evidence that the presence of aggregated proteins (even humanized or fully human proteins) can significantly increase the risk that a patient will develop an immune response to the active protein monomer, resulting in the formation of neutralizing antibodies and drug resistance, or other adverse side effects (Mahler J Pharm Sci. 2009 Sep;98(9):2909-34).

[0006] Protein-based pharmaceutical formulations are often lyophilized and stored in the solid state to help preserve the integrity of the protein, such as the antibody or bispecific antibody construct, in the formulation during storage. Many current methods of lyophilizing protein formulations, however, fail to result in solid state formulations that exhibit suitable stability over time. Thus, there is a need for new methods of producing lyophilized protein formulations that exhibit improved storage stability.

SUMMARY

[0007] In one aspect, the disclosure provides a method of preparing a lyophilized formulation, the method including (a) cooling a lyophilization chamber containing a liquid formulation comprising a bispecific antibody construct, a saccharide, and a surfactant to a temperature ranging from -35°C to -50°C to produce a frozen formulation, and holding the chamber at a temperature ranging from -40°C to -50°C for a time period of 2 hours to 24 hours; (b) heating the chamber to a temperature ranging from -30°C to -20°C and a pressure ranging from 25 mTorr to 100 mTorr to produce a primary dried formulation, and holding the chamber at a temperature ranging from -30°C to -20°C and a pressure ranging from 25 mTorr to 100 mTorr for a time period of 45 hours to 60 hours; (c) heating the chamber to a temperature ranging from 20°C to 35°C to produce a secondary dried formulation, and holding the chamber at a temperature ranging from 20°C to 30°C and a pressure ranging from 25 mTorr to 100 mTorr for a time period of 5 hours to 10 hours to produce the lyophilized formulation; wherein the liquid formulation has a pH or 3-7 and does not contain mannitol; and the method lacks an annealing step. In another example, the disclosure provides a method of preparing a lyophilized formulation, the method including (a) cooling a lyophilization chamber containing a liquid formulation comprising a protein , a saccharide, and a surfactant to a temperature ranging from about -35°C to about -50°C to produce a frozen formulation, and holding the chamber at a temperature ranging from about -40°C to about -50°C for a time period of about 2 hours to about 24 hours; (b) heating the chamber to a temperature ranging from about -30°C to about -20°C and a pressure ranging from about 25 mTorr to about 100 mTorr to produce a primary dried formulation, and holding the chamber at a temperature ranging from about -30°C to about -20°C and a pressure ranging from about 25 mTorr to about 100 mTorr for a time period of

about 45 hours to about 60 hours; (c) heating the chamber to a temperature ranging from about 20°C to about 35°C to produce a secondary dried formulation, and holding the chamber at a temperature ranging from about 20°C to about 30°C and a pressure ranging from about 25 mTorr to about 100 mTorr for a time period of about 5 hours to about 10 hours to produce the lyophilized formulation; wherein the liquid formulation has a pH or about 3-7 and does not contain mannitol; and the method lacks an annealing step.

[0008] In another aspect, the disclosure provides a lyophilized protein formulation prepared by the method of the disclosure.

[0009] Further aspects and advantages will be apparent to those of ordinary skill in the art from a review of the following detailed description. While the methods disclosed herein are susceptible of embodiments in various forms, the description hereafter includes specific embodiments with the understanding that the disclosure is illustrative, and is not intended to limit the invention to the specific embodiments described herein.

EXAMPLE 1

Lyophilization of Bispecific Antibody Construct Formulation Without An Annealing Step

[0097] A liquid protein formulation was prepared as described above and introduced into a lyophilization chamber. The chamber was cooled from ambient temperature to -45 °C at a rate of 0.5 °C/min, and held at -45 °C for 2 hours. The pressure of the lyophilization chamber was lowered from ambient to 70 mTorr, the chamber was heated to -25 °C at a rate of 0.33 °C/min, and held at -25 °C for 52 hours at 70 mTorr pressure. The chamber was then heated to 30 °C at a rate of 0.1 °C/min, and held at 30 °C for 8 hours at 70 mTorr. To enable vial stoppering, the temperature of the chamber was lowered to 5 °C, and the chamber was aerated with nitrogen at 500 mTorr. The vial containing the lyophilized protein formulation was removed from the lyophilization chamber and stored at 2-8 °C until further processing and analysis.

[0098] The lyophilized protein formulation was reconstituted with water for injection.

EXAMPLE 2

Lyophilization of Bispecific Antibody Construct Formulation With An Annealing Step

[0099] A liquid protein formulation was prepared as described above and introduced into a lyophilization chamber. The chamber was cooled from ambient temperature to -45 °C at a rate of 0.5 °C/min, and held at -45 °C for 2 hours. The temperature of the chamber was ramped to -12 °C at a rate of 0.5 °C/min, and held at -12 °C for 5 hours. The chamber was then cooled back to -45 °C at a rate of 0.5 °C/min, and held at -45 °C for 2 hours. The pressure of the chamber was lowered from ambient to 70 mTorr, the chamber was heated to -25 °C at a rate of 0.33 °C/min, and held at -25 °C for 52 hours at 70 mTorr pressure. The chamber was then heated to 30 °C at a rate of 0.1 °C/min, and held at 30 °C for 8 hours at 70 mTorr. To enable vial stoppering, the temperature of the chamber was lowered to 5 °C, and the lyophilization chamber was aerated with nitrogen at 500 mTorr. The vial containing the lyophilized protein formulation was removed from the lyophilization chamber and stored at 2-8 °C until further processing and analysis.

[0100] The lyophilized protein formulation was reconstituted with water for injection.

EXAMPLE 3

Comparison of the Stability of Liquid and Lyophilized Formulations of Bispecific Antibody Constructs Using rCE-SDS

[0101] Liquid formulations and lyophilized formulations (prepared with an annealing step) containing 1 mg/ml of BiTE A, BiTE B, BiTE C, or BiTE D were prepared as described above. The liquid and reconstituted lyophilized (reconstituted with water for injection) formulations at a protein concentration of 1 mg/mL were subjected to reduced capillary electrophoresis with sodium dodecyl sulfate (rCE-SDS) to determine the degree of clipping that occurred after one month of storage at 25 °C. As shown in Figure 1, all of the reconstituted lyophilized formulations demonstrated significantly less clipping than the liquid formulations, as evidenced by a lower percentage of low molecular weight species (LMS%), which indicates that the reconstituted lyophilized formulations have superior stability over liquid formulations. Further, liquid and reconstituted lyophilized formulations containing 1 mg/ml of BiTE B were subjected to rCE-SDS to determine the degree of clipping that occurred after one month of storage at 40 °C. As shown in Figure 2, the reconstituted lyophilized formulation demonstrated significantly less clipping than the liquid formulation, as evidenced by a lower LMS%, which indicates that the lyophilized formulation (prepared using an annealing step) has superior stability over the liquid formulation even at a higher temperature.

EXAMPLE 4

Effect of Concentration of the Stability of Lyophilized Formulations Using SE-UHPLC

[0102] Lyophilized formulations (prepared with an annealing step) having different concentrations of BiTE B (1 mg/mL, 5 mg/mL, 13 mg/mL, and 23 mg/mL) were subjected to SEC-UHPLC upon reconstitution after one month of storage at 40 °C to determine the degree of aggregation in the formulation, which was determined by the percentage of high molecular weight species (%HMW). As shown in Figure 3, there was no increase in %HMW under accelerated stress conditions, demonstrating that the lyophilization cycle is able to stabilize formulations having both low and high concentrations of bispecific antibody constructs.

EXAMPLE 5

Comparison of the Stability of Annealed and Non-Annealed Lyophilized Formulations of a Bispecific Antibody Construct Using SE-UHPLC

[0103] The amount of aggregation in annealed (prepared according to Example 2) and non-annealed (prepared according to Example 1) samples of lyophilized formulations containing 23 mg/ml of BiTE B after storage at frozen temperatures was determined using SE-UHPLC. The annealed sample was stored as follows: 45 °C for 48 hours, -12 °C storage for 5 hours, -45 °C for 5 hours, and -25 °C for 48 hours. The non-annealed sample was stored at -46 °C for 58

hours followed by -25 °C for 48 hours. As shown in Figure 4, the annealed sampled exhibited significantly more aggregation upon reconstitution, evidenced by a higher %HMW, than a non-annealed sample upon reconstitution or a control sample containing 10 mM glutamic acid, 9% (w/v/ sucrose, 0.01% (w/v) polysorbate 80 with a drying temperature of -10 °C. *See* Figure 5.

[0104] The amount of aggregation in annealed (prepared according to Example 2) and non-annealed (prepared according to Example 1) samples of lyophilized formulations containing 15 mg/ml of BiTE B was determined using SE-UHPLC before and after the lyophilization occurred. As shown in Table 1, below, the formulation that had been annealed during the lyophilization process exhibited significantly more aggregation upon reconstitution, as evidenced by a greater %HMW, than the sample that had not been annealed during the lyophilization process upon reconstitution.

Table 1.

%HMW (15 mg/ml of BiTE B)		
	Pre-lyophilization	Post-lyophilization (after reconstitution)
Lyophilization cycle with annealing (n=1)	0.437	1.182
Lyophilization cycle; without annealing (n=1)	0.820	0.788

[0105] The amount of aggregation in annealed (prepared according to Example 2) and non-annealed (prepared according to Example 1) samples of lyophilized formulations containing 20 mg/ml of BiTE A, BiTE C, or BiTE E (a bispecific antibody construct having a sequence set forth is SEQ ID NO: 122) was determined using SE-UHPLC before and after the lyophilization occurred. As shown in Table 2, below, reconstituted formulations that had been annealed during the lyophilization process exhibited significantly more aggregation, as evidenced by a greater %HMW, than reconstituted samples that had not been annealed during the lyophilization process.

Table 2

Bispecific Antibody Construct Having a Sequence Set Forth In	**Condition**	**%HMW**	**%Main**	**%LMW**
BiTE E	Pre-Lyophilization	2.37	96.19	1.44
	Post-Lyophilization (with annealing) after reconstitution	3.35	95.23	1.42
	Post-Lyophilization (no annealing) after reconstitution	2.38	96.24	1.37

WHAT IS CLAIMED:

1. A method of preparing a lyophilized formulation, the method comprising:

(a) cooling a lyophilization chamber containing a liquid formulation comprising a bispecific antibody construct, a saccharide, and a surfactant to a temperature ranging from -35 °C to -50 °C to produce a frozen formulation, and holding the chamber at a temperature ranging from -40 °C to -50 °C for a time period of 2 hours to 24 hours;

(b) heating the chamber to a temperature ranging from -30 °C to -20 °C and a pressure ranging from 25 mTorr to 100 mTorr to produce a primary dried formulation, and holding the chamber at a temperature ranging from -30 °C to -20 °C and a pressure ranging from 25 mTorr to 100 mTorr for a time period of 45 hours to 60 hours;

(c) heating the chamber to a temperature ranging from 20 °C to 35 °C to produce a secondary dried formulation, and holding the chamber at a temperature ranging from 20 °C to 30 °C and a pressure ranging from 25 mTorr to 100 mTorr for a time period of 5 hours to 10 hours to produce the lyophilized formulation;

wherein the liquid formulation has a pH of 3-7 and does not contain mannitol; and the method lacks an annealing step.

2. The method of claim 1, wherein, the cooling of step (a) occurs to a temperature of about -45 °C, the holding of step (a) occurs, at a temperature of about -45°C, or both.

3. The method of claim 1 or 2, wherein the cooling of step (a) occurs at a rate ranging from 0.5 °C/min to 1 °C/min, preferably 0.5 °C/min.

4. The method of any one of claims 1-3, wherein the heating of step (b) occurs to a temperature of about -25 °C, the holding of step (b) occurs at a temperature of about -25 °C, or both.

5. The method of any one of claims 1-4, wherein the heating of step (b) occurs at a rate ranging from 0.1 °C/min to 1 °C/min, 0.1 °C/min to 0.5 °C/min, or about 0.3 °C/min.

6. The method of any one of claims 1-5, wherein the heating of step (b), the holding of step (b), or both occurs at a pressure ranging from 70 mTorr to 100 mTorr, preferably about 70 mTorr.

7. The method of any one of claims 1-6, wherein the heating of step (c) occurs to a temperature of about 30 °C, the holding of step (c) occurs at a temperature of about 30°C, or both.

8. The method of any one of claims 1-7, wherein the heating of step (c) occurs at a rate ranging up to about 0.5 °C/min, for example at a rate ranging from 0.05 °C/min to 0.5 °C/min, preferably at a rate of about 0.1 °C/min.

9. The method of any one of claims 1-8, wherein the holding of step (c) occurs at a pressure ranging from 70 mTorr to 100 mTorr, preferably at about 70 mTorr.

10. The method of claim 1, wherein the bispecific antibody construct is a half-life extended (HLE) bispecific antibody construct, optionally comprising an amino acid sequence set forth in SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 33, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 55, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 55, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 87, SEQ ID NO: 97, SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, SEQ ID NO: 121, SEQ ID NO: 122, SEQ ID NO: 131, SEQ ID NO: 141, SEQ ID NO: 142, SEQ ID NO: 146, SEQ ID NO: 147, SEQ ID NO: 156, SEQ ID NO: 165, SEQ ID NO: 174, SEQ ID NO: 183, SEQ ID NO: 184, SEQ ID NO: 185, SEQ ID NO: 186, SEQ ID NO: 187, or SEQ ID NO: 188.

11. The method of any one of claims 1-10, wherein the bispecific antibody construct is present in the liquid formulation at a concentration ranging from 0.1 mg/mL to 100 mg/mL, or from 0.1 mg/mL to 70 mg/mL, or from 0.5 mg/mL to 30 mg/mL, or from 1 mg/ml to 20 mg/mL, or about 1 mg/mL.

12. The method of any one of claims 1-11, wherein the liquid formulation of step (a) has a pH of 4-6, or 4-5, and optionally further comprises a buffer wherein the buffer is an acetate buffer, a glutamate buffer, a citrate buffer, a lactate buffer, a succinate buffer, a tartrate buffer, a fumarate buffer, a maleate buffer, a histidine buffer, a phosphate buffer, a 2-(N-morpholino) ethanesulfonate buffer, or any combination thereof.

13. The method of claim 12, wherein the buffer is present at a concentration ranging from 5 mM to 200 mM, from 10 mM to 50 mM, or about 10 mM.

14. The method of any one of claims 1-13, wherein the saccharide is a monosaccharide or a disaccharide.

15. The method of any one of claims 1-14, wherein the saccharide is present in the liquid formulation at a concentration ranging from 1 to 15% (w/v), from 6% to 12% (w/v), or about 9% (w/v).

16. The method of any one of claims 1-15, wherein the surfactant is polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80, poloxamer 188, poloxamer 407, triton X-100, polyoxyethylene, PEG 3350, PEG 4000, or a combination thereof and the surfactant is present in the liquid formulation at a concentration ranging from 0.001% to 0.5% (w/v), from 0.001% to 0.01% (w/v), or about 0.01% (w/v).

17. The method of any one of claims 1-16, wherein the liquid formulation of step (a) has a pH of about 4.2 and comprises about 10 mM L-glutamic acid, about 9.0% (w/v) sucrose, and about 0.010% (w/v) polysorbate 80.

18. The method of any one of claims 1-17, wherein the lyophilized formulation, upon reconstitution, exhibits a 0.5% or less increase in the percentage of high molecular weight species after storage for one month at 40 °C, or exhibits a 0.3% or less increase in the percentage of high molecular weight species after storage for one month at 40 °C.

19. A lyophilized protein formulation prepared by the method according to any one of claims 1-18.