 200 pg size exclusion chromatography of the Q-HIC-eluate (AS90-94) and of the Q-HIC-flowthrough (AS95-100).

Prior to chromatography, Tris was added to the silk preparations to a final concentration of 50 mM Tris-HCl, pH 8.0. The silk was centrifuged and filtered before loading to the Q-Sepharose column, to remove any preformed aggregates. The silk compositions were loaded onto the Q-Sepharose column, and the flowthrough fraction was collected. The negatively charged silk compositions were eluted using high salt buffer (50 mM Tris, 500 mM CaCl₂). The eluted fractions were pulled together and are referred to as the Q-elution fraction. The Q-flowthrough fraction is colorless and tends to aggregate.

The Q-elution was further fractionated by using a Butyl ImpRes column, which separates polypeptides based on hydrophobicity. The chromatography was performed in the presence of 300 mM ammonium sulfate, to expose hydrophobic regions within the silk polypeptides. The highly charged flowthrough fraction (Q-HIC-flowthrough) was collected for further fractionation by size exclusion chromatography. The more hydrophobic, bound silk peptides were eluted using 50 mM Tris, pH 8.0 in the absence of ammonium sulfate, to reverse the exposure of the hydrophobic regions in silk polypeptides, which results in their release from the Butyl ImpRes column.

The Q-HIC-elution was further fractionated by the HiLoad 26/600 Superdex 200 pg, where the largest polypeptide compositions were eluted first, and each following fraction had a population of lower molecular weight of silk compositions (Table 25 and Figs. 51A- 51B). The fractions that were isolated are AS90-AS94. Then, the Q-HIC-flowthrough fraction was fractionated as well by the HiLoad 26/600 Superdex 200 pg, resulting in the generation of AS95-AS100.

The Q-HIC Elution fraction is composed of higher-molecular-weight peptide composition, while the silk peptides that compose the Q-HIC Flowthrough fraction are eluted later, indicating smaller molecular weights (Table 26, Figs. 51A- 51B and 52A- 52B). Unfractionated Low Skid silk has an average Mw of 41.2 kDa, indicating that most of the peptide population tends to have lower molecular weight than fractions AS90-AS94 (Table 26). Additionally, the PDI of unfractionated silk is significantly higher than the resultant fractions supporting that unfractionated Low Skid silk is composed of a much diverse polypeptide population compared to fractions AS90-AS100 (Table 26).

**Mid Skid silk/modified polypeptide compositions isolated by charge and size:
AS101-AS105**

To isolate AS101-AS105, Mid Skid silk/modified polypeptide compositions were fractionated using anion exchange chromatography (Q-Sepharose chromatography),
5 following HiLoad 26/600 Superdex 200 pg size exclusion chromatography of the Q-eluate. Prior to chromatography, Tris was added to the silk preparations to a final concentration of 50 mM Tris-HCl, pH 8.0. The silk was centrifuged and filtered before loading to the Q-Sepharose column, to remove any preformed aggregates. The silk compositions were loaded onto the Q-Sepharose column, and the flowthrough fraction
10 was collected. The negatively charged silk compositions were eluted using high salt buffer (50 mM Tris, 500 mM CaCl₂). The eluted fractions were pulled together and are referred to as the Q-elution fraction. The Q-elution was further fractionated by the HiLoad 26/600 Superdex 200 pg, where the largest polypeptide compositions were eluted first, and each following fraction had a population of lower molecular weight of
15 silk compositions. Mid Skid silk preparation solutions have a characteristic yellow hue. The Q-elution fraction has a strong yellow hue, while the flowthrough fraction is transparent, and tends to self-assemble very quickly. The Q-elution silk compositions that are fractionated by size exclusion also had a yellow hue.

When silk formulations AS101-AS105 are analyzed with an analytical SEC-MALS
20 (see materials and methods) with HPLC, each of the silk formulations demonstrates a unique average Mw and Polydispersity (PDI) value (Figs. 53A- 53B and Table 27). In general, AS101 has the highest Mw (101.5 kDa), while AS105 has the lowest Mw (48.9 kDa). Unlike Mw, there was no clear trend in PDI as related to fraction number. Unfractionated Mid Skid silk has an average Mw of 81.9 kDa, indicating that most of
25 the peptide population tends to have lower molecular weight than fractions AS101-AS105. The polydispersity of unfractionated Mid Skid silk is 1.697 – significantly higher than the values of fractions AS101-AS105. This indicated that the unfractionated Mid Skid silk is composed of a much diverse peptide population compared to fractions AS101-AS105, where the majority of the peptide populations have lower molecular
30 weight.

Mid Skid silk/modified polypeptide compositions isolated by size: AS106-AS111

To isolate AS106-AS111 Mid Skid silk/modified polypeptide compositions was fractionated using HiLoad 26/600 Superdex 200 pg size exclusion chromatography column (Figs. 46 & 49A-49B). Tris was added to the silk preparations to a final concentration of 50 mM Tris-HCl, pH=8.0. The silk was centrifuged and filtered before loading to the HiLoad 26/600 Superdex 200 pg column, to remove any preformed aggregates. The silk compositions were fractionated by the HiLoad 26/600 Superdex 200 pg, where the largest polypeptide compositions were eluted first, and each following fraction had a population of lower molecular weight of silk compositions (Figs. 51A-51B & 52A-52B). Mid Skid silk preparation solutions have a characteristic yellow hue, and the fractionated silk compositions had a light-yellow hue. When silk formulations AS106-AS111 are analyzed with an analytical SEC column (see materials and methods) with HPLC, each of the silk formulations demonstrates a different average molecular weight, and a different Polydispersity (PDI) value (Table 28 and Figs. 54A- 54B). In general, AS106 has the highest molecular weight (204.4 kDa), while AS111 has the lowest molecular weight (67.4 kDa). There is not a strong trend between PDI and fraction number. Unfractionated Mid Skid silk has the highest PDI, 1.697, indicating a broad and diverse peptide population sizes.

Analytical Methods

Analytical SEC-MALS

Analysis was performed with a PolySep GFC P-4000 LC Column, 300 mm x 7.8 mm (Phenomenex, Part No. CH0-9229) with guard column connected to an Agilent 1260 Infinity II HPLC system with Wyatt LS and RI detectors (Table 22).

Table 22. HPLC Instrument Configuration

Instrument Component	Vendor	Part Number
Sample Manager	Agilent	G5668A
Sample Thermostat	Agilent	G7167-60101
Quaternary Pump	Agilent	G5654A
Column Compartment	Agilent	G7116A
UV Detector	Agilent	G1315C
LS Detector	Wyatt	miniDAWN TREOS with online QELS
RI Detector	Wyatt	Optilab TrEX

The mobile phase used for the analysis was a solution of 12.5 mM Na₂HPO₄, 100 mM NaCl titrated to a final pH of 7.0 ± 0.2 with H₃PO₄. Before installing mobile phase on the HPLC, the solvent was filtered through a 0.22 µm PES filter into a clean glass bottle. A summary of critical method parameters is described in Table 23.

5 Table 23. SEC-MALS Critical Method Parameters

Critical Attribute	Value
Target Load Mass ¹ (injection volume)	50 µg (50 µL)
Flow Rate	0.75 mL/min
Column Temperature	25 °C
UV Detection Wavelength	280 nm
Run Time	24 min
Astra Collection Time	23 min
¹ Samples were 1 mg/mL in 20 mM Tris pH 8 unless otherwise noted.	

Data Analysis

All silk samples were processed in software ASTRA 7.3.2 following vendor recommended procedures. Reportable values of Mw, Mn, and PDI were derived from software ASTRA 7.3.2. Additional data analysis and production of figures were performed in GraphPad Prism 9 version 9.5.1 and Adobe Illustrator version 27.5.

10

Fractionated Low Skid Silk

Unfractionated Low Skid Silk had a Mw of 41.2 and PDI of 1.575.

Tables

Table 24. Summary of Mw and PDI for fractionated Low Skid Silk by Q-SEC (AS77-AS81)

15

Fractionating Method	Activated Silk Number	Mw (kDa)	PDI
None	<i>Low Skid Silk</i>	<i>41.2</i>	<i>1.575</i>
Q-SEC (Q-Elution)	AS77	118.2	1.069
	AS78	102.6	1.056
	AS79	78.9	1.054
	AS80	63.5	1.068
	AS81	61.1	1.062

Table 25. Summary of Mw and PDI for fractionated Low Skid Silk by SEC (AS82-AS89)

Fractionating Method	Activated Silk Number	Mw (kDa)	PDI
None	<i>Low Skid Silk</i>	<i>41.2</i>	<i>1.575</i>
SEC	AS82	100.9	1.050
	AS83	85.4	1.025
	AS84	71.2	1.029
	AS85	56.2	1.037
	AS86	51.5	1.048
	AS87	18.2	1.061
	AS88	18.5	1.089
	AS89	11.1	1.109

Table 26. Summary of Mw and PDI for fractionated Low Skid Silk by Q-HIC-SEC (AS90-AS100)

Fractionating Method	Activated Silk Number	Mw (kDa)	PDI
None	<i>Low Skid Silk</i>	<i>41.2</i>	<i>1.575</i>
Q-HIC-SEC (Q-Elution)	AS90	136.0	1.060
	AS91	100.5	1.068
	AS92	74.4	1.046
	AS93	74.0	1.024
	AS94	59.4	1.031
Q-HIC-SEC (Q-Flow Through)	AS95	44.0	1.040
	AS96	48.0	1.057
	AS97	48.7	1.130
	AS98	41.8	1.090
	AS99	28.4	1.057
	AS100	27.9	1.165

Table 27. Summary of Mw and PDI for fractionated Mid Skid Silk by Q-SEC (AS101-AS105)

Fractionating Method	Activated Silk Number	Mw (kDa)	PDI
None	<i>Mid Skid Silk</i>	<i>81.9</i>	<i>1.697</i>
Q-SEC (Q-Elution)	AS101	101.5	1.064
	AS102	68.6	1.030
	AS103	59.3	1.016

	AS104	53.1	1.050
	AS105	48.9	1.048

Table 28. Summary of Mw and PDI for fractionated Mid Skid Silk by SEC (AS106-AS111)

Fractionating Method	Activated Silk Number	Mw (kDa)	PDI
None	Mid Skid Silk	81.9	1.697
SEC	AS106	204.4	1.228
	AS107	131.5	1.145
	AS108	95.9	1.099
	AS109	100.7	1.286
	AS110	72.8	1.142
	AS111	67.4	1.229

Example 12: Laundry Pods with Activated Silk

- 5
- Three fabrics will be used for testing AS-319 moisture management properties and hand. With this study N=2 nylon is coated based and N=1 polyester based fabrics after application of Activated Silk AS-319 during the “Rinse” cycle in a front loader washing machine. The fabrics after drying will be characterized with absorbency, dry time/dry rate testing at T=0,1, 3, 5 and 7. The hand feel will be evaluated in-house at
- 10
- each of the time points.

Materials

- 319 lyophilized pieces made with 40% AS14A and 60% Capryl Glucoside in 2.5gr net weight pouches.
- Nylon blend fabrics:

15
 - Sculpt
 - Sculptek Lite
 - Darlington

Equipment:

- 20
- Electrolux front loader washing machine model W4105H
 - Drying rack for fabrics

Methods:

- Cut fabrics in 1 meter length.
- Add N=3 pouches of AS 319 pellets to the washing machine drum
- Add all the fabrics and run program Laundry Pods 25L
- The fabrics are laid on top of a drying rack

5 **Table 34.** Sample ID of fabrics

Sample ID	Reference #	AS-319 Recipe
715-A-25	Darlington Nylon	N=3 pellets with 40% AS14A and 60% Capryl Glucoside → mass 2.5gr each
715-A-48	Sculptek Lite	
715-A-61	Sculpt	
715-A-22	Darlington Nylon	Unfinished
715-A-58	Sculptek Lite	
715-A-70	Sculpt	

After the fabrics are rested overnight, they are delivered to Manufacturing Solution Center for testing. Returned fabrics after testing were screened for in-house hand assessment.

10 **Table 35.** Fabric Absorbency

Sample ID	Reference #	AS-319 Recipe	T=0 Ave	std ev	1	2	3	4	5
715-A-25	Darlington Nylon	N=3 pellets with 40% AS14A and 60% Capryl Glucoside → mass 2.5gr each	0.41	0.06	0.32	0.46	0.43	0.47	0.38
715-A-48	Sculptek Lite		0.58	0.18	0.81	0.63	0.66	0.47	0.34
715-A-61	Sculpt		0.25	0.04	0.31	0.25	0.25	0.19	0.27
715-A-22	Darlington Nylon	Unfinished	13.87	2.89	14.65	13.63	9.78	17.84	13.44
715-A-58	Sculptek Lite		14.13	1.19	12.59	15.43	13.18	14.69	14.78
715-A-70	Sculpt		2.25	0.73	2.34	3.03	1.69	2.85	1.34

Characterization

- Coated samples and unfinished fabrics are subjected to the absorbency, vertical wicking and dry rate according to AATCC test methods.

- Note, in-house environment is not humidity and temperature controlled.
- Results collected will only be used for internal assessment.

Table 36. Laundry Pod Experiment

	front loader	top loader
load (gallons)	12.5	19.6
load (L)	25	74
AS at 2.5gr (gr/L)	0.100	0.027
AS at 1.25gr (gr/L)	0.050	0.017
AS at 0.625gr (gr/L)	0.025	0.008

5 **Table 37.** Pellet Composition

CG (%)	61
AS (%)	39

Table 38. Pellet Composition and Activated Silk Rate

		percent	at application	AS rate gr/L
CG (gr)	3.9	61%	4.575	0.183
AS (gr)	2.5	39%	2.925	0.117
total (gr)	6.4		7.5	

Table 39. Wet Mass Pickup

	dry mass	wet mass	wet pick up
darlington	254.8	321.46	26%
Sculptek Lite	382.84	587.27	53%
Sculpt	279.31	448.79	61%

10

Example 13: Sericin Quantitation

Quantitation of Silk Peptides in Samples

Introduction

The following reports the findings using a LCMS method developed to quantify the relative abundance of the different silk fibroin and sericin chains.

15

Sample preparation

Enzymatic digestion

The samples were denatured, reduced and alkylated before 3 aliquots were taken in separate tubes for enzymatic digestion with trypsin.

20

Table 40. Percent Protein Chain

Protein	Cocoon Control	New Reactor & Process, 1 st Run	New Reactor & Process, 2 nd Run	New Reactor & Process, 3 rd Run
Chorion	0	0	0	0
Fibrohexamerin	9.58	0.95	1.74	1.5
Heavy Chain	27.05	29.47	43.01	36.27
Light Chain	23.92	69.58	55.25	62.22
Sericin	35.27	0	0	0
Trypsin Inhibitor	4.19	0	0	0

Example 14: Molecular Weight Analysis of Silk Protein by SEC-RI

This example defines the procedure for measuring the molecular weight of silk protein using size-exclusion chromatography with refractive index detection (SEC-RI). To create a calibration curve of Dextran Standards, with nominal molecular weights (Mw) of 5 kDa, 12 kDa, 25 kDa, 50 kDa, and 150 kDa, to determine the Molecular weight (Mw) and Polydispersity (PD) values of Evolved By Nature (EBN) processed silk protein.

Described herein is the procedure used to operate the high-performance liquid chromatography (HPLC) SEC-RI system, including the preparation of mobile phase and sample solutions, system startup, column equilibration, system shutdown, to create a calibration curve of Dextran standards using SEC data collection and analysis software; and to determine the molecular weight of silk protein and its derivatives.

The values obtained from this process will contribute to protein characterization for research and development purposes and quality assurance. The molecular weight can further be used as a requirement for batch-release of all future production of silk solutions.

Software:

- Agilent Technologies OpenLAB CDS ChemStation Edition for LC & LC/MS Systems software (Rev C. 01.08 [210]), or equivalent
- Cirrus SEC data collection and molecular weight analysis software, or equivalent

Materials:

- Silk solution
- Reverse Osmosis (RO) or Deionized (DI) water
- Sodium chloride (VWR, Part No. BDH9286 or equivalent)
- Sodium phosphate dibasic heptahydrate (VWR, Part No. BDH9296 or
5 equivalent)
- Phosphoric acid (VWR, Part No. BDH153155E or equivalent)
- Dextran molecular weight standard 5KDa (Sigma-Aldrich, Part No. 00269, or
equivalent)
- Dextran molecular weight standard 12KDa (Sigma-Aldrich, Part No. 00270,
10 or equivalent)
- Dextran molecular weight standard 25KDa (Sigma-Aldrich, Part No. 00271, or
equivalent)
- Dextran molecular weight standard 50KDa (Sigma-Aldrich, Part No. 00891, or
equivalent)
- Dextran molecular weight standard 150KDa (Sigma-Aldrich, Part No. 00893,
15 or equivalent)
- 5% w/v aqueous sodium azide solution (VWR Chemicals)

Equipment:

- 20 • HP Z240 Workstation computer, or equivalent
- HP EliteDisplay E190i LED monitor, or equivalent
- HPLC system (Agilent 1260 Infinity II LC System, or equivalent)
- Refractive Index Detector (Agilent G7162A RID, or equivalent)
- PolySep GFC P-4000 LC Column, 300 mm x 7.8 mm (Phenomenex,
25 Part No. CH0-9229)
- Mettler Toledo XP26 Delta Range micro analytical balance
- pH Meter (Mettler Toledo FiveEasy F20 or Thermo Scientific Orion
Star A221 or equivalent)
- pH standards
- 30 • Vacuum driven disposable filtration system, PES 0.2um (Millipore
Express)
- Vacuum pump (Vacuubrand, Part No. ME 1)
- Autosampler vials (clear glass, 2 mL) and vial caps

- Autosampler vial tray
- Syringe filter (PTFE membrane, 0.45 μm)
- 3 mL syringe
- 5 mL microcentrifuge tubes
- 5 • Graduated cylinder
- Glass beakers
- Glass media bottles
- Magnetic stir bars
- Disposable weigh boats
- 10 • Disposable pipettes
- Eppendorf pipets

Procedure:**15 Preparation of 1L Mobile Phase (0.1M sodium chloride solution in 12.5mM sodium phosphate buffer, pH 7).**

- Weigh 3.35 g of sodium phosphate heptahydrate and record the weight. Weigh 5.84 g of sodium chloride and record the weight. Transfer both solids to a beaker. Add 1000 mL of RO/DI water to the beaker and mix until solids are dissolved. Once dissolved adjust the pH of the solution to 7.0 ± 0.2 with phosphoric acid. Filter the solution through a 0.2 μm PES filter into a clean glass media bottle.
- 20 • Label the bottle 'MW Mobile Phase, 0.1M NaCl + 12.5mM Na₂HPO₄' and include the pH of the solution, the date prepared, notebook reference and the expiry of the solution. Store at room temperature for up to 1 week.
- 25

(Note: The volume of the solution can be varied as long as the concentration of sodium phosphate and sodium chloride remains as defined above.)

30 System startup and column equilibration.

1. Select the LC OpenLAB icon 'HPLC (online)' from the desktop to open the Agilent HPLC control software.

2. Load the pre-set method that will be used to run samples for silk molecular weight analysis. The method parameters that should be used for all silk molecular weight testing are listed in Table 1. If the same column and mobile phase that are currently installed are going to be used for this run, then proceed to step 7.2.15.

File → Load → Method → SEC-RI_MRL Method.M

3. Update the program with the correct volume of mobile phase connected to the system. (Always input at least 0.20L less volume for the *Actual volume* than the *Total Volume*). Under *Actions*, select and input '*Prevent analysis if level falls below 0.20 liter*' and '*Turn pump off if running out of solvent*'.

Right click *Quat. Pump* → *Bottle Fillings*

4. Manually open the purge valve located in the quaternary pump by turning the nozzle counterclockwise. The mobile phase will now flow directly to waste and not enter the column compartment.

5. Once the purge valve is open and the mobile phase is not flowing in the direction of the column compartment purge the mobile phase tubing at 5 mL/min until no bubbles are detected. Reduce the flow to 1 mL/min and close the valve.

6. Turn on both temperature controllers for the column compartment and refractive index detector. Do not turn on without flow going through the column.

Right click *Column Comp* → *Switch On*

Right click *RID* → *Switch On*

Table 41: SEC-RI_MRL Method parameters.	
Column:	PolySep GFC P-4000 (7.8 x 300 mm)
Column Temperature:	25°C
Detector:	Refractive Index Detector

Detector Temperature:	35°C
Injection Volume:	25 µL
Mobile Phase:	0.1 M Sodium Chloride 12.5mM Sodium phosphate buffer
Flow Rate:	1.0 mL/min
Run Time:	20.0 min

7. Open the RID purge valve to purge the reference cell with new mobile phase.

Right click *RID* → *Open Purge Valve*

- 5 8. If not already displayed, add both the pressure and RID signals to the *Online Plot* window.

Online Plot window → *Change...* → Under *Available Signals* select *PMP1A, Pressure (bar)* and *RID1A, RI Signal (nRIU)* → *Add*

10 **Preparation of 1 mg/mL Dextran molecular weight standard solutions**

1. Weigh 5 mg of each dextran standard into 5 separate 5 mL micro centrifuge tubes. Record the weight for each standard. Dissolve each standard in 5 mL of mobile phase. Let the solutions sit for at least 1 hour at room temperature to ensure homogeneity.
- 15 2. Slowly filter the standard solutions through a 0.45 µm syringe filters into HPLC vials and cap tightly.

Preparation of 1 mg/mL sample solutions

- 20 1. Dilute the silk solution with mobile phase to 1 mg/mL in a 1.5 mL microcentrifuge tube. For a 6% silk solution, weigh approximately 83 mg of silk solution into a 5 mL microcentrifuge tube and add 5 mL of mobile phase. Invert the microcentrifuge tube 10 times to mix the solution.

2. Slowly filter the sample solution through a 0.45 µm syringe filter into a glass HPLC vial and close with an HPLC vial cap.

(Note 1: Fast filtration may induce shearing in the sample)

(Note 2: If there is limited amount of sample, the volume of

5 sample solution can be scaled down as long as the final sample concentration remains at 1 mg/mL.)

Running Dextran standards and sample solutions.

1. Filter mobile phase into a HPLC vial to be used for blank runs.
- 10 2. Place each vial into the autosampler.
3. Edit the sequence table and list the order of samples that are going to be run. Input the sample location, name, and method. Always run standard solutions at the beginning of the run, after 12 injections, and at the end of the run. Make the last run of the sequence a blank run
- 15 using the *FlowDecrease* method. *Sequence Window* → right click blue box → *Sequence Table...*
4. Close the RID purge valve. Right click RID → *Close Purge Valve*
5. Once all of the boxes under the *Instrument Control* tab turn green, the system is ready to run the sample sequence. Select *Start Sequence* to
- 20 begin the run. If a message appears stating, '*Method SEC-RI_MRL Method.M has changed. Save current changes?*', select *No* to ensure the method saved in the software does not change.
6. Once the run is complete, the mobile phase will continue to flow through the system at the flow rate set for the wash method, 0.05
- 25 mL/min, as it is the last run of the sequence. If the experiment is done, then the system can be shutdown according to the procedure noted in 7.6.

System Shutdown

1. If the system does not need prolonged storage, the flow rate can be set to 0
- 30 ml/min.
2. For prolonged storage, rinse and store the system and column in 0.05% sodium azide in RO/DI water. Rinse the system with filtered RO/DI water prior to flushing with sodium azide solution.

To make a 1L 0.05% sodium azide solution in water, add 10 mL of 5% sodium azide solution to 990 mL of RO/DI water.

3. Once the column and system has been rinsed with sodium azide at 1 mL/min for at least 1 hour, the flow rate can be placed to 0 mL/min and/or the column can be disconnected.

Calibration Curve Procedure:

1. Select the LC OpenLAB icon 'HPLC (offline)' from the desktop to open and view collected data.
2. Open the Cirrus analysis software.
Data Analysis → select the sample set to be analyzed → *Cirrus* → *GPC Analysis*
3. Create a calibration method.
 - Make a new method to be used for creating a calibration curve and analyzing a set of samples.
Method Browser → right click on the table → *New Method...*
 - Under the *General* tab, input a detailed name of the method and include in the comments the mobile phase, column information, and flow rate. Select *Do Data Processing* and input 60 secs for the *Time between chromatograms*.
 - Under the *Column Calibration* tab, next to *Generate Using* select *Narrow Standard*. Input *Apply a Polynomial* curve, of *order 3*. For *Calibrant Automation*, select *Manual Processing* and check *Detect Peaks Automatically*.
 - Under the *Sample Analysis* tab, for *Automation* select *Manual Processing* and check *Detect Peaks Automatically*. Under *Calculation* check *Perform GPC Calculations* and select *Area Integration* for the *Calculation Method* using *1 data point in 1*. Check *Calculate MW Ranges* and select the *Details* option.
 - A new window will appear containing a MW Ranges table. Input the M_w value listed on the COA of the Dextran Standard with the highest M_w under the *High MW Limit* column and input the M_w value listed in the COA with the lowest M_w value under the *Low MW Limit* column.

- Under the *Peak Detection* tab, input the values listed in **Table 52** into each column of the *Peak Detection* table. Under *Baseline Assignment*, check *Apply Horizontal Baseline* and *Start of First Peak*.

Type	System detects
Start [mins]	5.00
End [mins]	10.00
Slope	0.1000
Separation Width [secs]	8
Noise Tolerance	5.0000
Negative Peaks	Unchecked box
Min. Height [mV]	0.0000
Min. Area [mV.s]	0.0000
Valley-to-Valley	Uncheck box
Skim Ratio	8.0000

Table 42: Peak Detection table input values.

5

- Leave the tables shown under the *Reporting* and *User Programs* tabs blank. Select *OK* to exit out of the window. The method should now appear in the *Methods Browser* list and have a symbol indicating that the method has not been calibrated yet and cannot be used.

10

4. Create a calibration curve using Dextran Standards.

- Select *Analysis Runlist* and clear any previous samples that are listed in the runlist table .
 - Select the table → *Runlist* → *Clear Runlist*
- The Open the folder that contains the Dextran standard runs that will be used for the calibration curve.
- Add the RI signal for each Dextran standard (5 kDa, 12 kDa, 25 kDa, 50 kDa, and 150 kDa) to the runlist table by double clicking on the RI signal file.
- For each standard in the runlist table, input Sample Type = Narrow Standard and Cal. Version = Create. Add the method

15

20

that was previously created to be used for the calibration curve.
Leave all other columns as default.

- Double click Method box next to first standard →
Choose Method → Fill Down
- 5 ■ Add the first standard to the *Analysis* window by selecting the *Next Analysis* icon. The text of the first standard on the list will turn from blue to green when selected.
- View the chromatogram under the *Analysis* tab and select *Peak Summary*. Delete all of the auto-labeled peaks.
- 10 • Highlight each peak in the *Analysis* window → hit *Delete* on keyboard
- Visually analyze the chromatogram of each standard. The graph should show a single narrow curved peak with a flat baseline. Make sure impurities are limited and do not
- 15 significantly interfere with the main peak. If the peak is broad and/or the baseline is uneven, there is something wrong with the column or system that needs to be addressed before the samples can be analyzed.
- Identify and label the main peak of the chromatogram.
- 20 • *Analysis → Detect Peak Max →* click on the top of the peak on the chromatogram.
- Double check the sample name by selecting the *Sample Summary* tab. Return to the *Peak Summary* tab and input the *Peak Name* and record the *Max. RT* listed in the table.
- 25 ■ Enter the M_p value listed on the COA for the Dextran Standard of that chromatogram in the *Peak MW* column. The rest of the columns will autofill in from internal calculations of the software.
- Add the value to the calibration curve by select the *Add to Calibration* icon. An error may appear indicating Insufficient
- 30 Data Points; select OK to continue. The error will not appear as more points are added. The next sample chromatogram in the runlist will automatically appear with a point on the graph

indicating the start of the calibration curve. If the next chromatogram does not appear, select the *Next Analysis* icon for it to show.

- 5 ▪ Repeat steps 7.4.6-7.4.10 until all of the Dextran standards have been added to the calibration curve.
- To Check and make sure that the method has been calibrated, select the *Method Browser* tab. The calibration status should now show an icon indicating the method has been calibrated.
- 10 ▪ To analyze the calibration curve details, select the *Calibration Browser* tab. Make sure the Coefficient of Determination value is at least 0.99.
 - Select the method → Right click on the table → *Show Calibration Details*

15 **Sample Analysis Procedure:**

1. After creating the calibration curve, add sample runs to the analysis window.
 - Add the sample RI signal to the runlist table by double clicking on the RI signal file. Analyze all standard samples first, then silk lot samples.
 - 20 ○ In the runlist table, make sure the sample type is listed as *Unknown* and add the calibration method that was previously created to be used for analysis. Leave all other columns as default.
 - Double click Method box next to first standard → Choose Method → Fill Down or OK
 - 25 ○ Add the first standard to the *Analysis* window by selecting the *Next Analysis* icon. The text of the first standard on the list will turn from blue to green when selected.
 - View the chromatogram under the *Analysis* tab and select *Peak Summary*. Delete all of the auto-labeled peaks.
 - 30 Highlight each peak listed in the *Peak Summary* table → hit *Delete* on keyboard

- Visually analyze the chromatogram and report any additional peak formations that could be signs of protein aggregation or impurities, such as excess lithium bromide.
- 5 2. Integrate the RI peaks for each sample solution and determine the Mw and PD values through the software.
- Identify the main positive peak of the silk protein solution found within the retention time of 5-10 min. A majority of the peak should fall within range of calibration curve between the high and
- 10 low limits.
- Integrate the peak from left to right, aligning the baseline at both ends of the peak. Do not include the solvent mis-match peak in the integration. Zoom into the peak if needed. Afterwards, a red line will appear at the baseline and the peak will be labeled and added
- 15 to the *Peak Summary* table.
- Analysis → Zoom →* drag cursor along the chromatogram to box area of interest and zoom-in
- Analysis → Detect Peaks and Baseline*
- Select *Calculate Results* to fill in the rest of the table.
- 20 ○ Record the following values on the batch record: Mw and PD.

Example 15: Comparative Silk Protein (e.g., Silk Fibroin) Processing Parameters

Table 43. Processing Parameters

Unit Op	Process Parameter	Parameter Variables and Process Operating Ranges that can be used to create single specific weight average Molecular Weight	Gradient (if applicable)	Standard Dev (if applicable)	Available in original BT process	Example of Specific Conditions and Ranges to Create Specific Fibroin Fragment Populations that have a single weight average Molecular Weight Average (MW) and poly dispersity below 3	Example of Specific Conditions to Create Specific Fibroin Fragment Populations with a single weight average Molecular Weight Average (MW) and poly dispersity below 3						
						“Mid Range” of specific single MW fragment population created from range of process conditions : 39.7 kDa single population to 33.0 kDa single MW population by SEC-RI	“Low Range” of specific single MW fragment population created from range of process conditions : 27.2 kDa single population to 11.9 kDa single MW population by SEC-RI	“High Range” of specific single MW fragment population created from range of process conditions : 95.1 kDa single population to 67.6 kDa single MW population by SEC-MALS	Specific single “Mid” MW fragment population n: 47 kDa by SEC-RI and 84 kDa by SEC-MALS	Specific single “Low” MW fragment population n: 22 kDa by SEC-RI and 37 kDa by SEC-MALS	Specific single “High” MW fragment population n: 53 kDa by SEC-RI and 95 kDa by SEC-MALS	Specific single “High” MW fragment population n: 145 kDa by SEC-RI and 250 kDa by SEC-MALS	Specific single “Low” MW fragment population n: 1 kDa by SEC-RI and 1 kDa by SEC-MALS

		Example of Specific Conditions to Create Specific Fibroin Fragment Populations with a single weight average Molecular Weight (MW) and poly dispersity below 3					Example of Specific Conditions and Ranges to Create Specific Fibroin Fragment Populations that have a single weight average Molecular Weight (MW) and poly dispersity below 3				
	equal to or lower than 3										
Fibroin isolation or extraction	Equipment style	Stationary bed w/ continuous solvent spray, moving bed w/ counter current solvent feed (Bollman or Hildebrandt), stationary bed w/ unagitated solvent, dispersed solid with agitated solvent	-	-	Yes	Dispersed solid with agitated solvent. Solid held within permeable secondary containment.	Dispersed solid with agitated solvent. Solid held within permeable secondary containment.	Dispersed solid with agitated solvent. Solid held within permeable secondary containment.	Dispersed solid with agitated solvent. Solid held within permeable secondary containment.	Dispersed solid with agitated solvent. Solid held within permeable secondary containment.	Dispersed solid with agitated solvent. Solid held within permeable secondary containment.
Fibroin isolation or extraction	Process pressure	0.145 psia - 1450 psia	-	0.05 psia - 10 psia	Range is limited for BT	atmospheric	atmospheric	atmospheric	atmospheric	atmospheric	atmospheric
Fibroin isolation or	Agitation	Stationary bed (no intentional agitation),	-	-	Range is limited for BT	Dispersed by drum rotation	Dispersed by drum rotation	Dispersed by drum rotation	Dispersed by drum rotation	Dispersed by drum rotation	Dispersed by drum rotation

										Example of Specific Conditions and Ranges to Create Specific Fibroin Fragment Populations that have a single weight average Molecular Weight Average (MW) and poly dispersity below 3	Example of Specific Conditions and Ranges to Create Specific Fibroin Fragment Populations with a single weight average Molecular Weight Average (MW) and poly dispersity below 3						
extraction		moving bed, or dispersed (fully or partially) in solvent															
Fibroin isolation or extraction	Agitation Duration or Frequency	Intermittent; continuous	-	No						Intermittent	Intermittent	Intermittent	Intermittent	Intermittent			Intermittent
Fibroin isolation or extraction	Secondary containment	Yes or no	-	Yes						yes	yes	yes	yes	yes		yes	yes
Fibroin isolation or extraction	Solvent concentration	0.1%wt - 1%wt	-	Yes						0.94%wt	0.94%wt	0.94%wt	0.94%wt	0.94%wt		0.705%wt	1.88%wt
Fibroin isolation or extraction	Cocoon to solvent ratio	0.01 kg/kg to 0.1 kg/kg	-	Yes						0.042kg/kg	0.042kg/kg	0.042kg/kg	0.042kg/kg	0.042kg/kg		0.068kg/kg	0.042kg/kg
Fibroin isolation or extraction	Extraction solvent temperature	30 C - 370 C	0.1 C - 10 C	Range is limited for BT						100 °C	100 °C	100 °C	100 °C	100 °C		60C	100 °C

							Example of Specific Conditions and Ranges to Create Specific Fibroin Fragment Populations that have a single weight average Molecular Weight Average (MW) and poly dispersity below 3	Example of Specific Conditions to Create Specific Fibroin Fragment Populations with a single weight average Molecular Weight Average (MW) and poly dispersity below 3						
Fibroin isolation or extraction	Temperature control style	Internal (electrical/flow medium heating/cooling element), external (circulation of heat transfer fluid through jacket, conduction against electrically heated jacket, conduction against electrically heated plates or walls), indirect (vessel temperature not maintained, but solvent is heated in a recirculation or feed).	-	0.05 min	Yes	Range is limited for BT	internal	internal	internal	internal	internal	internal	internal	internal
Fibroin isolation or extraction	Extraction Time	5 min - 60 min	-	0.05 min	Yes		30 min	30 min	30 min	30 min	30 min	30 min	15 min	60 min

		Example of Specific Conditions and Ranges to Create Specific Fibroin Fragment Populations that have a single weight average Molecular Weight Average (MW) and poly dispersity below 3					Example of Specific Conditions to Create Specific Fibroin Fragment Populations with a single weight average Molecular Weight Average (MW) and poly dispersity below 3				
							0.037 kg/kg	0.037 kg/kg	0.037 kg/kg	0.037 kg/kg	0.037 kg/kg
Fibroin isolation or extraction	Cocoon to rinse water ratio	0.005 kg/kg - 0.04 kg/kg	-	0.001 kg/kg - 0.005 kg/kg	Yes	0.037 kg/kg	0.037 kg/kg	0.037 kg/kg	0.037 kg/kg	0.037 kg/kg	0.037 kg/kg
Fibroin isolation or extraction	Rinse water temperature	1 C - 370 C	0.1 C - 10 C	0.1 C - 10 C	Range is limited for BT	60C	60C	60C	60C	60C	60C
Fibroin isolation or extraction	Rinse single stage time	5 min - 60 min	-	0.05 min	Yes	10min x 3	10min x 3	10min x 3	10min x 3	10min x 3	10min x 3
Fibroin isolation or extraction	Excess water removal	Centrifugal, vacuum, heating (up to 60 C), dry air flow, rinsing w/ volatile solvent and ambient drying, or any combination of these	0.1 C - 10 C	0.1 C - 10 C	Yes	centrifuga l, thermal at 60 C	centrifuga l, thermal at 60 C	centrifuga l, thermal at 60 C	centrifuga l, thermal at 60 C	centrifuga l, thermal at 60 C	centrifuga l, thermal at 60 C
Fibroin solvation and dissolution	Equipment style	Dispersed solid w/agitated solvent, stationary bed w/ recirculated solvent (compress	-	-	Yes	Dispersed solid w/agitated solvent	Dispersed solid w/agitated solvent	Dispersed solid w/agitated solvent	Dispersed solid w/agitated solvent	Dispersed solid w/agitated solvent	Dispersed solid w/agitated solvent

		Example of Specific Conditions and Ranges to Create Specific Fibroin Fragment Populations that have a single weight average Molecular Weight Average (MW) and poly dispersity below 3					Example of Specific Conditions to Create Specific Fibroin Fragment Populations with a single weight average Molecular Weight Average (MW) and poly dispersity below 3				
Fibroin solvation and dissolution		fibroin into cage, pump solvent such that it strips fibroin as it passes through the cage)	0.145 psia - 1450 psia	-	0.05 psia - 10 psia	Range is limited for BT	atmospheric	atmospheric	atmospheric	atmospheric	atmospheric
Fibroin solvation and dissolution		Stationary bed (no intentional agitation), or dispersed (fully or partially) in solvent by some means		-	-	Range is limited for BT	Solid fully dispersed in solvent	Solid fully dispersed in solvent	Solid fully dispersed in solvent	Partially dispersed by manual agitation	Solid fully dispersed in solvent
Fibroin solvation and dissolution		Intermittent; continuous		-	-	Range is limited for BT	Continuous	Continuous	Continuous	Intermittent	Continuous
Fibroin solvation and dissolution		Yes or no		-	-	Yes	No	No	No	No	No
Fibroin solvation and		10%wt - 70%wt		-	0.1%wt	Yes	51.5%wt LiBr	51.5%wt LiBr	51.5%wt LiBr	51.5%wt LiBr	51.5%wt LiBr
		Solvent concentration									

									Example of Specific Conditions to Create Specific Fibroin Fragment Populations with a single weight average Molecular Weight Average (MW) and poly dispersity below 3				
disolutio n													
Fibroin solvation and disolutio n	Fibroin to solvent ratio	0.02 kg/kg to 0.2 kg/kg	-	0.005 kg/kg - 0.05 kg/kg	Yes	0.159 kg/kg	0.159 kg/kg	0.159 kg/kg	0.159 kg/kg	0.159 kg/kg	0.159 kg/kg	0.159 kg/kg	0.159 kg/kg
Fibroin solvation and disolutio n	Solvent temperature	20C - 370 C	0.1 C - 10 C	0.1 C - 10 C	Range is limited for BT	100 °C	123 C	100 °C	100 °C	123 C	100 °C	60C	135 C (boiling)
Fibroin solvation and disolutio n	Temperature control style	Internal (electrical/flo w medium heating/cooling element), external (circulation of heat transfer fluid through jacket, conduction against electrically heated jacket, conduction against electrically heated plates or walls), indirect (vessel temperature	-	-	Range is limited for BT	external (held in oven)	external	external	external (held in oven)	external (held in oven)	external	external (held in oven)	external

		Example of Specific Conditions to Create Specific Fibroin Fragment Populations with a single weight average Molecular Weight (MW) and poly dispersity below 3					Example of Specific Conditions and Ranges to Create Specific Fibroin Fragment Populations that have a single weight average Molecular Weight (MW) and poly dispersity below 3					Example of Specific Conditions to Create Specific Fibroin Fragment Populations with a single weight average Molecular Weight (MW) and poly dispersity below 3				
Fibroin solvation and dissolution	not maintained, but solvent is heated in a recirculation or feed).	Fibroin loading time	0 min - 60 min	-	0 min - 15 min	Range is limited for BT	45 min	45 min	0 min	45 min	45 min	45 min	0 min	0 min	45 min	45 min
Fibroin solvation and dissolution	1 min - 1440 min	Reaction time	1 min - 1440 min	-	0.05 min	Yes	From 1 min to 63 min	From 17 min to 360 min	From 15 min to 60 min	20 min	46 min	15 min	15 min	0 min	480 min	
Fibroin solvation and dissolution	Internal (flow medium cooling element), external (circulation of heat transfer fluid through jacket), system flow through heat exchanger, ambient cooling after discharge	Cooling style		-	-	Range is limited for BT	Ambient cooling after discharge	Ambient cooling after discharge	Ambient cooling after discharge	Ambient cooling after discharge	Ambient cooling after discharge	Ambient cooling after discharge	Ambient cooling after discharge	Ambient cooling after discharge	Ambient cooling after discharge	Ambient cooling after discharge

Example of Specific Conditions to Create Specific Fibroin Fragment Populations with a single weight average Molecular Weight (MW) and poly dispersity below 3		Example of Specific Conditions and Ranges to Create Specific Fibroin Fragment Populations that have a single weight average Molecular Weight (MW) and poly dispersity below 3					Example of Specific Conditions to Create Specific Fibroin Fragment Populations with a single weight average Molecular Weight (MW) and poly dispersity below 3				
Fibroin purification and dissolution	Product cooling time	15 min - 1440 min	-	3 min - 15 min	Range is limited for BT	1440 min	1440 min	1440 min	1440 min	1440 min	1440 min
Fibroin purification	Concentrate filtration style (dead end)	Filter press, shell and leaf filter, in-line filter, belt filter, centrifugal (rotating drum) filter, vacuum filter	-	-	Range is limited for BT	In-line filter	In-line filter	In-line filter	In-line filter	In-line filter	In-line filter
Fibroin purification	Diluted fibroin concentration	1%wt - 10%wt	-	0.05%wt	Yes	2.5%wt	4.5%wt	2.5%wt	4.5%wt	2.5%wt	4.5%wt
Fibroin purification	Diluted fibroin filtration style (dead end)	Filter press, shell and leaf filter, in-line filter, belt filter, centrifugal (rotating drum) filter, vacuum filter	-	-	Range is limited for BT	In-line filter	In-line filter	In-line filter	In-line filter	In-line filter	In-line filter
Fibroin purification	LiBr removal style	Diafiltration (TFF), dialysis, adsorption (chromatography)	-	-	Range is limited for BT	Diafiltration on (TFF)	Diafiltration on (TFF)	Diafiltration on (TFF)	Diafiltration on (TFF)	Diafiltration on (TFF)	Diafiltration on (TFF)
Fibroin purification	Diafiltration/concentration (TFF)	4 C - 60 C	2 C	2 C	Range is	20 C - 25 C	20 C - 25 C	20 C - 25 C	20 C - 25 C	20 C - 25 C	30 C - 40 C

		Example of Specific Conditions to Create Specific Fibroin Fragment Populations with a single weight average Molecular Weight Average (MW) and poly dispersity below 3					Example of Specific Conditions and Ranges to Create Specific Fibroin Fragment Populations that have a single weight average Molecular Weight Average (MW) and poly dispersity below 3					Example of Specific Conditions to Create Specific Fibroin Fragment Populations with a single weight average Molecular Weight Average (MW) and poly dispersity below 3				
		processing temperature	limited for BT													
Fibroin purification	Temperature control style	Internal (electrical/flow medium heating/cooling element), external (circulation of heat transfer fluid through jacket, conduction against electrically heated jacket, conduction against electrically heated plates or walls), system flow through heat exchanger	Range is limited for BT	-	-		External	External	None	External	External	None	None	None	None	External
Fibroin purification	Diafiltration/concentration (TFF) selective layer rejection	3 kDa - 80 kDa	Yes	-			10 kDa	10 kDa	10 kDa	10 kDa	10 kDa	10 kDa	10 kDa	10 kDa	10 kDa	RO membrane (salt rejection only)
Fibroin purification	Diafiltration/concentration (TFF) TMP	2 psi - 40 psi	Yes	0.5 psi - 5 psi			12.5 psi	12.5 psi	35 psi	12.5 psi	12.5 psi	35 psi	35 psi	35 psi	35 psi	-
Fibroin purification	RO membrane	20-100psi	no	5-10psi	-		-	-	-	-	-	-	-	-	-	70 psi

							Example of Specific Conditions to Create Specific Fibroin Fragment Populations with a single weight average Molecular Weight Average (MW) and poly dispersity below 3				
							Example of Specific Conditions and Ranges to Create Specific Fibroin Fragment Populations that have a single weight average Molecular Weight Average (MW) and poly dispersity below 3				
Fibroin purification	Diafiltration/concentration (TFF) flow rate	200 lpm - 900 lpm	-	5 lpm	No		300 lpm	300 lpm	Variable (control to TMP)	300 lpm	Variable (control to TMP)
Fibroin purification	Concentration style	TFF, pervaporation, thin film evaporation (vacuum, or thermal, or both), reverse osmosis, dead end membrane filtration	-	-	Range is limited for BT		TFF	TFF	TFF	TFF	TFF
Fibroin purification	Product concentration	5%wt - 40%wt	-	1%	Range is limited for BT		6%wt	6%wt	6%wt	6%wt	6%wt
Fibroin purification	Secondary separation	size exclusion column; RODI membrane type					NA	NA	NA	NA	NA

Example 16: Construction of Silk Fibroin-Nanoclay Composite Films and/or Coatings, Used to Regulate Water Vapor (and other gas) Permeability Across the Material Surface

5 This example encompasses the construction of silk fibroin-nanoclay composite films and/or coatings, used to regulate water vapor (and other gas) permeability across the material surface.

Films composed of regenerated silk fibroin (RSF) and nanoclays such as bentonite clay exhibit lower water vapor permeability (WVP) properties compared to films cast using equivalent quantities of RSF or nanoclay alone. The mechanism through
10 which incorporation of bentonite clay reduces WVP will also serve to reduce the permeability of other gases. The extremely low permeability of the nanoclay platelets, which act as a filler in the RSF film matrix, increases the diffusive pathway for any permeant through the RSF/nanoclay membrane. While properties of polymer-nanoclay composites are well-documented, this disclosure represents a novel characterization of
15 regulation of water vapor permeability specific to an RSF/nanoclay composite. The type of clay used for this discovery include both Elementis Bentone Hydroclay 1100 (1100) and Elementis Bentone Hydroclay 2001 (2001), both of which are forms of bentonite clay. Throughout this disclosure, the term “nanoclay” will serve to encompass the general class of bentonite clays.

20 Synthetic polymers are currently used as liquid bandage materials in products such as Nexcare Liquid Bandage, New Skin Liquid Bandage, Curad Flex Seal, and others. Naturally occurring polymers (such as silk fibroin) are desirable for their increased biocompatibility, biodegradability, and good mechanical strength. One of the main ways that a liquid bandage promotes wound healing is to regulate the rate of WVP
25 from damaged skin or an open wound. In a liquid bandage application, this invention could be used to target a specific WVP for an RSF/nanoclay liquid bandage film at a given thickness.

Such a reduction in permeability can be applied anywhere improved barrier properties are desirable, not just as a liquid bandage. Other potential applications include
30 corrosion resistant coatings, flame retardant coatings, topical skin ointments, biodegradable food packaging, and others.

Additional additives such as plasticizing agents can be incorporated into silk-nanoclay materials across a range of pH conditions to augment material properties (such as strength, flexibility, etc), further expanding their potential applications.

Properties

5 Films can be prepared with water-based solutions of RSF and clay. A solution with the desired concentration of both silk and clay is made by combining stock solutions of silk fibroin and clay. The clay is dissolved into water via a high shear mixer. A centrifuge may be used for controlled acidic solutions (pH <4.0), but it is not necessary when the solution is neutral (pH ~7.0). Once the desired solution is obtained,
10 the solution is cast onto a PDMS slab and dried in an environmental chamber at 35C, 50% RH.

 Water vapor permeability was benchmarked for silk-only and clay-only films using a method adapted from ASTM E96. The method of measurement was adapted by the author to specifically target this class of films. Tables of results are presented below.
15 Silk type was kept consistent as 33B, or “mid skid” silk, (MW 24-40kDa, PD 1.8-2.3) for this IDF due to a larger data set. However, the same behavior documented in this example is present in 27P, or “low skid” silk (MW 13-16kDa, PD 1.7-2.4) as well.

Table 44: WVP values of RSF/nanoclay films of various concentrations. All measurements were taken at 35C and 50% RH. RSF type is 33B for all samples.

	Percent Clay	Water Vapor Permeance [g/m ² *Pa*24h]	StDev
Silk Only	0%	0.515	0.035
Clay Only	100%	0.804	0.017
2001	20%	0.348	0.019
	33%	0.309	0.010
	50%	0.295	0.011
	66%	0.260	0.036
1100	20%	0.397	0.048
	33%	0.352	0.013
	50%	0.304	0.031
	66%	0.293	0.005

Table 45: Effect of nanoclay content on WVP.

Percent Clay	2001 % Reduction	1100 % Reduction
0%	0.0	0.0
20%	-32.4 ± 3.7%	-23.0 ± 9.3%
33%	-39.9 ± 1.9%	-31.6 ± 2.5%
50%	-42.8 ± 2.1%	-41.1 ± 6.1%
67%	-49.5 ± 7.1%	-43.1 ± 1.0%
100%	+56.3 ± 3.3%	ND

Mechanism

Bentonite Clay refers to a class of Tetrahedral-Octahedral-Tetrahedral (TOT) minerals. The outer sheets of the clay are composed of a tetrahedral SiO₄ structure, while the inner sheet sandwiched between the tetrahedral layers is composed of octahedral alumina (Al₂O₃). The outer layer bears a negative charge resulting from cation substitutions in the tetrahedral silica structure. The clay is susceptible to swelling as water penetrates into the inner layers of the system. Each outer tetrahedral layer of the clay exhibits very low permeability to water vapor and gasses.

In solution, clay clusters break down to single nanoscale-thick layers. Upon drying, these nanoclay layers arrange to form a network of parallel sheets, as confirmed via SEM (see *Figure 64*). Water vapor and other gasses can readily diffuse through the space between these clay sheets due to their irregular stacking and available free volume in the film.

In contrast, films cast with pure RSF exhibit a comparatively homogenous structure (see *Figure 65*). As is typical with polymer films, pure RSF films contain amorphous, semicrystalline and crystalline regions, densely packed, and free volume space throughout the film.

SEM images of 1:1 RSF/nanoclay films confirms a composite network that maintains the layered pattern of the exfoliated clay distributed throughout the RSF. RSF/nanoclay films cast under neutral conditions appear to retain more of the layered structure (*Figure 66*), while the identical solution cast under acidic conditions appear to form more ribbonlike structures (*Figure 67*). This could be due to the stronger electrostatic attractive forces between the RSF and nanoclay that exist under acidic

conditions, while interactions between RSF and nanoclay under neutral conditions are driven by ion-dipole forces between negatively charged functional groups in the RSF and the exchangeable cations in the nanoclay inner layer.

Due to the extremely low permeability of the nanoclay sheets, water vapor is forced to diffuse around the clay layers within the film. In contrast with a pure nanoclay film, RSF decreases the free volume between the nanoclay layers and further restricts the motion of water vapor. Through this stacking and filling structure, the RSF and the nanoclay create a more tortuous pathway for any diffusing molecules to follow and resultingly yield a less permeable film (*Figure 68*). Up to concentrations of 67%, clay content and permeability for a film of a given area density are inversely proportional. Beyond this concentration, the physical properties of RSF/nanoclay solutions begin to substantially differ, posing a challenge for repeatable film casting and measurement.

FTIR scans have been taken to investigate the conformation of silk in a silk/nanoclay composite film. Specifically, the amide I ($1700\text{-}1600\text{ cm}^{-1}$) and amide II ($1600\text{-}1500\text{ cm}^{-1}$) peaks were analyzed. In neutral conditions (pH of ~ 7.0), increasing the relative nanoclay concentration decreased the relative silk beta sheet content and increased the random coil structure. (*Figure 69*).

Example 17: Stabilizers for Silk Compositions

Stabilizers for all compositions disclosure herein may include:

- Emulsifiers (e.g. polysaccharide, etc.)
- Surfactants (e.g. polysorbate, glycosides, etc.)
- Buffering agents (e.g. PBS, etc.)
- Amino acids (e.g. arginine, etc.)
- Sugar (e.g. Trehalose, glucose, sucrose, etc.)
- KCl
- NaCl
- MgCl_2
- CaCl_2
- PBS
- Tris
- Polysorbate 20

- Polysorbate 80
- Capryl Glucoside
- Sucrose
- Histidine (buffer)
- 5 • Glycine (buffer)
- Arginine

Excipient Overview and Mechanisms

- Ionic strength/Salts
 - 10 ○ Directly influence conformational integrity and stability
 - Have been shown to dramatically affect the amount of protein aggregation induced by temperature and agitation
 - Anions > cations in terms of influence on protein structure
- Buffering agents (pH)
 - 15 ○ Maintain solution pH (which greatly impacts protein higher-order structure)
 - Can have specific ion effects, impacting chemical and conformational stability.
- Surfactants
 - 20 ○ Outcompete/shield protein molecules from hydrophobic surfaces (like air-water interfaces), which prevents protein unfolding
 - May directly interact with hydrophobic regions in protein molecules
- Sugars/Osmolytes
 - 25 ○ Stabilize proteins through preferential hydration (increasing the amount water molecules can associate with protein surface, increasing solubility)
 - Protect protein structure during stressful events (like lyophilization)
- Amino Acids
 - Stabilize via a variety of mechanisms: preferential hydration, direct binding, buffering capacity, antioxidant properties

Example 18: Preparation of Powder of Silk Fibroin Protein Fragments (SPF Powder)

30 **Freeze Drying Process:**

Each of the 650 mL of aqueous solution of low-MW and mid-MW silk fibroin protein fragments as prepared above was added to a 1 L round bottom glass bottle. The two bottles loaded with silk solutions were placed inside a freezer and were

allowed stay inside the freezer overnight to provide fully frozen silk solutions. The two bottles containing frozen silk solutions were removed from the freezer. The bottles were left open and the openings were covered with Kimwipe paper tissues and were placed inside a lyophilizer. The pressure inside the lyophilizer is reduced to 0.02 mbar. The collector temperature was set at -65 °C. After 24 hours of lyophilization, the two bottles were removed from the lyophilizer and were immediately capped to avoid the contacting the dried silk solid with moisture. The coarse powders immediately from the lyophilization were grinded with a mortar and pestle to produce fine powders of silk fibroin protein fragments with even size distribution. The further grinding/processing may be performed to produce silk solid particle with desired particle size.

The coarse solids of low-MW silk was very easy to break down using the mortar and pestle, resulting in a very fine powder. As it became smaller, the lyophilized silk revealed a lamellar-looking appearance (approximately a couple of millimeters in length and width, but extremely thin, almost see-through). These small particles are somewhat similar to mica, in the sense that they are very thin sheets that shimmer in the light (See Figs. 91A-91C).

As the solid silk were ground more and the particle size was reduced, the powder lost its shimmer. Based on the appearance and the way it tends to fly at the slightest air movement, the particle size can be between a few microns and a few hundred microns.

The solids of mid-MW silk (Fig. 92) did not crumble immediately upon grinding (as was the case for the low-MW solid silk). Other silk drying methods that could be employed include, but are not limited to, spray drying, polar drying, and thin film evaporation.

Thin Film Evaporation Process:

Aqueous solutions of Low-MW or mid-MW silk fibroin protein fragments as prepared herein were placed inside a thin film evaporator. Water was continuously removed from silk solutions inside the thin film evaporator under reduced pressure, using gentle heating, resulting in a solid of variable particle size (Fig. 93). The particle size can be adjusted by varying the process parameters, such as, but not limited to pressure, temperature, rotational speed of the cylinder, thickness of the liquid film in the evaporator.

Microparticles Prepared by Aqueous Solution Precipitation Process:

Salt-out method: A 1.0 M phosphate buffer solution was prepared and the pH value was adjusted to 8. To a gently stirring silk solution of 5.0 mg/ml concentration, phosphate buffer was added in a 1:5 ratio (v/v). Samples were reacted for 5 minutes and then were placed inside a refrigerator to promote the precipitation of silk particles. The resulting silk solid suspension was then centrifuged to collect solid particles. The silk particles were washed three times with deionized water and dried to give solid particles of silk fibroin protein fragments (SPF powder).

PVA-assisted method: A 3.0 wt. % stock silk solution was mixed with a 5.0 wt. % solution of polyvinyl alcohol (PVA) in a 1:4 ratio (v/v). The resulting solution mixture was stirred gently for 2 hours. The solution mixture was then sonicated followed by casting to a substrate to allow formation of film. The film was reconstituted in minimal amount of D.I. water and centrifuged. The supernatant was removed and additional D.I. water was added. This process was repeated two times. After two washes, the liquid was removed from the flask to provide wet silk microparticles. Then a small volume of methanol was added to the wet microparticles in the flask (the methanol annealing). The particle suspension inside the flask was swirled. The particle suspension was then poured over a large cloth filter to isolate the microparticles (See Figs. 94A-94B).

Example 19: Pre-Dosed Delivery Packaging for Lyophilized Silk Fibroin

Reconstitution

As described in this Example, solid silk fibroin can be enclosed in a pouch to allow users to reconstitute (i.e. dissolve or solubilize) solid dry Activated Silk™ into water without any added specialty or custom equipment.

Liquid Activated Silk™ is difficult to transport and store due to its shelf life requirements and limitations. One solution is to convert the Liquid Activated Silk™ into powder form, however when dried and prepared into a solid, Activated Silk™ presents a number of technical problems to the end user. Firstly, a user will have to handle, transfer, and weigh out the loose flakes of dried silk. An accurate weight measurement of the material (in addition to the water) is paramount to obtaining a liquid of desired concentration that will perform and function as designed. Secondly, the milled flakes/particles of Activated Silk™ may or may not be riddled with static. This will make weighing out the material cumbersome but will also make the transfer into the reconstitution vessel difficult. Transferring the loose material to a container

for reconstitution may result in incidental loss of material. This will result in a diminished reconstituted yield and inability to achieve the desired concentration of solids dissolved in water.

This present disclosure provides a pre-dosed packing of pre-weighed material.
5 This eliminates the need to handle loose dry Activated Silk™ at point of use, which may help in more consistent reconstitution by minimizing loss on handling.

In one iteration, which used cryo-pelletized material, the solid, dry Activated Silk™ is extremely low density such that handling and material transfer becomes difficult. Fitting the desired mass of solid, dry material into a container for
10 reconstitution becomes a non-trivial process – as the material tends to be too large for a reasonably sized container. Additionally, the static issue of this material is compounded due to its low density. Due to the static and low weight, it is not uncommon for this material to shift out of place or fly out of the container in response to nearby movement. For this reason, the cryo-pelletized material is pressed into a
15 puck using a mold and handpress. This compressed puck of specific and predetermined dimensions is then able to be handled without difficulty.

Another significant technical problem is filtration. Reconstituting solid, dry Activated Silk™ frequently results in a miniscule amount of remaining solid particles. It is recommended to remove all solid particles prior to use, as any visible particles
20 may result in an inconsistent application of the liquid Activated Silk™. Gravity and vacuum filtration through cloth, paper filters, or mesh are commonly employed as a method for removing solid particles. All of these filtration methods require additional equipment and additional time-consuming processing steps. This invention discloses a product with built-in filtration, such that the no additional equipment or processing
25 steps are necessary. The loose solid, dry Activated Silk™ is enclosed in a nylon mesh pouch with 100 µm pore size. The nylon mesh acts as a filter for the undissolved material, which can be easily removed after the desired reconstitution. The nylon mesh pouch does not hinder the rate of reconstitution in any way, and its contribution/effect to the process is limited to the benefit it provides regarding
30 filtration. This removes the need for additional equipment or additional processing steps.

The following is a description for preparation of a pre-dosed packaging solution with built-in filtration including 3.5 g of dry, solid Activated Silk™. The resulting dimensions of the nylon pouch with 3.5 g material enclosed are 6 cm x 6 cm.

First, 15 cm x 12 cm 100 μ m nylon mesh was cut out. The nylon mesh was folded over so that it was 15 cm tall and a heat sealing apparatus was then used to seal the bottom most and side of the folded nylon. With two applied heat seals, the only remaining opening on the nylon pouch was the top – by which material was loaded.

- 5 Then the 3.5 g dry, solid Activated SilkTM was weighed out. When tray-frozen lyophilized Activated SilkTM was used, the loose material of particle size 1-3 mm was loaded into the pouch. A heat seal was then applied to the top of the pouch to make the 6 cm x 6 cm sealed off area (Fig. 96A). The excess nylon can be trimmed off with a scissor and discarded. For use in reconstitution, the pouch can then be placed into a
10 vessel, followed by the addition of 100 mL of water to produce a solution of roughly 3% in 2 hr. After 2 hr, the pouch can be removed and discarded. Note that the pouch may contain some foam or small particulates that have not completely solubilized.

- In the iteration where cryo-pelletized material is used, 3.5 g of dry, solid material was weighed and molded using a hand press and mold such that the resulting
15 press had a diameter of 4 cm and a height 2 cm. The loose & unpressed material was extremely low density, presenting issues with handling and static, as described previously. The molding of the material into a tight and cohesive puck or disc is a proven successful method for minimizing handling issues. Once the puck was removed from the mold, it was loaded into the top of the pouch. Then the top portion
20 of the pouch was sealed using the heat sealer to produce a sealed off area of 6 cm x 6 cm (Fig. 96B). The excess nylon can be trimmed off with a scissor and discarded. For use in reconstitution, the pouch can then be placed into a vessel, followed by the addition of 100 mL of water to produce a solution of roughly 3% in 2 hr. After 2 hr, the pouch can be removed and discarded. Note that the pouch may contain some foam
25 or small particulates that have not completely solubilized.

Example 20: Densification of Lyophilized Silk Fibroin

- Reconstituting solid, dry Activated Silk frequently results in an amount of remaining solid particles. It is recommended to remove all solid particles prior to use, as any visible particles may result in an inconsistent application of the liquid
30 Activated Silk. Gravity and vacuum filtration through cloth, paper filters, or mesh are commonly employed as a method for removing solid particles. All of these filtration methods require additional equipment and additional time-consuming processing steps by the end user. In a second iteration where these filtration methods are not

readily available, the tablet Activated Silk is enclosed in a nylon mesh pouch with 100 μm pore size. The nylon mesh acts as a filter for the undissolved material, which can be easily removed after the desired reconstitution. The nylon mesh pouch does not hinder the rate of reconstitution and its contribution/effect to the process is limited to the benefit it provides regarding filtration. This removes the need for additional equipment or additional processing steps.

Activated Silk used in these applications can be a composition of polypeptides that are derived from *B. mori* silkworm cocoons comprising of natural and/or unnatural polypeptides.

10 Preparation

Twenty-five grams of cryo-pelletized and lyophilized silk pellets are loaded into a cylindrical mold capable of holding the low bulk density volume pellets (90 mm ID, 160 mm height). A plunger is used to press the silk pellets into a tablet form targeting a density of 0.22 ± 0.02 g/ml. Acceptable range of density is above 0.2 g/ml and less than 0.27 g/ml. This density has been optimized to ensure integrity of the tablet and optimized reconstitution. Density above 0.32 g/ml has significant reduction in reconstitution in the desired time. Density below 0.2 g/ml tends to fall apart during handling. Alternatives shapes and/or weight can be used. Cylindrical shape was selected due to removal ease from manual press and mold.

The following is a description for preparation of a pre-dosed packaging solution with built-in filtration for a 3.5 g of lyophilized Activated Silk tablet. The tablet is contained in a 6 cm x 7.5 cm nylon pouch. Alternative material of constructions such as polylactic acid (PLA), muslin and other hydrophilic materials are also appropriate for this method. For use in reconstitution, the pouch can be placed into a vessel, followed by the addition of water to produce target concentration. The reconstitution is static and does not require manual or mechanical stirring. After 2 hr, the pouch can be removed and discarded. Note that the pouch may contain some foam or small particulates that have not completely solubilized. The pouch is to the removal of particulates. Concentration of up to 20% w/w have been made using this method. The final product is shown in FIG. 96B.

Example 21: Methods of Natural and Unnatural Silk Polypeptide Lyophilization

Currently liquid Activated Silk is prepared at 6% concentration and has a shelf life of from about 5 months at 4 °C to about 45 days at 25 °C. Without wishing to be bound by any particular theory, it is believed that either active or passive cooling could be required to ensure silk solution quality for a period of time, for example during transportation.

A solid, dry form of Activated Silk would weigh less due to the removal of water. Water is also the solvent that enables the interactions which contribute to a shorter shelf life. Therefore, dry version of Activated Silk would have a significantly longer shelf stable at room temp, and could be easily reconstituted/resolubilized in water at point of use. Activated Silk used in these applications can be a composition of polypeptides that are derived from *B. mori* silkworm cocoons comprising of natural and/or unnatural polypeptides.

There are two alternate pathways identified to produce a viable solid, dry Activate Silk that achieves the needs listed previously. The general pathway that represents both paths include 1) freezing, 2) primary drying, and 3) secondary drying. The primary way in which the two alternate pathways deviate is in relation to the freezing.

Path 1:

The first path towards a lyophilized solid, dry Activated Silk is tray freezing the material. Generally, the liquid Activated Silk is poured into deep trays where they are frozen. Due to bulk freezing, occasional uneven freezing has been observed. Once frozen, the trays are transferred into a lyophilizer that is capable of pulling vacuum and applying heat. Once dried, the material is milled and particle size distribution can be isolated. The methods and general procedure for tray-frozen lyophilization are in continued optimization. In an earlier procedure, using 20195 as an example batch, the liquid is frozen to roughly -30 °C. Primary drying then includes a rapid ramp of 10 °C to 35 °C shelf temperature, which roughly correlates to -30 °C to -10 °C product temperature. The secondary drying then occurs at 35 °C for several hours, wherein the product temperature rapidly rises from -10 °C to 35 °C. The material produced by these rapid changes in temperature was shelf stable and capable of reconstitution, however another round of optimization was necessary to

increase the rate of reconstitution to meet user needs. Without wishing to be bound by any particular theory, it was hypothesized that rapid temperature increase leads to heterogeneity in the lattice structure of the final product. This would present issues in the goal to reconstitute the product into water but also in scaling the procedure to larger quantities.

A tray-frozen lyophilization round most notably included a slower rate of temperature increase for primary and secondary drying. Specifically, the shelf temperature was increased at a rate of 0.6 °C/hr for the first 35 hr. It was hypothesized that the faster temperature ramp of the previous run negatively impacted time required to reconstitute due to the denser and heterogenous morphology that occurs when temperature jumps occur. Therefore, the aim with the 20316 tray-freezing lyophilization run was to have a very gradual primary and secondary drying time. Without wishing to be bound by any particular theory, it is believed that a slower and more gentle drying rate can be correlated with a looser and less dense morphology and cake structure, this looser cake structure can be more rapidly wetted and increase the rate of reconstitution. The freeze/dry procedure used for the 20195 produced a bulk density of **0.23 g/mL**, whereas the more gradual and controlled procedure used for the latter 20316 produced a bulk density of **0.16 g/mL**.

The optimization of slow temperature ramp during lyophilization does not address the uneven freezing observed which also has a negative impact to the morphology. This leads to the second path of lyophilization.

Path 2:

The second path of lyophilization is cryo-pelletization. This is a process that drips liquid Activated Silk at a controlled rate into a pool of liquid nitrogen, where it immediately freezes after exposure to about -196 °C. Frozen balls and/or pellets are formed from this process. These pellets are then transferred into a lyophilization chamber for lyophilization. The pellets provide additional surface areas for sublimation during the lyophilization process and in general can lyophilize the same amount of Activated Silk in less than half the time when compared to tray lyophilization.

In one example, the shelf temperature is brought to 38 °C and held there for 6 hr. The temperature of the shelf is then increased to 60 °C and maintained for 8 hrs.

For the final drying, the temperature is decreased to 27 °C and maintained until completely dry (< 4% moisture content).

5 In a second example, the cryo-pellets were put into a lyophilization chamber, brought below the triple point of the silk solution and was slowly ramped up to 140 F over the course of 30 – 50 hrs. The material is held at 140 F until the product temperature is equivalent to the shelf temperature in the lyophilizer. The time is further extended by at least 2 hrs to ensure completion of dryness in the full chamber. Vacuum level/vapor check is also confirmed to be stabilized. A pressure rise test can be performed to ensure material has gone through primary drying by quickly
10 isolating the chamber from the condenser for a short time (< 30 sec) and analyzing for pressure rise. If there is little or no pressure rise, this fulfills the criterion for primary drying. Additional heat up to 200 F can be added for secondary drying phase. Lower shelf temperature at 120 F during primary drying has also been used. Production size up to 200 L has been performed using this method.

15 In a third example, the cryo-pellets were brought down below the triple point of the silk solution and was slowly ramped up to 200 F over the course of 50 – 100 hrs. The material is held at 200 F until the product temperature is equivalent to the shelf temperature in the lyophilizer. Vacuum level/vapor check is confirmed to be stabilized. Production size up to 800 L has been performed using this method.

20 Meltback, which is a form of cake collapse, can take place if there is incomplete sublimation. Lyophilized silk that exhibits meltback do not reconstitute well or at all. Vacuum level stabilization is critical to ensure no meltback occurs when the vacuum is released from the chamber.

The benefit of instantaneous freezing at extreme temperature and controlling
25 the temperature in the lyophilization process is the ability to produce pellet that is low-density with no meltback. By freezing at this rate and temperature, the product can then be dried using slightly more extreme conditions when compared to the tray-frozen lyophilization and still maintain the loose pellet structure, with bulk density of < 0.06 g/mL. This is beneficial in scaling the process, by shortening cycle time and
30 allowing for more temperature flexibility.

Example 22: Densification of Lyophilized Silk Fibroin Minimizing/Eliminating Static

Liquid Activated Silk is difficult to transport and store due to its shelf-life requirements and limitations. One solution is to convert the Liquid Activated Silk into solid form. However, cryo-pelletized and then lyophilized solid Activated Silk presents some challenges to the end user. The dried solid has a bulk density of <0.6 g/ml and can be riddled with static. Due to the static and low weight, it is not uncommon for this material to shift out of place or fly out of the container in response to nearby movement. This makes weighing out the material cumbersome and could result in inaccurate weight measurement that is paramount to obtaining a liquid of desired concentration that will perform and function as designed. Secondly, due to the low bulk density, fitting the desired mass of solids into a mixing vessel becomes a non-trivial process. Thirdly, due to the low bulk density, the lyophilized solid tends to remain above liquid level without stirring and takes a long time to fully reconstitute. Without wishing to be bound by any particular theory, stirring is not recommended as it is believed it could initiate fibril formation. A silk fibroin tablet version was created to resolve this issue. By densifying the pellets into a tablet, it enables the tablet silk to be submerged below the liquid level, facilitating the reconstitution, and reducing the reconstitution time.

The following is a description of a process for making a densified version that is optimized for handling and to minimizes reconstitution time, including forming beads which enable handling without difficulty and minimize/eliminate static. Activated Silk used in these applications can be a composition of polypeptides that are derived from *B. mori* silkworm cocoons comprising of natural and/or unnatural polypeptides.

25 Preparation

Liquid Activated Silk is concentrated from an initial concentration of about 6% w/w up to about 50% w/w. Any other higher concentrations are used. Concentration can be achieved via conventional methods such as diafiltration and thin film evaporation, etc., or by reconstituting AS tablets. The liquid silk can be silk/natural polypeptide or silk/unnatural polypeptides.

The high concentration silk is then processed through cryo-pelletization. The liquid silk is dripped into a liquid nitrogen bath at a controlled rate to freeze into small beads. Beads are achieved by controlling the flow rate of the silk, viscosity of

the silk and product feed nozzle size. The material then can be lyophilized. Depending on the starting concentration of the liquid silk, different sizes and density of beads can be achieved. As the density of the bead increases, the static is reduced to further facilitate handling.

5 In the tablet form where the starting AS concentration is 6% w/w, the bulk density of the pellet is ≤ 0.06 g/ml with average density of 0.04 g/ml. The pellets must be densified into a tablet. By using higher concentration silks up to 30 %w/w, densities up to 0.22 g/ml were achieved. Full reconstitution was achieved under 2 hours with no agitation and the lower density beads can fully reconstituted as quickly
10 as 5-10 minutes. This result is illustrated in Fig. 100.

Example 23. Silk Fractionalization

The fractionation of silk is based on three chromatography principles:

- Size
- Charge
- 15 - Hydrophobicity
- And the combination of size, charge, and hydrophobicity

There are three pipelines of silk fractionalization:

1. Size exclusion chromatography as shown in Fig. 58.
- 20 2. Anion exchange chromatography followed by size exclusion chromatography as shown in Fig. 59.
3. Anion exchange chromatography followed by hydrophobic interactions chromatography and size exclusion chromatography shown in Fig. 60.

25 Table 46: Pipelines of Silk Fractionalization

	Low Skid Silk	Mid Skid Silk
Size exclusion chromatography	Generated	Generated
Anion exchange + size exclusion chromatography	Generated	Generated

Anion exchange + hydrophobic interactions + size exclusion chromatography	Generated	-
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Characterization Methods

- SDS PAGE
 - Dynamic light scattering (DLS)
 - Mw (Molecular weight) and PDI (Polydispersity) determination
 - Self-assembly assay
- 5

Table 47: Z-averages of Low Skid silk/modified polypeptide composition

Low Skid Silk/ Unnatural Polypeptide Composition	Z-Average (d. nm)
AS82	18.147
AS83	15.659
AS84	14.836
AS85	13.473
AS86	13.467
AS87	8.577
AS88	7.843
AS89	7.681

Example 24. Lyophilized Silk Stability

- Stability testing of tablets was conducted under accelerated aging conditions. The testing documented is based on multiple lots of AS-3003-103 and AS-5003-103 stored in 60 °C and 40 °C incubators packaged in vacuum sealed mylar bags. The real-time equivalent of the accelerated aging time points were calculated using the following formulas below. All formulas can be referenced in ASTM F1980 -
- 10
- 15
- Accelerated aging of sterile barrier systems for medical devices.

$$\text{Accelerated Aging Factor (AAF)} = Q_{10}^{\frac{T_a - T_s}{10}}$$

Where T_a = accelerated temp., T_s = storage temp. (25°C), and Q_{10} is estimated to be 2.

Therefore, the AAF for 60°C is 11.3 ($AAF = 2^{\frac{60-25}{10}} = 11.3$), and the AAF for 40°C is 2.8 ($AAF = 2^{\frac{40-25}{10}} = 2.8$)

5 **Table 48:** Lyophilized Silk Stability Test Method

Parameter	Acceptance Criteria	Test Method
Visual Inspection (Form and Color)	White to Off-White Solid	Visual
Reconstitution yield in DI water	>90%	Measured after 2 hr static (no agitation) reconstitution targeting a 5% solution Theoretical percent solids = 5% Measured percent solids = x % (oven method) Percent yield = x/5 * 100

Table 49: Lyophilized Silk Reconstitution Yield

Lot	Ground v. Pellet	Reconstitution Yield in DI water*
0075-23177-001	Pellet	96.7
0075-23177-001	Ground	79.1
0092-23193-001	Pellet	95.3
0092-23193-001	Ground	93.0

10

As shown in Fig. 103, particle size and reconstitution method will impact reconstitution yield. The finer grind silk powder stayed above liquid level and did not wet out for reconstitution using static method vs. the rougher grind.

15 **Aerosolized Activated Silk**

Activated Silk™ with preservatives was filled in bag on valve system aerosol aluminum canisters. Preservatives were added to prevent microorganism growth from impacting the aerosol testing. The system is a 1” continuous valve for bag on valve system with a bag length of 125 mm by COSTER. Spray rate was measured by

recording mass of aerosol can before and after spraying for 3 seconds. Spray rate (g/s) was monitored and is stopped when spray rate is <70% of the original value.

Table 50. Activated Silk with Preservatives

Solution Number	Low Mw Silk (mg/mL)	[Preservatives] (mg/mL)
1	10	9 mg/mL Aspen Bark Extract 5 mg/mL Sodium Anisate

5 Table 51. Aerosolized Activated Silk Microorganism Growth Results

Solution Number	Condition	Status after 1-month of daily spray	Status after 2.5 month with 1.5 month of paused spraying
1	Room Temperature #1	Good	Good
	Room Temperature #2	Good	No Good
	40C	No Good Occulsion occurred at day 12	N/A

*Measured after 2 hr static (no agitation) reconstitution targeting a 5% solution

Figs. 104A- 104C demonstrate the ability to spray Activated Silk™ and the dispersion of the droplets.

10

Table 52: Lyophilized Silk Stability

Silk Type	Low Mw Silk	Low Mw Silk	Low Mw Silk	Mid Mw Silk	Mid Mw Silk	Mid Mw Silk
Sample Lot #	VDF 494	LYO-004-002-L	LYO-004-003-L	MV-7	LYO-004-004-M	LYO-004-005-M
Storage Temp.	40 °C	60 °C	60 °C	40 °C	60 °C	60 °C
Simulated Age (Years)	4.7	9.2	13.6	4.8	6.8	4.0
Visual Inspection	White / Off-White	White / Off-White	White / Off-White	White / Off-White	White / Off-White	White / Off-White
Reconstitution yield in DI water	95%	96%	93%	94%	99%	99%
Result	Pass	Pass	Pass	Pass	Pass	Pass

The longest time point at 60° C was 430 days for low MW silk (equivalent to 13.3 years). The longest time point at 40° C was 621 days for low MW silk (equivalent to 4.8 years). The longest time point at 40 °C was 634 days for Mid MW silk (equivalent to 4.9 years). Based on accelerated shelf-life study, lyophilized silk is shown to be stable for 4+ year.

SEM images of Lyophilized Silk with Different Processing Conditions

Figures 101A- 101G show that different processing conditions enable unique silk structures. By varying pore structure, it is possible to tune reconstitution rate/release and utilize the structure as carrier for additives.

Example 25. Critical Material Attributes for Activated Silk™ freeze drying

Tg of frozen Activated Silk™ solution: -30C to -20C (DSC)

Tcollapse ~ -10C (Freeze Drying Microscopy)

15

Freeze drying approaches:

Bulk tray freeze drying:

Tray frozen lyophilization is performed by filling long shallow trays with 5 – 7% w/w concentrated liquid Activated Silk™. The trays are brought down to temperature -40 to -20°C (below Tg) to freeze. Once the liquid is frozen, vacuum and heat are applied to sublime the water from the bulk. Once removed, the solid cake is processed/milled to smaller particles that can be handled and used for reconstitution. Shrinkage during lyophilization is relatively common. This is caused by tensile stress built up within the bulk when the water is removed. However, for large scale processing, when the bulk “heaves” away from the tray and is no longer in contact with the heated surface of the tray, this could lead to ice remaining and creating meltback when the product is exposed to ambient condition. The uneven drying would also vary drying time.

The initial slow freezing (20-25hrs) also enables large ice crystals to grow. The large pores created once the ice crystals start to sublime could allow for structure collapse. The collapse creates a denser product with the potential of trapping moisture inside the structure, creating an environment for beta sheet formation and could

ultimately lead to poor reconstitution. Bulk tray freezing can produce acceptable product but variation is expected at commercial scale with this method (see Table 56).

Cryo freeze drying:

Activated Silk™ solution at 5 – 7% is first fed into a cryogenic bath to flash freeze the liquid solution. Spherical droplets are preferred to allow instantaneous and uniform freezing and drying, but alternative shapes such as elongated (elliptical) pellets may also be acceptable depending on applications. The droplet formation is influenced by various formulation factors such as viscosity, surface tension and solid content. Optimization of feed rate, drop height, nozzle size and residence time in the cryogenic bath based on the formulation factors is critical to ensuring pellet shape uniformity.

Table 53 Feed Rate Impact to Pellet Uniformity

	Feed Rate 1		2X Feed Rate 1	
	Diameter (mm)	AR	Diameter (mm)	AR
Average PS	4.68	1.17	6.23	1.19
Standard Deviation	0.86	0.18	1.58	0.15
Min PS	1.28	1.00	3.51	1.02
Max PS	9.24	2.33	12.36	1.67

Flash freezing at cryogenic temperature facilitates small ice crystal formation, providing a good structure to prevent collapse. The large surface area of the numerous pellets reduces drying time, improves product uniformity and reduces the risk of meltbalk during the drying process compared to the bulk tray freeze method. Common cryogenic fluids such as liquid nitrogen and liquid helium can be used. For Activated Silk™ cryopelletization, liquid nitrogen was selected based on boiling temperature, availability and cost. The cryo pellets can be stored below Tg to ensure the structure is maintained before lyophilization. It is preferable to pre-chill lyophilization trays during pellet loading to minimize any melting. Commercial scale freeze dryers are used for lyophilization. Freeze dryers need to have shelf temperature control and have product temperature and pressure monitoring for the expected Critical Process Parameters. Additional features such as front/back loading for dry/clean room processing or ability for wash down/sanitization are

beneficial. Units from Manufacturers such as Cuddon Freeze Dry and Parker Freeze Dry or others can be used depending on product and application requirements. Critical Material Attributes such as T_g and T_c are considered for the lyophilization process. At the start of lyophilization, the pellets are brought below the T_g to maintain structure integrity and the condition of the chamber is monitored to confirm it is below the triple point (0.01°C, 0.00603 atm) before heating is started. The chamber is held at this condition for an extended amount of time (30 - 120 min) to ensure proper sublimation will take place. During the initial primary drying phase, the shelf temperature is slowly ramped up to drive sublimation. Initial temperature difference between shelf and product temperature does not need to be very large in order for sublimation to occur. It is typical for the product temperature to be within 5 – 10 degrees of the shelf temperature. However, as the product dries and becomes more insulated, it requires more energy (heat) to maintain the sublimation. Temperature difference between shelf and products can be >40 degrees. Various temperature profiles were trialed to optimize Critical Process Parameters (freezing temperature/rate, temperature and time) to ensure product meets Critical Quality Attributes (appearance, water content, reconstitution time). Profile examples are provided in Figures 109 and 110. Lyophilization is determined to be completed once the product temperature reaches shelf temperature and the water vapor pressure detected by the Pirani gauge has plateaued (< 280mTorr) and is similar to the pressure detected by the capacitance manometer. Due to the batch size, heating is held for an additional 2 hours after this condition is met to ensure uniform lyophilization in the chamber.

Typically, it is preferable to process products below T_c to ensure physical structure, protein functionality and stability of final product. This was done for Activated Silk™ solution at bench scale. However, to ensure product has a reasonable cycle time at commercial scale (190kg to 850kg), the current process for the cryopelletized Activated Silk™ does dry above the T_c during mid drying. *(Increasing the product temperature by 1°C. during lyophilization could reduce the primary drying time by 13%. The Critical Formulation Temperature in Freeze Drying.*

Optimization by changing temperature rate, shelf hold temperature and time below T_c was performed to minimize/eliminate meltback (Figure 108) and discoloration (Figure

109). An example of the profile 5 is shown in Table 54 with the critical quality attributes of the final products being met shown in Table 56.

Table 54 Activated Silk™ Processing Example Profiles

Vendor	VDF (Low and Mid Hw New Silk)	VDF (Pilot), Udon and ERN (Low Hw New Silk)	ERN (Low Hw and Mid Hw New Silk)
Equipment style	Profile 1 (Equipment 1)	Profile 2 (Equipment 2)	Profile 5 (Equipment 2)
Profile	Start at -40°F	Start at -40°F	Start at -40°F
	30 min vacuum pull with no heating	30 min vacuum pull with no heating	120 min vacuum pull with no heating
	30 minute ramp to 100°F	300 min ramp to 200°F	600 min ramp to 120°F
	4 hour ramp to 180°F	Hold at 200°F until complete	180 min hold at 120°F
	30 minute ramp to 120°F and hold until dry		120 min ramp to 140°F
Kg/hr (Normalized Process Rate)	0.43	0.44	0.32
Observation	Meltback observed in a few trays	Meltback observed in a few trays	No meltback observed
Approximate hrs below Tc	21 - 24*	4 - 6	9 - 11

* Batch size is 4% compared to others

Table 55 Activated Silk™ Processing Example Profiles

Vendor	VDF (Low and Mid Hw New Silk)	Nidrauka (Mid Hw New Silk)	Nidrauka (Mid Hw New Silk)	ERN (Low Hw and Mid Hw New Silk)
Equipment style	Profile 1 (Equipment 1)	Profile 3 (Equipment 2)	Profile 4 (Equipment 2)	Profile 5 (Equipment 2)
Profile	Start at -40°F	Start at -40°F	Start at -40°F	Start at -40°F
	30 min vacuum pull with no heating	30 min vacuum pull with no heating	30 min vacuum pull with no heating	120 min vacuum pull with no heating
	30 minute ramp to 100°F	300 min ramp to 180°F	190 min ramp up from -40°F to 40°F	600 min ramp to 120°F
	4 hour ramp to 180°F	300 min hold at 180°F	300 min ramp to 100°F (hold for 5 hrs)	180 min hold at 120°F
	30 minute ramp to 120°F and hold until dry	180 min ramp to 140°F and hold until dry	300 min hold at 100°F	120 min ramp to 140°F
Kg/hr (Normalized Process Rate)	0.43	0.44	0.22	0.32
Observation	Meltback observed in a few trays	Meltback and product discoloration	Product discoloration	No meltback observed
Approximate hrs below Tc	21 - 24*	1 - 3	3 - 5	9 - 11

* Batch size is 4% compared to others

Table 56 Critical Quality Attributes of Lyophilized Activated Silks™

Batch	Method	Profile	Freezing Temp of Activated Silk™ (°C)	Bulk Density (g/ml)	Moisture Content (%)	50ug/ml Idle Reconstitution % yield at 120 min	Observation
Product Target	-	-	-	<0.06	<4%	>90%	Homogenous color/appearance
20195	Tray	Tray	-30	0.23	7.83	86.0 ± 6.7	Meltback
20280T	Tray	Tray	-20	0.36	7.12	78.6 ± 18.0	Meltback
20280C	Cryo	1	-195	0.05	1.6	95.2 ± 2.6	Meltback
L-EBN09822	Cryo	2	-195	0.04	1.7	93.0 ± 1.1	Meltback
M-EBN11222	Cryo	3	-195	0.04	0.8	96.9 ± 1.1	Meltback and discoloration
M-EBN13422	Cryo	4	-195	0.04	1.3	95.0 ± 5.7	Slight discoloration
LYO-004-001-M	Cryo	5	-195	0.04	1.1	93.9 ± 3.9	White pellet

To determine material handling for end users at large scale, flow characteristics of lyophilized Activated Silk™ products were assessed. The Hausner ratio is an indirect

measure of the property of a bulk material (bulk density) to reduce its volume under mechanical influence (tapped density) as well as a measure of the interaction between particles. Factors such as particle shape (e.g. non-spherical vs. spherical) and cohesiveness of particles (e.g. electrostatic, van der waals) will influence how well a powder or granule can flow or fluidize.

Table 57 Flow characteristics of lyophilized Activated Silk™ products

Batch	Method	Hausner Ratio	% Solid of Feed during cryopelletization
20195	Tray	1.26	6
20280T	Tray	1.24	6
LYO-004-004-M	Cryo	1.22	6
LYO-004-003-L	Cryo	1.34	6

Table 58 Chart reproduced from Fitzpatrick, John (2013). Powder properties in food production system.

Flow Character	Hausner Ratio
Excellent	1.00 – 1.11
Good	1.12 – 1.18
Fair	1.19 – 1.25
Passable	1.26 – 1.34
Poor	1.35 – 1.45
Very poor	1.46 – 1.59
Very, very poor	> 1.60

The flowability of the tray lyophilized samples was mainly influenced by the particle shape. Even though the samples were sieved (125 – 1180 micron), the flowability of the final product is only passable. Further work can be done to narrow the distribution of the particle size to improve the flowability.

The cryopelletized samples were tested without further processing and had a diameter range of 4000 – 9000 micron. These samples were very heavily influenced by the cohesiveness of the pellets. The low density and low moisture pellets had high static charge and tend to adhere to surfaces. Static charge removal or neutralization techniques can be used to minimize this issue. Alternatively, a higher density and/or higher moisture pellets can also be produced to eliminate the problem.

One method of increasing the density of the lyophilized pellets is to increase the concentration of the Activated Silk solution before cryopelletization. Liquid Activated Silk can be concentrated from the commercially available 6% w/w up to 50% w/w. Concentration can be achieved via conventional methods such as tangential flow filtration (TFF), thin film evaporation (TFE) and/or reconstituting lyophilized Activated silk to the desired concentration.

Thin Film Evaporation:

Thin film evaporation was assessed to concentrate Activated Silk™.

At bench scale, Activated Silk™ at ~6%w/w was concentrated using a Buchi Rotovapor R-100 to simulate a thin film evaporation process. Activated Silk™ was processed up to 49C for over 100 min. No degradation was observed. Molecular weight and polydispersity were found to be within expected range and close to the control, which was made reconstituting lyophilized Activated Silk™.

Table 59 Molecule Weight of Activated Silk™ at different process conditions

Trial	Max Temp (°C)	Time (min)	% Solids after Concentration	Molecular Weight (Target 14000 - 30000 Da)	Polydispersity (<3.4)
Control	N/A	N/A	18.0	16995	2.5
1	49	104	12.2	15672	2.5
2	45	70	14.3	15750	2.5
3	45	100	15.1	15025	2.5

A horizontal mechanically-aided thin-film evaporator (Artisan Rototherm E) was used to assess commercial scale viability. The flow is once-through and product residence time is controlled. Material fed into the rototherm is held against the heated wall by means of the centrifugal force exerted by the rotor blades and material is constantly renewed as the more concentrated material is displaced towards the bottom discharge nozzle by the incoming feed. The concentrated products exit the bottom outlet of the unit and the vapors exit the rototherm through a vapor outlet where it is connected to a condenser.

Table 60 Rototherm E Processing Conditions

Sample ID	Date	8/21/2019	8/22/2019	8/23/2019
	Time	10:55 AM	1:14 PM	12:03 PM
	Run #	1	2	3
	Sample #	1001	1002	2001
Temperatures [F]	2 Rototherm Film	91	101	120
	3 Vapor	91	92	93
	4 Distillate	51	53	52
	5 Hot Oil Supply to Rototherm	229	270	229
	7 Hot Oil Return to Rototherm	327	266	227
	8 Chilled Water Supply to Condenser	33	33	31
	9 Chilled Water Return from Condenser	37	37	35
Pressures	PT101 System [Torr]	40	40	40
	Feed Line [psig]	3	3	6
Rates	Feed Setpoint [mL/min]	114	114	164
	Feed Setpoint [lb/hr]	15	15	20
	Bottoms [lb/hr]	5.625	0.930	13.200
	Distillate [lb/hr]	9.525	14.085	7.440
	Feed Calculated [lb/hr]	15.150	15.015	20.640
Other	Run Duration [minutes]	20	20	10
	% Rotor Speed	100	80	70
	Rotor Power Draw [kW]	0.73-0.82	0.58-0.68	0.62-0.66
	% Evaporation	63%	94%	36%
	% Solids in Feed	2.3%	2.3%	6.0%
	% Solids in Bottoms	6.2%	37%	9%
	N2 Sparge [lb/hr]	-	-	-

- 5 Based on the test results, the use of thin film evaporation is a viable method of concentrating Activated Silk™. This was achieved at a contact temperature lower than 49 °C. No degradation is expected based on the rotovap experiment.

Tangential flow filtration (TFF):

Refer to Activated Silk™ Purification Methods.

10 Comparison of cryopelletized lyophilized Activated Silk™

ImageJ, an image processing program was used to evaluate lyophilized samples:

Table 61 Aspect ratio of lyophilized pellets at different concentration feed

	6% Cryo Lyophilized pellets		10% Cryo Lyophilized pellets		17% Cryo Lyophilized pellets	
	Diameter (mm)	Aspect Ratio	Diameter (mm)	Aspect Ratio	Diameter (mm)	Aspect Ratio
Average	6.1	1.10	5.1	1.06	5.1	1.06

Standard Deviation	1.0	0.09	0.8	0.05	0.5	0.06
Min	4.1	1.01	4.1	1.01	4.3	1.01
Max	8.6	1.45	8.1	1.34	6.4	1.31

Aspect ratios of the lyophilized pellets showed spherical to elliptical in shape with a trend towards more spherical as the concentration increases. It is expected that changes in feed rate, drop height, nozzle size can also be optimized to achieve a more spherical shape.

The flowability of the lyophilized pellets improved with increased concentration during cryopelletization:

Table 62 Hausner Ratio of lyophilized pellets at different concentration feed

% Solid of Feed during cryopelletization	Hausner Ratio
6	1.34
10	1.09
17	1.10

The specific surface area of the cryopelletized and lyophilized pellets were measured via Brunauer-Emmett-Teller (BET) analysis of volumetric nitrogen adsorption isotherms. The large surface areas along with the large pore size of the pellets provide considerable contact area to enable fast reconstitution. Pore diameters also show that the pellets are mesoporous (pore size between 2 to 50 nm). Mesoporous particles have a solid framework with porous structure and large surface area, which may allow the attachment of different functional group for use in formulated work in the future.

Method:

Instrument: 3 Flex Surface Characterization Tool

Degas at 80C for 12 hours

Table 631 BET Specific Surface Area

Sample	BET surface area (m2/g)	Pore size (nm)	Pore Volume (cm3/g)
Lyo-004-001M (6%)	3.86 ± 0.65	8.55 ± 4.1	0.00370 ± 0.0021
0092-23193-001 (10%)	4.93 ± 0.50	5.96 ± 1.5	0.00520 ± 0.0010
0075-23177-001 (16.7%)	5.22 ± 0.58	4.77 ± 0.4	0.00754 ± 0.0023

5 Note: Samples were tested in triplicate.

In the SEM micrograph, the highly porous structure was confirmed and show radially orientated microchannels. This is resulted from the ice growth that started from the outer surface when the pellets come into contact with the liquid nitrogen and grow inward to the core. Lot 0075-23177-001 had largest surface area and lot Lyo004-001M had the largest pore size.

10

Conclusions

15 Process development has resulted in a commercially viable product that is easy to handle and meets critical quality attributes.

Example 26. Densification beads of Lyophilized Silk Fibroin for Reconstitution

The densification of lyophilized silk fibroin minimizing/eliminating static and improving use by end users.

20 This disclosure provides a densified version that is optimized for handling and to minimizes reconstitution time. These beads enable handling without difficulty and minimize/eliminate static. Activated Silk used in these applications can be a composition of polypeptides that are derived from *B. mori* silkworm cocoons comprising of natural and/or unnatural polypeptides.

25 The following is a description for the preparation of the Activated Silk (AS) bead form:

Liquid Activated Silk is concentrated from the commercial concentration of 6% w/w up to 50% w/w. Concentration can be achieved via conventional methods such as diafiltration and thin film evaporation, etc., or by reconstituting AS tablets. The liquid silk can be silk/natural polypeptide or silk/unnatural polypeptides.

- 5 The high concentration silk is then processed through cryo-pelletization. The liquid silk is dripped into a liquid nitrogen (LN2) bath at a controlled rate to freeze into small beads. Beads are achieved by controlling the flow rate of the silk, viscosity of the silk and product feed nozzle size. The material then can be lyophilized. Depending on the starting concentration of the liquid silk, different sizes and density of beads can be
- 10 achieved. As the density of the bead increases, the static is reduced to further facilitate handling.

- In the tablet form where the starting AS concentration is 6%w/w, the bulk density of the pellet is $\leq 0.06\text{g/ml}$ with average density of 0.04g/ml . The pellets must be densified into a tablet. By using higher concentration silks up to 30%w/w, densities up to 0.22g/ml were
- 15 achieved (the target of the tablet silk in previous filing). Full reconstitution was achieved under 2 hours with no agitation and the lower density beads can fully reconstituted as quickly as 5-10 minutes.

Example 27. Stressed Silk: In Pursuit of a Stable Solution

- 20 Described herein is method development to quantify formulation stability, improve shelf life, and enable an aerosol product.

Goal:

- Develop a silk aerosol having robust shelf-life stability profile and consistent spray performance
- 25 • Slow aggregation process of aq. silk suspensions
- Understand effects of excipients/formulation additives on silk stability

Approach:

- Researched excipients commonly used in FDA-approved vaccines/therapeutic protein solutions
- 30 • Subject silk/excipient solutions to accelerated aging conditions
- Capture and quantify formation of pre-gelation aggregates in solution over time using DLS
 - Target: Minimize / slow increase in particle size over time

- Next: Benchmark formulation testing in aerosol system

Methodology

Dynamic Light Scattering (DLS) allows for the quantification of the rate of aggregation of silk solutions and the impact of various excipients to system stability. DLS measures the fluctuations in scattered light to determine the size and motion of particles or molecules in a solution. Using DLS, the “z-average” particle size can be measured, which is an intensity-weighted measurement of mean hydrodynamic diameter. In this way, samples can be subjected to accelerated aging conditions, and z-average can be measured over time as pre-gelation aggregates (1nm to ~1µm) start to form through self-association.

Key Learnings:

Buffering agents like PBS (and other ionic solutions) drastically slow the rate of aggregation of 33B over a wide concentration range (1 - 30 mg/mL silk). Incubation temperature and Silk Type (27P and 33B) have the largest impact on aggregation rate.

Aggregation rate of silk-only systems appears relatively consistent across replicate runs having equivalent input factors, but the time-to-gelation and z-avg size at or just before gelation are variable (33B). 33B is the best model system in which to test excipient formulations (fastest aggregation rate → quicker observed impact of excipient).

Data Summary:

Figure 118 illustrates in silk-only controls, 27P is unsurprisingly orders of magnitude more stable than 33B over a range of temperatures and concentrations (Below Left, Top Right). However, 33B’s faster aggregation makes it a useful model system in which to study excipient impact on aggregation rate (Below Right). (n = 3 replicates unless otherwise noted).

Figures 120 and 121 illustrate that buffering is relatively concentrated (10mg/mL) 33B silk in PBS drastically reduces rate of aggregation under accelerated conditions. Data hints at interesting interaction effects when surfactants like Polysorbate-80 are added into PBS-buffered silk solutions.

Figure 122 illustrates results from analyzing other salts alongside PBS confirm stabilizing properties of ions in solution. When ionic strength was held constant, other salts matched (or beat) PBS's stabilizing ability.

Excipient Overview and Mechanisms

5 Ionic strength/Salts

- Directly influence conformational integrity and stability
- Have been shown to dramatically affect the amount of protein aggregation induced by temperature and agitation
- Anions > cations in terms of influence on protein structure

10 Buffering agents (pH)

- Maintain solution pH (which greatly impacts protein higher-order structure)
- Can have specific ion effects, impacting chemical and conformational stability.

Surfactants

- #### 15
- Outcompete/shield protein molecules from hydrophobic surfaces (like air-water interfaces), which prevents protein unfolding
 - May directly interact with hydrophobic regions in protein molecules

Sugars/Osmolytes

- #### 20
- Stabilize proteins through preferential hydration (increasing the amount water molecules can associate with protein surface, increasing solubility)
 - Protect protein structure during stressful events (like lyophilization)

Amino Acids

- Stabilize via a variety of mechanisms: preferential hydration, direct binding, buffering capacity, antioxidant properties

25

Figure 126 illustrates concentration dependent aggregation. In this experiment, higher concentration lead to faster aggregation.

DLS Method:

- #### 30
- In solution, particles are constantly moving due to interaction with random movement of solvent molecules (Brownian motion).

DLS measures the diffusion rate of these particles in solution, which yields a proportional scattered light signature. This diffusion speed is proportional to particle size (larger particles → slower diffusion → unique scattered light signature).

5 Scattered light is measured as a spectra based on the distribution of various-sized particles in solution (Intensity curves).

Intensity curves are reflective of hydrodynamic diameter (spherical equivalent of particle + surrounding electric double layer in solution) that corresponds to the measured rate of diffusion. Z-average particle size is the intensity weighted mean hydrodynamic diameter. All this to say: DLS can be used to measure particle sizes
10 (~1nm to 1µm) in solution.

Measurement summary: Solution is hit with a laser; Scattered light is measured; DLS software reads light signature, makes assumptions about particles, and back-calculates particle size distribution that correspond to the scattered light signature

Benefits of DLS:

- 15
- Easy to use
 - Relatively fast; ~8 minutes per sample
 - Non-destructive measurement: samples can be measured again and again to track changes (less variability!)
 - Temperature control available; may be able to accelerate testing more than the
20 current technique (40C → 70C)
 - Great for identifying trends in particle size over time

Limitations of DLS:

- 25
- Long run times across solutions using current methodology
 - Assumes particles are perfect spheres in solution to simplify curve fitting; proteins are not perfect spheres
 - Hydrodynamic diameter is not the same as particle size; HD is particle PLUS surrounding electric double layer, which can be influenced by ionic strength and viscosity of solution.

30

 - Despite this, trends over time still hold
 - When particle size reaches a certain threshold, measurements get noisy
 - Only one sample can be measured at a time

- Control samples for every formulation iteration need to be tracked alongside silk samples
- This quickly balloons the number of samples required to measure one excipient condition

5

Interpreting One-Phase Association Model Metrics

As shown in Figures 133A- 133C, 70C curves were analyzed via the One-Phase Association model in Prism software to extract better quantitative data from samples. Figure 132 shows the model used.

$$10 \quad y = y_0 + (\text{Plateau} - y_0) * (1 - e^{-kx})$$

y_0 : starting z-average value of silk+excipient. Since the z-average particle size is heavily influenced by the excipients in solution (anything that affects the electric double layer of particles in solution), this value will vary for a given particle size. However, generally; the smaller y_0 , the more “room to run” a silk solution has before aggregation.

15

Plateau: The higher the plateau, the higher the particle size in solution and closer to aggregation. Lower plateaus = more stable solution.

20 k : reflects the shape of the graph. Smaller k = slower initial aggregation = more “linear” appearing graph. Larger k = faster initial aggregation = more “curved” looking graph

Span: reflects the total projected change in z-average from 70C exposure. Smaller span = smaller total change in aggregation = more stable solution.

25

Figures 134- 137 show results of the One-Phase Association Model. The results show that salts have potential to stabilize otherwise aggregation-prone solutions.

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CLAIMS

1. A plurality of substantially solid silk fibroin particles comprising silk fibroin fragments, the particles being characterized by at least one of: bulk density,
5 surface area, pore size, pore volume, aspect ratio, and/or Hausner ratio.
2. The plurality of substantially solid silk fibroin particles of claim 1, wherein the substantially solid silk fibroin particles are annealed.
- 10 3. The plurality of substantially solid silk fibroin particles of claim 1 or 2, wherein the substantially solid silk fibroin particles are grounded.
4. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 3, wherein the substantially solid silk fibroin particles have a bulk density of less
15 than 0.03 g/ml, less than 0.04 g/ml, less than 0.05 g/ml, less than 0.06 g/ml, less than 0.07 g/ml, less than 0.08 g/ml, less than 0.09 g/ml, less than 0.10 g/ml, less than 0.11 g/ml, less than 0.12 g/ml, less than 0.13 g/ml, less than 0.14 g/ml, less than 0.15 g/ml, less than 0.16 g/ml, less than 0.17 g/ml, less than 0.18 g/ml, less than 0.19 g/ml, less than 0.20 g/ml, less than 0.21 g/ml, less than 0.22 g/ml, less than 0.23 g/ml, less than
20 0.24 g/ml, or less than 0.25 g/ml, less than 0.26 g/ml, less than 0.27 g/ml, less than 0.28 g/ml, less than 0.29 g/ml, less than 0.30 g/ml, less than 0.31 g/ml, less than 0.32 g/ml, less than 0.33 g/ml, less than 0.34 g/ml, or less than 0.35 g/ml.
5. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 3, wherein the substantially solid silk fibroin particles have an average bulk
25 density of about 0.03 g/ml, about 0.04 g/ml, or about 0.05 g/ml, about 0.03 g/ml, about 0.04 g/ml, about 0.05 g/ml, about 0.06 g/ml, about 0.07 g/ml, about 0.08 g/ml, about 0.09 g/ml, about 0.10 g/ml, about 0.11 g/ml, about 0.12 g/ml, about 0.13 g/ml, about 0.14 g/ml, about 0.15 g/ml, about 0.16 g/ml, about 0.17 g/ml, about 0.18 g/ml,
30 about 0.19 g/ml, about 0.20 g/ml, about 0.21 g/ml, about 0.22 g/ml, about 0.23 g/ml, about 0.24 g/ml, about 0.25 g/ml, about 0.26 g/ml, about 0.27 g/ml, about 0.28 g/ml, about 0.29 g/ml, about 0.30 g/ml, about 0.31 g/ml, about 0.32 g/ml, about 0.33 g/ml, about 0.34 g/ml, or about 0.35 g/ml.

6. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 5, wherein the substantially solid silk fibroin particles have a Hausner ratio of between 1.00 and 1.11, between 1.12 and 1.18, between 1.19 and 1.25, between 1.26 and 1.34, or between 1.35 and 1.45.

5

7. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 6, wherein the substantially solid silk fibroin particles have an average diameter of between about 3 mm and about 10 mm.

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8. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 6, wherein the substantially solid silk fibroin particles have an average diameter of about 3 mm, about 4 mm, about 5 mm, about 6 mm, about 7 mm, about 8 mm, about 9 mm, or about 10 mm.

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9. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 8, wherein the substantially solid silk fibroin particles have an aspect ratio between 1 and about 1.45.

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10. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 8, wherein the substantially solid silk fibroin particles have an aspect ratio of 1, about 1.10, about 1.15, about 1.20, about 1.25, about 1.30, about 1.35, about 1.40, or about 1.45.

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11. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 8, wherein the substantially solid silk fibroin particles are substantially spherical.

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12. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 11, wherein the substantially solid silk fibroin particles are mesoporous.

13. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 11, wherein the substantially solid silk fibroin particles have a BET (Brunauer–Emmett–Teller) surface area between about 2.50 m²/g and about 6.50 m²/g.

14. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 11, wherein the substantially solid silk fibroin particles have a BET (Brunauer–Emmett–Teller) surface area of between about 2.50 m²/g and about 3.00 m²/g, about 3.00 m²/g and about 3.50 m²/g, about 3.50 m²/g and about 4.00 m²/g, about 4.00 m²/g and about 4.50 m²/g, about 4.50 m²/g and about 5.00 m²/g, about 5.00 m²/g and about 5.50 m²/g, about 5.50 m²/g and about 6.00 m²/g, or about 6.00 m²/g and about 6.50 m²/g.

15. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 14, wherein the substantially solid silk fibroin particles have an average pore size between about 25 Å and about 500 Å.

16. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 14, wherein the substantially solid silk fibroin particles have an average pore size of about 25 Å to about 30 Å, about 30 Å to about 35 Å, about 35 Å to about 40 Å, about 40 Å to about 45 Å, about 45 Å to about 50 Å, about 50 Å to about 55 Å, about 55 Å to about 60 Å, about 60 Å to about 65 Å, about 65 Å to about 70 Å, about 70 Å to about 75 Å, about 75 Å to about 80 Å, about 80 Å to about 85 Å, about 85 Å to about 90 Å, about 90 Å to about 95 Å, about 95 Å to about 100 Å, about 100 Å to about 105 Å, about 105 Å to about 110 Å, about 110 Å to about 115 Å, about 115 Å to about 120 Å, about 120 Å to about 125 Å, about 125 Å to about 130 Å, about 130 Å to about 135 Å, about 135 Å to about 140 Å, about 140 Å to about 145 Å, or about 145 Å to about 150 Å.

17. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 16, wherein the substantially solid silk fibroin particles comprise a plurality of radially orientated microchannels.

18. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 17, wherein the substantially solid silk fibroin particles further comprise an emulsifier, a surfactant, a buffering agent, an amino acid, or a sugar.

19. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 17, wherein the substantially solid silk fibroin particles further comprise a

polysaccharide, a polysorbate, a glycoside, PBS, arginine, trehalose, glucose, or sucrose.

20. The plurality of substantially solid silk fibroin particles of any one of
5 claims 1 to 17, wherein the substantially solid silk fibroin particles further comprise a surfactant selected from sucrose ester, cetearyl glucoside, caprylyl/capryl glucoside, sucrose laurate, sucrose palmitate, sucrose stearate, sucrose cocoate, sorbitan monostearate, and combinations thereof.

10 21. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 17, wherein the substantially solid silk fibroin particles further comprise an additional protein or peptide, a C12-C24 fatty alcohol, a glycolipid, or a lipid.

15 22. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 17, wherein the substantially solid silk fibroin particles further comprise a protein, a peptide, a sugar surfactant, a biosurfactant, a lipid, or a combination.

20 23. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 17, wherein the substantially solid silk fibroin particles further comprise a sugar fatty acid ester, a sugar fatty acid monoester, a sugar fatty diester, a sugar fatty triester, or a sugar fatty and polyester.

25 24. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 17, wherein the substantially solid silk fibroin particles further comprise a sucrose fatty acid ester, a sorbitan or sorbitol fatty acid ester, an alkyl glucoside, an alkyl polyglucoside, or a combination thereof.

30 25. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 17, wherein the substantially solid silk fibroin particles further comprise KCl, NaCl, MgCl₂, CaCl₂, PBS, Tris, Polysorbate 20, Polysorbate 80, Capryl Glucoside, Sucrose, Histidine, Glycine, or Arginine.

26. A silk fibroin nanoclay composite or film, comprising silk fibroin fragments and a clay, wherein the % (w/w) of clay in the composite is from about 1% to about 99%.

5 27. The nanoclay composite or film of claim 26, wherein the clay is a bentonite clay.

28. The nanoclay composite or film of claim 26 or 27, wherein the concentration of clay in the composite or film is from about 20% (w/w) to about 33%
10 (w/w), from about 33% (w/w) to about 50% (w/w), or from about 50% (w/w) to about 67% (w/w).

29. The nanoclay composite or film of any one of claims 26 to 28, wherein the water vapor permeance (WVP) of the composite is inversely proportional to the
15 concentration of clay in the composite.

30. The nanoclay composite or film of any one of claims 26 to 28, wherein water vapor permeance (WVP, $\text{g/m}^2 \cdot \text{Pa} \cdot 24\text{h}$) of the composite is from about 0.20 to about 0.30, from about 0.30 to about 0.35, from about 0.35 to about 0.40, from about
20 0.40 to about 0.45, from about 0.45 to about 0.50, from about 0.50 to about 0.55, from about 0.55 to about 0.60, from about 0.60 to about 0.65, from about 0.65 to about 0.70, from about 0.70 to about 0.75, from about 0.75 to about 0.80, or from about 0.80 to about 0.85.

25 31. A stabilized silk fibroin solution comprising silk fibroin fragments and a stabilizer, wherein:

- i) the solution has a z-average value lower than a substantially similar silk fibroin solution comprising silk fibroin fragments but excluding the stabilizer; and/or
- ii) the solution has a z-average plateau value lower than a substantially similar
30 silk fibroin solution comprising silk fibroin fragments but excluding the stabilizer.

32. The stabilized silk fibroin solution of claim 31, wherein the z-average is measured after a period of time after the silk fibroin fragments and the stabilizer are co-formulated, wherein the period time ranges from 1 hour to 250 hours, from 1 hour

to 350 hours, from 1 hour to 450 hours, from 1 hour to 550 hours, from 1 hour to 650 hours, or from 1 hour to 1000 hours.

33. The stabilized silk fibroin solution of claim 31, wherein the z-average is
5 measured after a period of time after the silk fibroin fragments and the stabilizer are co-formulated, wherein the period time ranges from 1 minute to 10 minutes, from 1 minute to 20 minutes, from 1 minute to 30 minutes, from 1 minute to 40 minutes, from 1 minute to 50 minutes, from 1 minute to 60 minutes, from 1 minute to 70 minutes, or from 1 minute to 80 minutes.

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34. The stabilized silk fibroin solution of any one of claims 31 to 33, wherein the stabilizer is an emulsifier, a surfactant, a buffering agent, an amino acid, or a sugar.

15 35. The stabilized silk fibroin solution of any one of claims 31 to 33, wherein the stabilizer is a polysaccharide, a polysorbate, a glycoside, PBS, arginine, trehalose, glucose, or sucrose.

20 36. The stabilized silk fibroin solution of any one of claims 31 to 33, wherein the stabilizer is a surfactant selected from sucrose ester, cetearyl glucoside, caprylyl/capryl glucoside, sucrose laurate, sucrose palmitate, sucrose stearate, sucrose cocoate, sorbitan monostearate, and combinations thereof.

25 37. The stabilized silk fibroin solution of any one of claims 31 to 33, wherein the stabilizer is an additional protein or peptide, a C12-C24 fatty alcohol, a glycolipid, or a lipid.

30 38. The stabilized silk fibroin solution of any one of claims 31 to 33, wherein the stabilizer is a protein, a peptide, a sugar surfactant, a biosurfactant, a lipid, or a combination.

39. The stabilized silk fibroin solution of any one of claims 31 to 33, wherein the stabilizer is a sugar fatty acid ester, a sugar fatty acid monoester, a sugar fatty diester, a sugar fatty triester, or a sugar fatty and polyester.

40. The stabilized silk fibroin solution of any one of claims 31 to 33, wherein the stabilizer is a sucrose fatty acid ester, a sorbitan or sorbitol fatty acid ester, an alkyl glucoside, an alkyl polyglucoside, or a combination thereof.

5 41. The stabilized silk fibroin solution of any one of claims 31 to 33, wherein the stabilizer is KCl, NaCl, MgCl₂, CaCl₂, PBS, Tris, Polysorbate 20, Polysorbate 80, Capryl Glucoside, Sucrose, Histidine, Glycine, or Arginine.

10 42. The stabilized silk fibroin solution of any one of claims 31 to 41, wherein the solution is sprayable.

 43. A liquid in air suspension comprising a plurality of droplets comprising the stabilized silk fibroin solution of claim 42, wherein the droplets are sufficiently stable after being sprayed, for a period of time necessary to reach a surface.

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 44. A plurality of drops or droplets comprising the stabilized silk fibroin solution of any one of claims 31 to 41, wherein the droplets or drops are sufficiently stable after being formed, for a period of time necessary to reach a surface.

20 45. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 25, the silk fibroin nanoclay composite or film of any one of claims 26 to 30, the stabilized silk fibroin solution of any one of claims 31 to 42, the liquid in air suspension of claim 43, or the plurality of drops or droplets of claim 44, wherein the silk fibroin fragments have a weight average molecular weight selected from between
25 about 1 kDa and about 5 kDa, from between about 5 kDa and about 10 kDa, from between about 6 kDa and about 17 kDa, from between about 10 kDa and about 15 kDa, from between about 14 kDa and about 30 kDa, from between about 15 kDa and about 20 kDa, from between about 17 kDa and about 39 kDa, from between about 20 kDa and about 25 kDa, from between about 25 kDa and about 30 kDa, from between
30 about 30 kDa and about 35 kDa, from between about 35 kDa and about 40 kDa, from between about 39 kDa and about 54 kDa, from between about 39 kDa and about 80 kDa, from between about 40 kDa and about 45 kDa, from between about 45 kDa and about 50 kDa, from between about 50 kDa and about 55 kDa, from between about 55 kDa and about 60 kDa, from between about 60 kDa and about 100 kDa, from between

about 80 kDa and about 144 kDa, from between about 144 kDa and about 250 kDa, or from between about 250 kDa and about 350 kDa, and a polydispersity from 1 to about 5.

5 46. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 45, wherein the polydispersity is from 1 to about 1.5, from about 1.5 to about 2.0, from about 2.0 to about 2.5, from about 2.5 to about 3.0, from about 3.0 to about 3.5, from about 3.5 to
10 about 4.0, from about 4.0 to about 4.5, or from about 4.5 to about 5.0.

 47. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 45, further comprising about
15 0.001% (w/w) to about 10% (w/w) sericin relative to the silk fibroin fragments.

 48. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of any one of claims 45 to 47,
20 wherein the silk fibroin fragments do not spontaneously or gradually gelate and do not visibly change in color or turbidity when in an aqueous solution for at least 10 days prior to being formulated into the substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, or the stabilized silk fibroin solution.

25 49. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 25, the silk fibroin nanoclay composite or film of any one of claims 26 to 30, the stabilized silk fibroin solution of any one of claims 31 to 42, the liquid in air suspension of claim 43, or the plurality of drops or droplets of claim 44, wherein the silk fibroin fragments comprise a plurality of amino acids selected from M, R, V, K,
30 T, F, I, L, C, A, Q, Y, N, D, E, G, S, H, P, and W, wherein at least one of the amino acids is modified, substituted, or replaced.

 50. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air

suspension, or the plurality of drops or droplets, of claim 49, wherein the fibroin is a fibroin heavy chain, a fibroin light chain, or a fibrohexamerin.

51. The plurality of substantially solid silk fibroin particles, the silk fibroin
5 nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 49 or 50, wherein a silk fibroin fragment comprises between about 2 and about 100 amino acids.

52. The plurality of substantially solid silk fibroin particles, the silk fibroin
10 nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 49 to 51, wherein a silk fibroin fragment comprises between one and five modifications, substitutions, and/or replacements.

53. The plurality of substantially solid silk fibroin particles, the silk fibroin
15 nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of any one of claims 49 to 52, wherein a modification, substitution, and/or replacement is selected from an asparagine to aspartic acid modification, substitution, and/or replacement, a glutamine
20 to glutamic acid modification, substitution, and/or replacement, and a methionine to methionine oxide modification, substitution, and/or replacement.

54. The plurality of substantially solid silk fibroin particles, the silk fibroin
nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air
25 suspension, or the plurality of drops or droplets, of any one of claims 49 to 53, wherein the fibroin is a fibroin heavy chain, and wherein a modification, substitution, and/or replacement is at a position corresponding to any one position from 1 to 5263 of the fibroin heavy chain.

55. The plurality of substantially solid silk fibroin particles, the silk fibroin
30 nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 54, wherein a modification, substitution, and/or replacement is at Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, and/or N5262.

56. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of any one of claims 49 to 53, wherein the fibroin is a fibroin light chain, and wherein a modification, substitution, and/or replacement is at a position corresponding to any one position from 1 to 262 of the fibroin light chain.

57. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 56, wherein a modification, substitution, and/or replacement is at N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, and/or Q255.

58. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of any one of claims 49 to 53, wherein the fibroin is a fibrohexamerin (p25), and wherein a modification, substitution, and/or replacement is at a position corresponding to any one position from 1 to 220 of the fibrohexamerin (p25).

59. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 58, wherein a modification, substitution, and/or replacement is at Q62, N93, M120, N149, N172, N174, and/or N202.

60. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of any one of claims 49 to 59, wherein each modification, substitution, and/or replacement is independently ranging between about 1% to about 99% in the silk fibroin fragments portion of the substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, or the stabilized silk fibroin solution composition.

61. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 61, wherein a % modification, substitution, and/or replacement is defined as (number of peptide or protein fragments comprising a modification, substitution, and/or replacement at a specific position, divided by the total number of peptide or protein fragments which include the specific position, whether comprising a modification, substitution, and/or replacement, or not) x 100.

62. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 25, the silk fibroin nanoclay composite or film of any one of claims 26 to 30, the stabilized silk fibroin solution of any one of claims 31 to 42, the liquid in air suspension of claim 43, or the plurality of drops or droplets of claim 44, wherein the silk fibroin fragments are included in one or more fractions, each fraction independently comprising a plurality of fibroin heavy chain fragments, a plurality of fibroin light chain fragments, and/or a plurality of fibrohexamerin (p25) fragments.

63. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the silk fibroin fragments have a weight average molecular weight (M_w) selected from between about 1 kDa and about 20 kDa, from between about 20 kDa and about 40 kDa, from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, from between about 120 kDa and about 140 kDa, or from between about 140 kDa and about 160 kDa, from between about 160 kDa and about 180 kDa, from between about 180 kDa and about 200 kDa, or from between about 200 kDa and about 250 kDa, and a polydispersity between 1 and about 1.7.

64. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the silk fibroin fragments have a weight average molecular weight (M_w) selected from between about 10 kDa and about 20 kDa, from between about 20 kDa and about 40 kDa, from

between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, from between about 120 kDa and about 140 kDa, from between about 140 kDa and about 160 kDa, or from between about 160 kDa and about 180 kDa, and a polydispersity between 1 and about 1.1, or 1 and about 1.2.

65. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from between about 10 kDa and about 20 kDa, from between about 20 kDa and about 40 kDa, from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, or from between about 120 kDa and about 140 kDa, and a polydispersity between 1 and about 1.1, or 1 and about 1.2.

66. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, or from between about 100 kDa and about 120 kDa, and a polydispersity between 1 and about 1.1.

67. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from between about 10 kDa and about 20 kDa, from between about 20 kDa and about 40 kDa, from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, or from between about 100 kDa and about 110 kDa, and a polydispersity between 1 and about 1.1, or 1 and about 1.2.

68. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from
5 between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, or from between about 120 kDa and about 140 kDa, and a polydispersity between 1 and about 1.1.

69. The plurality of substantially solid silk fibroin particles, the silk fibroin
10 nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from between about 20 kDa and about 40 kDa, or from between about 40 kDa and about 60 kDa, and a polydispersity between 1 and about 1.1, or 1 and about 1.2.

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70. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the one or more fractions are selected from AS77, AS78, AS79, AS80, and AS81.

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71. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the one or more fractions are selected from AS82, AS83, AS84, AS85, AS86, AS87, AS88, and AS89.

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72. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the one or more fractions are selected from AS90, AS91, AS92, AS93, and AS94.

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73. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the one or more fractions are selected from AS95, AS96, AS97, AS98, AS99, and AS100.

74. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the silk fibroin fragments have a weight average molecular weight (M_w) selected from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, from between about 120 kDa and about 140 kDa, from between about 140 kDa and about 160 kDa, from between about 160 kDa and about 180 kDa, from between about 180 kDa and about 200 kDa, or from between about 200 kDa and about 220 kDa, and a polydispersity between 1 and about 1.7.

75. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, from between about 120 kDa and about 140 kDa, from between about 140 kDa and about 160 kDa, from between about 160 kDa and about 180 kDa, from between about 180 kDa and about 200 kDa, or from between about 200 kDa and about 210 kDa, and a polydispersity between 1 and about 1.2, or 1 and about 1.3.

76. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from between about 40 kDa and about 60 kDa, from between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, or from between about 100 kDa and about 110 kDa, and a polydispersity between 1 and about 1.1, or 1 and about 1.2

77. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the silk fibroin fragments in a fraction have a weight average molecular weight (M_w) selected from

between about 60 kDa and about 80 kDa, from between about 80 kDa and about 100 kDa, from between about 100 kDa and about 120 kDa, from between about 120 kDa and about 140 kDa, from between about 140 kDa and about 160 kDa, from between about 160 kDa and about 180 kDa, from between about 180 kDa and about 200 kDa, or from between about 200 kDa and about 210 kDa, and a polydispersity between 1 and about 1.2, or 1 and about 1.3.

78. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the one or more fractions are selected from AS101, AS102, AS103, AS104, and AS105.

79. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 62, wherein the one or more fractions are selected from AS106, AS107, AS108, AS109, AS110, and AS111.

80. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of any one of claims 62 to 79, wherein the silk fibroin fragments comprise one or more amino acid modifications, substitutions, or replacements of an amino acid is selected from M, R, V, K, T, F, I, L, C, A, Q, Y, N, D, E, G, S, H, P, and W.

81. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of any one of claims 62 to 80, wherein the silk fibroin fragments comprise between about 2 and about 100 amino acids.

82. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 80 or 81, wherein a silk

fibroin fragment comprise between one and five modifications, substitutions, and/or replacements.

83. The plurality of substantially solid silk fibroin particles, the silk fibroin
5 nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air
suspension, or the plurality of drops or droplets, of any one of claims 80 to 82,
wherein the fibroin is a fibroin heavy chain, and wherein a modification, substitution,
and/or replacement is at a position corresponding to any one position from 1 to 5263
of the fibroin heavy chain.

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84. The plurality of substantially solid silk fibroin particles, the silk fibroin
nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air
suspension, or the plurality of drops or droplets, of any one of claims 80 to 82,
wherein the fibroin is a fibroin light chain, and wherein a modification, substitution,
15 and/or replacement is at a position corresponding to any one position from 1 to 262 of
the fibroin light chain.

85. The plurality of substantially solid silk fibroin particles, the silk fibroin
nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air
20 suspension, or the plurality of drops or droplets, of any one of claims 80 to 82,
wherein the fibroin is a fibrohexamerin (p25) chain, and wherein a modification,
substitution, and/or replacement is at a position corresponding to any one position
from 1 to 220 of the fibrohexamerin (p25) chain.

25 86. The plurality of substantially solid silk fibroin particles, the silk fibroin
nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air
suspension, or the plurality of drops or droplets, of any one of claims 80 to 85,
wherein a modification, substitution, and/or replacement is selected from an
asparagine to aspartic acid modification, substitution, and/or replacement, a glutamine
30 to glutamic acid modification, substitution, and/or replacement, and a methionine to
methionine oxide modification, substitution, and/or replacement.

87. The plurality of substantially solid silk fibroin particles, the silk fibroin
nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air

suspension, or the plurality of drops or droplets, of any one of claims 80 to 86, wherein a modification, substitution, and/or replacement is at fibroin heavy chain position selected from Q58, M64, N68, N70, N77, M80, N93, M103, Q125, N132, Q139, Q275, N4191, Q5216, and/or N5262.

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88. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of any one of claims 80 to 86, wherein a modification, substitution, and/or replacement is at fibroin light chain position selected from N23, Q24, N28, M69, N105, N108, N118, N136, N138, Q149, N186, N200, Q202, N204, N240, N248, and/or Q255.

89. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of any one of claims 80 to 86, wherein a modification, substitution, and/or replacement is at fibrohexamerin (p25) position selected from Q62, N93, M120, N149, N172, N174, and/or N202.

90. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of any one of claims 80 to 89, wherein each modification, substitution, and/or replacement is independently ranging in the composition between about 1% to about 99%.

91. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of claim 90, wherein the % modification, substitution, and/or replacement is defined as (number of peptide or protein fragments comprising a modification, substitution, and/or replacement at a specific position, divided by the total number of peptide or protein fragments which include the specific position, whether comprising a modification, substitution, and/or replacement, or not) x 100.

92. The plurality of substantially solid silk fibroin particles, the silk fibroin nanoclay composite or film, the stabilized silk fibroin solution, the liquid in air suspension, or the plurality of drops or droplets, of any one of claims 45 to 91, wherein a molecular weight is determined by MALS.

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93. A pouch or a laundry pod comprising the plurality of substantially solid silk fibroin particles of any one of claims 1 to 92.

94. The laundry pod of claim 93, wherein the plurality of substantially solid
10 silk fibroin particles are compressed in a multi-particulate puck.

95. The laundry pod of claim 93 or 94, further comprising a dissolvable enclosure comprising polyvinylalcohol (PVA) or a derivative of PVA.

15 96. The pouch of claim 93, further comprising an enclosure comprising one or more of nylon, polyglycolide (PGA), polylactic acid (PLA), poly(lactide-co-glycolide) (PLGA), polycaprolactone (PCL), poly(butylene succinate) (PBS), polybutylene succinate adipate, poly(p-dioxanone) (PPDO), poly(butylene adipate-co-terephthalate) (PBAT), a copolyester of terephthalic acid and lactic acid, a copolyester
20 of terephthalic acid and glycolic acid, a copolyester of terephthalic acid and succinic acid, poly(hydroxybutyrate), poly(hydroxyvalerate), polyhydroxyhexanoate, a poly(hydroxyalkanoate) (PHA), polymethylene adipate/terephthalate.

97. A method of making the plurality of substantially solid silk fibroin
25 particles of any one of claims 1 to 92, the method comprising dripping a solution comprising a plurality of the silk fibroin fragments into liquid nitrogen.

98. The method of claim 97, further comprising a lyophilization step.

30 99. The method of claim 97 or 98, wherein the concentration of silk fibroin fragments in the solution is from about 3% (w/w) to about 50% (w/w).

100. The method of any one of claims 97 to 99, wherein the silk fibroin fragments comprise one or more of a molecular weight, polydispersity, and/or a

modification, substitution, and/or replacement at a specific amino acid position, as defined in any one of claims 45 to 91.

101. The method of any one of claims 97 to 100, wherein the solution is
5 stabilized as defined in any one of claims 31 to 41.

102. A method of reconstituting a silk fibroin fragments solution comprising dissolving the plurality of substantially solid silk fibroin particles of any one of claims 1 to 92 in a solvent, wherein the particles have a reconstitution yield of more than
10 90%.

103. The plurality of substantially solid silk fibroin particles of any one of claims 1 to 92, wherein the particles have a reconstitution yield in DI water of more than 90%.
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104. The method of claim 102, or the plurality of substantially solid silk fibroin particles of claim 103, wherein a reconstitution rate of at least 90% is preserved after a stability testing comprising simulated ageing of a plurality of substantially solid silk fibroin particles of any one of claims 1 to 92, the ageing
20 comprising storage a temperature between about 40 °C and about 60 °C, for a period of time ranging from about 400 days to about 650 days.

105. The method of claim 104, wherein the simulated age ranges from about 4 years to about 15 years.
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